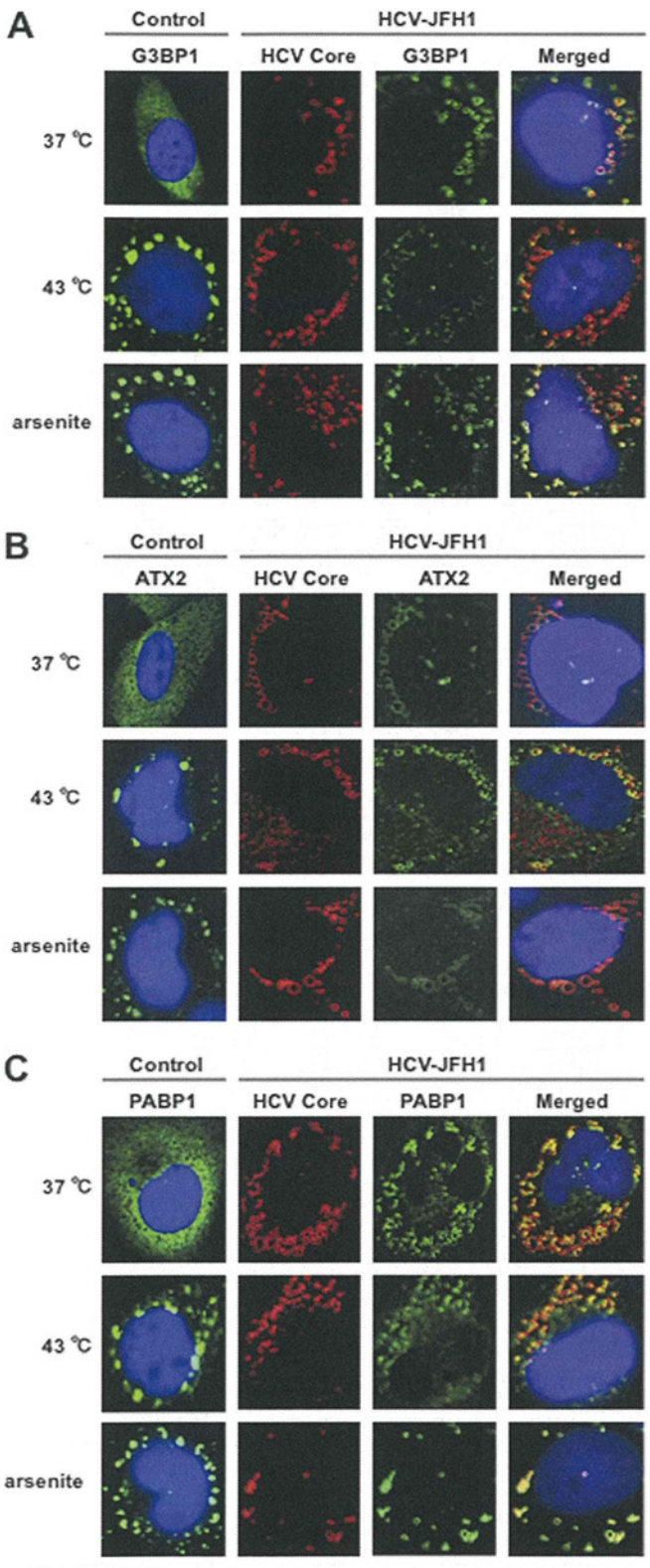

FIG. 5. HCV suppresses stress granule formation in response to heat shock or treatment with arsenite. Naïve RSc cells or HCV-JFH1-infected RSc cells at 72 h postinfection were incubated at 37°C or 43°C for 45 min. Cells were also treated with 0.5 mM arsenite for 30 min. Cells were stained with anti-HCV core and anti-G3BP1 (A), anti-ATX2 (B), or anti-PABP1 (ab21060) (C) antibodies and were examined by confocal laser scanning microscopy.

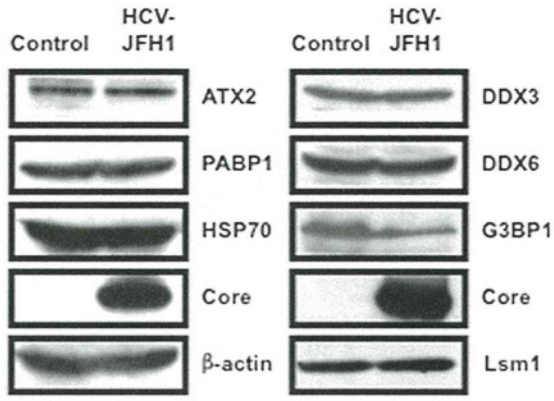

FIG. 6. Host protein expression levels in response to HCV-JFH1 infection. The results of the Western blot analyses of cellular lysates with anti-ATX2/SCA2 antibody (A301-118A), anti-PABP1 (ab21060), anti-HSP70 (610607), anti-HCV core, anti-β-actin, anti-DDX3 (54257 [NT] and 5428 [IN] mixture), anti-DDX6 (A300-460A), anti-G3BP1 (611126), or anti-LSM1 (LS-C97364) antibody in HCV-JFH1-infected RSc cells at 72 h postinfection as well as in naïve RSc cells are shown.

production (33). Since HCV harbors the internal ribosome entry site (IRES) structure in the 5′-UTR of the HCV genome instead of a cap structure, unlike HIV-1, DCP2 may not be recruited on the HCV genome and utilized for HCV replication. Otherwise, DCP2 may determine whether or not DDX6 and miRNAs positively or negatively regulate target mRNA.

Furthermore, we have demonstrated that HCV infection hijacks the P-body and stress granule components around LDs (Fig. 1, 2, 4, and 5). We have found that the P-body formation of DDX6 began to be disrupted at 36 h postinfection (Fig. 4). Consistently, G3BP1 formed stress granules at 36 h postinfection. We then observed the ringlike formation of DDX6 or G3BP1 and colocalization with the HCV core protein after 48 h postinfection, suggesting that the disruption of P-body formation and the hijacking of P-body and stress granule components occur at a late step of HCV infection. Furthermore, HCV infection could suppress stress granule formation in response to heat shock or treatment with arsenite (Fig. 5). In this regard, West Nile virus and dengue virus, of the family *Flaviviridae*, interfere with stress granule formation and P-body assembly through interactions with T cell intracellular antigen 1 (TIA-1)/TIAR (11). Moreover, PABP1 and G3BP1, stress granule components, are known to be common viral targets for the inhibition of host mRNA translation (34, 39). In fact, HIV-1 and poliovirus proteases cleave PABP1 and/or G3BP1 and suppress stress granule formation during viral infection (34, 39). On the other hand, HCV infection transiently induced stress granules at 36 h postinfection (Fig. 4) and did not cleave PABP1 (Fig. 6); however, HCV could suppress stress granule formation in response to heat shock or treatment with arsenite through hijacking their components around LDs, the HCV production factory (Fig. 5). Consistently, Jones et al. showed that HCV transiently induces stress granules of enhanced green fluorescent protein (EGFP)-G3BP at 36 h after infection with the cell culture-generated HCV (HCVcc) reporter virus Jc1FLAG2 (p7-nsGluc2A); however, those authors did not show the recruitment of EGFP-G3BP to LDs (18). Although we do not know the exact reason for this apparent discrepancy,

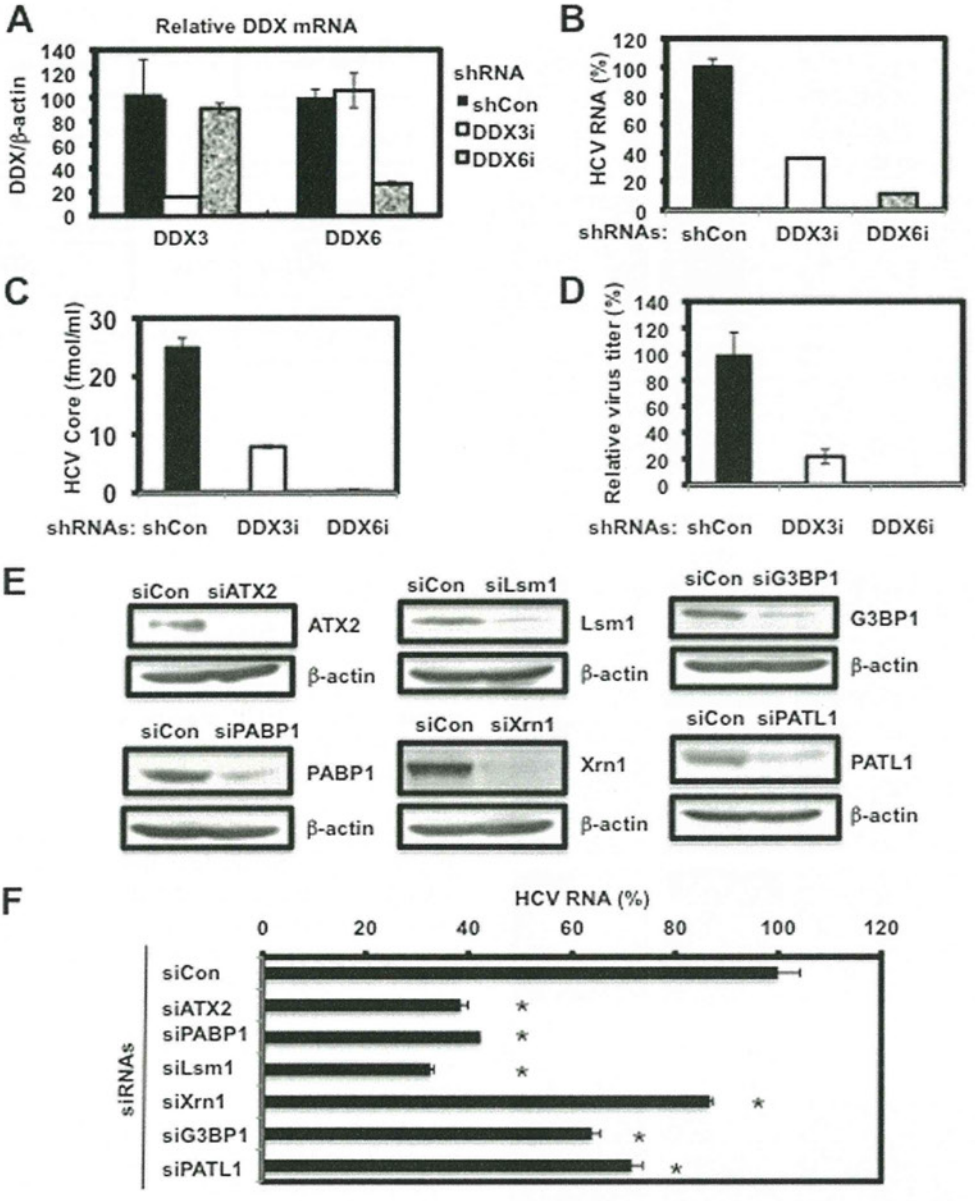

FIG. 7. Requirement of P-body and stress granule components for HCV replication. (A) Inhibition of DDX3 or DDX6 mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for DDX3 or DDX6 was also performed for β-actin mRNA in RSc cells expressing shRNA targeted to DDX3 (DDX3i) or DDX6 (DDX6i) or the control nontargeting shRNA (shCon) in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with the control nontargeting lentiviral vector (shCon), which was assigned as 100%. Error bars in this panel and other panels indicate standard deviations. (B) Levels of intracellular genome-length HCV-JFH1 RNA in the cells at 24 h postinfection at an MOI of 4 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments are shown. Each HCV RNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon), which was assigned as 100%. (C) The levels of HCV core in the culture supernatants from the stable knockdown RSc cells 24 h after inoculation of HCV-JFH1 at an MOI of 4 were determined by ELISA. Experiments were done in triplicate, and columns represent the mean core protein levels. (D) The infectivity of HCV in the culture supernatants from stable-knockdown RSc cells 24 h after inoculation of HCV-JFH1 at an MOI of 4 was determined by a focus-forming assay at 24 h postinfection. Experiments were done in triplicate, and each virus titer was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon), which was assigned as 100%. (E) Inhibition of ATX2, PABP1, Lsm1, Xrn1, G3BP1, or PATL1 protein expression by 72 h after transient transfection of RSc cells with a pool of control nontargeting siRNA (siCon) or a pool of siRNAs specific for ATX2, PABP1, Lsm1, Xrn1, G3BP1, or PATL1 (25 nM), respectively. The results of Western blot analyses of cellular lysates with anti-ATX2, anti-PABP1, anti-Lsm1, anti-Xrn1, anti-G3BP1, anti-PATL1, or anti-β-actin antibody are shown. (F) Levels of intracellular genome-length HCV-JFH1 RNA in the cells at 48 h postinfection at an MOI of 1 were monitored by real-time LightCycler RT-PCR. RSc cells were transiently transfected with a pool of control siRNA (siCon) or a pool of siRNAs specific for ATX2, PABP1, Lsm1, Xrn1, G3BP1, and PATL1 (25 nM). At 48 h after transfection, the cells were inoculated with HCV-JFH1 at an MOI of 1 and incubated for 2 h. The culture medium was then changed and incubated for 22 h. Experiments were done in triplicate, and each HCV RNA level was calculated relative to the level in RSc cells transfected with a control siRNA (siCon), which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment (*, $P < 0.01$).

several possible explanations can be offered. First, those authors examined the localization of EGFP-G3BP within 48 h postinfection, and we observed it at later times (Fig. 4). Second, they used only EGFP-tagged G3BP instead of endogenous G3BP1. Third, they used a Jc1FLAG2 (p7-nsGluc2A) clone, and an HCV-JFH1 clone could markedly induce the recruitment of the core protein to LDs compared to that of Jc1. Also, Jangra et al. failed to observe the recruitment of DDX6 to LDs at 2 days after infection with HJ3-5 virus (16). Accordingly, we also observed that most of the DDX6 still formed intact P bodies at earlier times (12 h or 24 h postinfection). Importantly, we observed the recruitment of DDX6 to LDs 48 h later (Fig. 4). Furthermore, those authors did not show the ringlike structure formation of the HJ3-5 core protein around LDs, unlike the JFH1 core protein that we used in this study. The interaction of the HCV core protein with DDX6 may explain the recruitment of P-body components to LDs. However, we do not yet know whether the P-body function(s) can be performed on LDs. At least, HCV infection did not affect the translation of several host mRNAs with 5′ caps and 3′ poly(A) tails despite the disruption of P-body formation at 72 h postinfection (Fig. 6), suggesting that HCV does not affect P-body function and that HCV recruits functional P bodies to LDs.

We need to address the potential role of stress granule components, such as PABP1, in HCV replication/translation, since the HCV genome does not harbor the 3′ poly(A) tail. Intriguingly, we have found that the accumulation of HCV RNA was significantly suppressed in PABP1 knockdown RSc cells (Fig. 7F). In this regard, Tingting et al. demonstrated previously that G3BP1 and PABP1 as well as DDX1 were identified as the HCV 3′-UTR RNA-binding proteins by proteomic analysis and that G3BP1 was required for HCV RNA replication (35). Yi et al. also reported that G3BP1 was associated with HCV NS5B and that G3BP1 was required for HCV RNA replication (42). We observed a moderate effect of siG3BP1 on HCV RNA replication (Fig. 7F). In contrast, the accumulation of HCV RNA was significantly suppressed in ATX2 and Lsm1 knockdown cells as well as in PABP1 knockdown cells (Fig. 7F), suggesting that ATX2, Lsm1, and PABP1 are required for HCV replication.

Taking these results together, this study has demonstrated for the first time that HCV hijacks P-body and stress granule components around LDs. This hijacking may regulate HCV RNA replication and translation. Indeed, we have found that the accumulation of genome-length HCV-O (genotype 1b) (14) RNA was markedly suppressed in DDX6 knockdown O cells (data not shown). More importantly, these P-body and stress granule components may be involved in the maintenance of the HCV RNA genome without 5′ cap and 3′ poly(A) tail structures in the cytoplasm for long periods, since the hijacking of P-body and stress granule components by HCV occurred at later times.

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Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. HuH-7 hepatoma-derived cells are widely used as the only cell-based HCV replication system for HCV research, including drug assays. Recently, using different hepatoma Li23-derived cells, we developed an HCV drug assay system (ORL8), in which the genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase replicates efficiently. In this study, using the HuH-7-derived OR6 assay system that we developed previously and the ORL8 assay system, we evaluated 26 anti-HCV reagents, which other groups had reported as anti-HCV candidates using HuH-7-derived assay systems other than OR6. The results revealed that more than half of the reagents showed different anti-HCV activities from those in the previous studies, and that anti-HCV activities evaluated by the OR6 and ORL8 assays were also frequently different. In further evaluation using the HuH-7-derived AH1R assay system, which was developed using the AH1 strain of genotype 1b, several reagents showed different anti-HCV activities in comparison with those evaluated by the OR6 and ORL8 assays. These results suggest that the different activities of anti-HCV reagents are caused by the differences in cell lines or HCV strains used for the development of assay systems. Therefore, we conclude that plural HCV assay systems developed using different cell lines or HCV strains are required for the objective evaluation of anti-HCV reagents.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virologic response [2]. HCV is an enveloped virus with a positive single-stranded RNA virus of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3,4].

To date, HuH-7 hepatoma-derived cells are used as the only cell culture system for robust HCV replication in HCV research, including drug assays. We have also developed a HuH-7-derived drug assay system (OR6), in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [5]. Recently, we found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication [6], and we showed that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells, although both cell lines had similar liver-specific expression profiles [7]. In that study, we identified three genes (New York esophageal squamous cell carcinoma 1, β-defensin-1, and galectin-3) showing Li23-specific expression profiles by a comparative analysis using several other hepatic cell lines [7]. We further developed Li23-derived drug assay systems (ORL8 and ORL11), which are relevant to the HuH-7-derived OR6 assay system [6]. During the process of evaluating the ORL8 and ORL11 assay systems using anti-HCV reagents such as IFNs, we noticed that these assay systems were frequently more sensitive to anti-HCV reagents than the OR6 assay system [6]. Furthermore, we recently found that ribavirin at clinically achievable concentrations (approximately 10 μM) effectively inhibited HCV RNA replication in both the ORL8 and ORL11 assay systems, but not in the OR6 assay system [8]. This finding led to the clarification of the anti-HCV mechanism of ribavirin, and we demonstrated that ribavirin's anti-HCV activity was mediated by the inhibition of inosine monophosphate dehydrogenase, a key enzyme in the guanosine biosynthetic pathway [8]. From these findings, we supposed that the anti-HCV reagents reported to date might show different activities among the different drug assay systems. To test this assumption, we evaluated 22 anti-HCV reagents that were reported using HuH-7-derived assay systems other than OR6, using the OR6 and ORL8 assay systems. Four additional

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reagents predicted by antiviral activity other than HCV were also evaluated. Furthermore, a recently developed HuH-7-derived AH1R assay system (AH1 strain of genotype 1b derived from a patient with acute hepatitis) (Mori et al., in preparation) was also used for the evaluation. Here, we report that plural assay systems derived from different cell lines and different HCV strains are required for the objective evaluation of anti-HCV reagents.

2. Materials and methods

2.1. Cell cultures

HuH-7-derived OR6 and AH1R cells were maintained in medium containing G418 (0.3 mg/ml) as described previously [5]. Li23-derived ORL8 cells were also maintained in medium containing G418 (0.3 mg/ml) as described previously [6].

2.2. Reagents

Acetylsalicylic acid, cephalotaxine, clemizole, crucumin, isoliquiritigenin, nitazoxanide, and tizoxanide were purchased from Sigma–Aldrich (St. Louis, MO). Cantharidin, 2′-deoxy-5-fluorouridine, griseofulvin, guanazole, homoharringtonine, resveratrol, and Y7632 were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Artemisinin and bisindoly maleimide 1 were purchased from Alexis Biochemicals (San Diego, CA). Artesunate and silibinin A were purchased from Lkt Laboratories (St. Paul, MN). Esomeprazole and nelfinavir were purchased from Toronto Research Chemicals (North York, ON, Canada). Cinanserin hydrochloride and HA1077 were purchased from Tocris Bioscience (Bristol, UK). 6-Azauridine was purchased from MP Biomedicals (Solon, OH). Carvedilol was purchased from Calbiochem (San Diego, CA). Hemin was purchased from Alfa Aesar (Ward Hill, MA). Methotrexate was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cinanserin hydrochloride, guanazole, HA11077, and Y27632 were dissolved in the culture medium for Li23-derived cells. Artesunate was dissolved in 0.5% $NaHCO_3$ solution. Other reagents were dissolved in dimethyl sulfoxide.

2.3. RL assay

RL assay was performed as described previously [6]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

2.4. WST-1 cell proliferation assay

The cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of each reagent was determined.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [9]. The antibodies used in this study were those against HCV Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15, Sigma–Aldrich) as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin–Elmer Life Sciences, Boston, MA).

2.6. Selective index (SI)

The SI value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

3. Results

3.1. Evaluation of 26 reagents for anti-HCV activity using OR6 and ORL8 assay systems

To obtain candidates for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems, we first searched the literature in the PubMed database using the key words (HCV or hepatitis C) and (inhibit or antiviral or suppress or block); this yielded approximately 4500 reports published between January 2003 and April 2010. From these results, we further selected the reports in which the EC_{50} values of reagents were determined or estimated by the HuH-7-derived HCV assay systems using the Con-1 strain (genotype 1b) [10], N strain (genotype 1b) [11], or HCV JFH-1 strain (genotype 2a) [12]. We finally chose 22 commercially available reagents for the evaluation of anti-HCV activity using OR6 and ORL8 assay systems. Four reagents predicted from the antiviral activity (hepatitis B virus, cytomegalovirus, etc.) other than HCV were also included in the evaluation study. The 26 selected reagents and their references are listed in Supplementary Table S1.

For each of the 26 reagents, we determined the EC_{50} value by RL assay and the CC_{50} value by WST-1 assay using the OR6 or ORL8 assay system, and calculated the SI value by dividing the CC_{50} value by the EC_{50} value. For each reagent, we first compared the EC_{50} value obtained from the OR6 or ORL8 assay with that of the previous study. Consequently, we classified the 26 reagents into five classes, A to E (Table 1). Eight reagents (methotrexate, artemisinin, artesunate, clemizole, hemin, 6-azauridine, acetylsalicylic acid, and isoliquiritigenin with the order of the SI value in the ORL8 assay) belonged to class A, in which the EC_{50} value obtained by either the OR6 or ORL8 assay was less than one-third of that in the previous study (Supplementary Table S1 and Table 1). Artesunate, an artemisinin-derivative possessing antiviral activity against cytomegalovirus, herpesvirus, Epstein-Barr virus etc., was included in class A by the comparison with the data on anti-cytomegalovirus activity. In this class, we especially noticed that methotrexate (an anti-cancer drug) showed very strong anti-HCV activity (EC_{50} 0.1 μM; $CC_{50} > 200$ μM; SI > 2000) in the ORL8 assay (upper panel in Fig. 1A and Table 1), whereas methotrexate showed very weak anti-HCV activity ($EC_{50} > 200$ μM; $CC_{50} > 200$ μM) in the OR6 assay as well as in a previous report [13] (upper panel in Fig. 1A and Table 1). This drastic difference was confirmed by Western blot analysis (lower panels in Fig. 1A). These results indicate that only the ORL8 assay is drastically sensitive to methotrexate, and suggest that the anti-HCV activity of methotrexate depends on the types of hepatic cells. The comparison of the EC_{50} values of other reagents belonging to class A revealed that the ORL8 assay was more sensitive than the OR6 assay (1.9–15-fold) to artemisinin, artesunate, clemizole, acetylsalicylic acid, and 6-azauridine, and conversely the OR6 assay was more sensitive than the ORL8 assay (2–2.5-fold) to hemin and isoliquiritigenin (Table 1). Furthermore, the CC_{50} values of clemizole and 6-azauridine also differed more than twofold between the OR6 and OR8 assays (Table 1). These results suggest that the anti-HCV activities of these reagents are affected by the kind of assay systems used. Especially, we noticed that artemisinin and artesunate (antimalarial drugs) showed higher SI values in the

Table 1
Anti HCV activities of 26 reagents evaluated in this study.

	Assay	[a]		OR6		ORL8		AHIR	
	Cell origin	HuH-7		HuH-7		Li23		HuH-7	
	HCV strain	Con-1, N, JFH-1, etc.		O		O		AH1	
Class	Reagent	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI	$\frac{CC_{50}}{EC_{50}}$	SI
A	Methotrexate	$\frac{>100}{>100}$	–	$\frac{>200}{>200}$	–	$\frac{>200}{0.1}$	>2000	$\frac{170}{>200}$	<0.9
A	Artemisinin	$\frac{>177}{>78}$	>2.3	$\frac{380}{81}$	4.7	$\frac{370}{23}$	16	$\frac{310}{5.3}$	58
A	Artesunate[b]	$\frac{>15}{3.9}$	>3.8	$\frac{6.1}{2.3}$	2.7	$\frac{3.4}{0.22}$	15	$\frac{4}{0.81}$	4.9
A	Clemizole	$\frac{>20}{8}$	>2.5	$\frac{11}{22}$	0.5	$\frac{22}{2.0}$	11	$\frac{7.3}{>25}$	<0.3
A	Hemin	$\frac{>52}{22}$	>2.4	$\frac{10}{1.2}$	8.3	$\frac{18}{2.4}$	7.5	$\frac{7.2}{1.1}$	6.5
A	6-Azauridine	$\frac{>100}{100}$	>1.0	$\frac{10}{5.7}$	1.8	$\frac{1.5}{0.37}$	4.1	$\frac{14}{3.3}$	4.2
A	Acetylsalicylic acid	$\frac{8^d}{4^d}$	2.0	$\frac{2.6^d}{1.6^d}$	1.6	$\frac{2.4^d}{0.83^d}$	2.9	ND	–
A	Isoliquiritigenin	$\frac{<24}{24}$	<1.0	$\frac{12}{3.9}$	3.1	$\frac{15}{9.8}$	1.5	ND	–
B	Nelfinavir	$\frac{>10}{9.9}$	>1.0	$\frac{26}{11}$	2.4	$\frac{68}{12}$	5.7	ND	–
B	2′-Deoxy-5-fluorouridine	$\frac{<15}{15}$	<1.0	$\frac{31}{32}$	1.0	$\frac{36}{14}$	2.6	$\frac{13}{86}$	0.2
B	Resveratrol	$\frac{>10}{10}$	>1.0	$\frac{35}{4.3}$	8.1	$\frac{42}{16}$	2.6	$\frac{76}{9.9}$	7.7
B	Cantharidine[c]	$\frac{3.5}{0.3}$	12	$\frac{1.5}{0.28}$	5.4	$\frac{1.8}{0.69}$	2.6	ND	–
B	Homoharringtonine[c]	$\frac{0.5}{30^e}$	17	$\frac{38^e}{18^e}$	2.1	$\frac{0.11}{45^e}$	2.4	$\frac{22^e}{19^e}$	1.2
B	Crucumin	$\frac{>15}{15}$	>1.0	$\frac{18}{14}$	1.3	$\frac{19}{11}$	1.7	ND	–
B	Griseofulvin	$\frac{207}{6.1}$	34	$\frac{16}{4.4}$	3.6	$\frac{14}{8.6}$	1.6	ND	–
B	Cinanserin hydrochloride	$\frac{>10}{>10}$	–	$\frac{33}{25}$	1.3	$\frac{39}{35}$	1.1	ND	–
B	Cephalotaxine[c]	$\frac{>100}{60}$	>1.7	$\frac{35}{29}$	1.2	$\frac{38}{47}$	0.8	$\frac{4.8}{41}$	0.1
C	Tizoxanide	$\frac{15}{0.15}$	100	$\frac{11}{2.4}$	4.6	$\frac{24}{9.6}$	2.5	ND	–
C	Nitazoxanide	$\frac{38}{0.21}$	181	$\frac{11}{2.8}$	3.9	$\frac{17}{9.2}$	1.8	$\frac{7.2}{2.2}$	3.3
D	Guanazole	$\frac{<100}{>100}$	<1.0	$\frac{200}{>200}$	<1.0	$\frac{170}{>200}$	<0.9	$\frac{173}{>200}$	<0.9
D	HA1077	$\frac{50}{15}$	3.3	$\frac{>50}{>50}$	–	$\frac{>50}{>50}$	–	$\frac{>50}{>50}$	–
E	Bisindoly maleimide 1	$\frac{ND}{5}$	–	$\frac{8.1}{6.2}$	1.3	$\frac{15}{15}$	1.0	$\frac{14}{9.1}$	1.5
E	Esomeprazole	$\frac{ND}{>10}$	–	$\frac{67}{67}$	1.0	$\frac{27}{27}$	1.0	$\frac{20}{25}$	0.8
E	Y27632	$\frac{>50}{50}$	>1.0	$\frac{>80}{>80}$	–	$\frac{>80}{>80}$	–	$\frac{39}{>80}$	<0.5
E	Carvedilol	$\frac{17}{4.5}$	3.8	$\frac{4.4}{3.7}$	1.2	$\frac{6.6}{8.8}$	0.8	$\frac{6.3}{6.2}$	1.0
E	Silibinin A	$\frac{ND}{23}$	–	$\frac{12}{85}$	0.1	$\frac{26}{89}$	0.3	$\frac{28}{96}$	0.3

ND, not determined.
[a] Assay used in previous reports.
[b] Reported as anti-cytomegalovirus reagent.
[c] Reported as anti-hepatitis B virus reagent. EC_{50} and CC_{50} values are indicated by the order of μM except 'd' (μM) and 'e' (nM).

ORL8 assay than previously reported [14,15]. The anti-HCV profiles of artemisinin and artesunate in the OR6 and ORL8 assays are shown in Fig. 1B and Supplementary Fig. 1A, respectively. In addition, the comparison of SI values revealed that the OR6 assay was more sensitive to hemin and isoliquiritigenin than the HuH-7-derived assays (Con-1 and N strains) used in the previous reports (Supplementary Table S1), suggesting that the HCV strains used in the assay systems affect the evaluation of anti-HCV reagents.

Nine reagents (nelfinavir, 2′-deoxy-5-fluorouridine, resveratrol, cantharidin, homoharringtonine, crucumin, griseofulvin, cinanserin hydrochloride, and cephalotaxine with the order of SI value in the ORL8 assay) were placed in class B, in which the EC_{50} values obtained by the OR6 and ORL8 assays were similar (more than one-third to less than threefold) to those in the previous study (Table 1 and Supplementary Table S1). Cantharidin, homoharringtonine, and cephalotaxine, all of which possess anti-hepatitis B virus activity, were placed in class B by the comparison with the data on anti-hepatitis B virus activity (Supplementary Fig. 1).

Tizoxanide and nitazoxanide belonged to class C, in which the EC_{50} values obtained by both the OR6 and ORL8 assays were more than threefold higher than in the previous study (Table 1 and Supplementary Table S1). Guanazole and HA1077 were placed in class D, in which there was no anti-HCV activity in both the OR6 and ORL8 assays (Table 1). No anti-HCV activity of guanazole and HA1077 was also confirmed by Western blot analysis (data not shown). Lastly, five reagents (Bisindoly maleimide 1, esomeprazole, Y27632, carvedilol, and silibinin A) were placed in class E, in which pro-HCV activity was exhibited in both OR6 and ORL8 assays. We unexpectedly observed that these reagents enhanced the HCV RNA replication level. As a

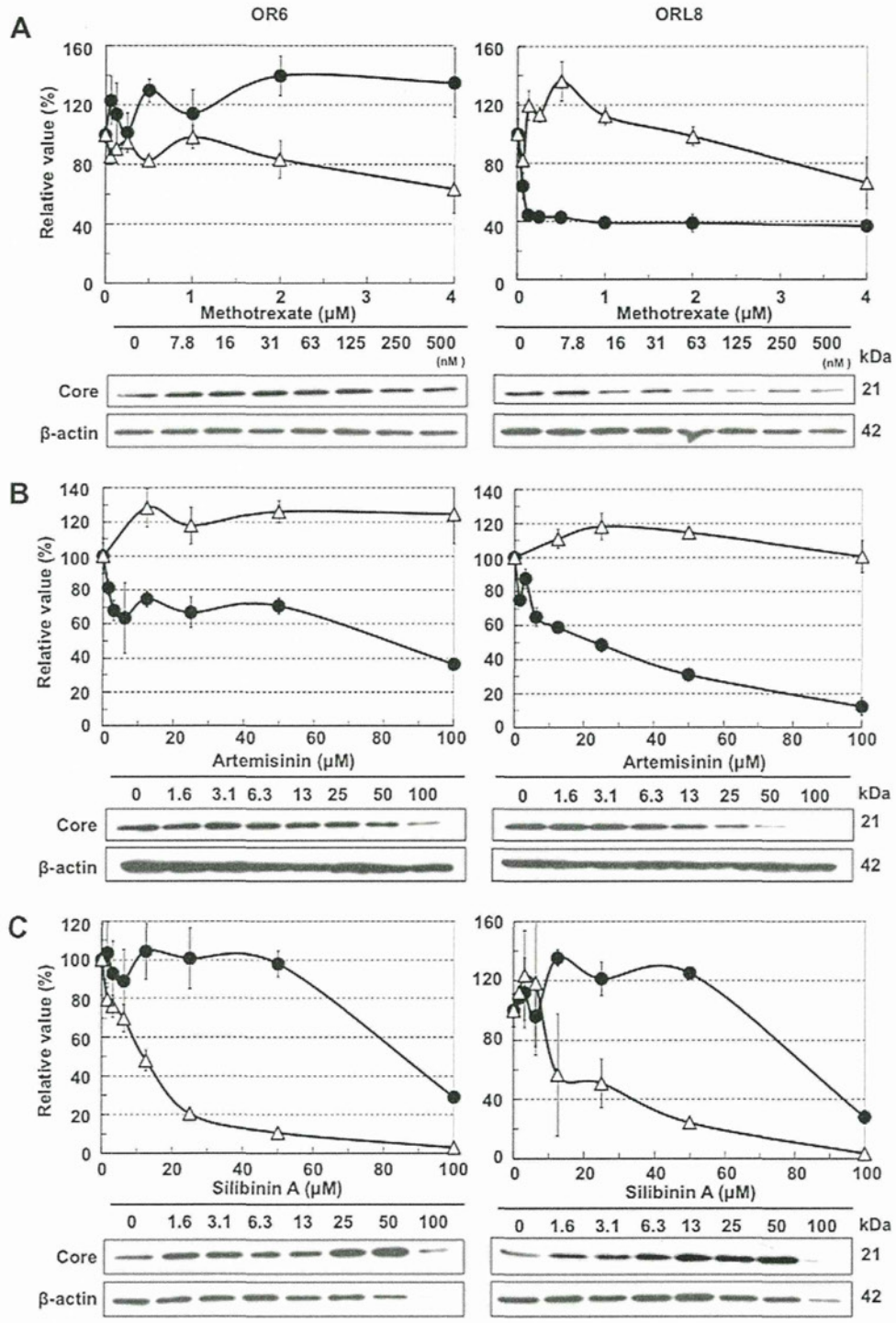

Fig. 1. Anti-HCV profiles of representative reagents in the OR6 and ORL8 assay systems. (A) Methotrexate sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. OR6 and ORL8 cells were treated with methotrexate for 72 h, followed by RL assay (black circle in the upper panel) and WST-1 assay (open triangle in the upper panel). The relative value (%) calculated at each point, when the level in nontreated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (lower panel). (B) Artemisinin sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A). (C) Silibinin A sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. RL assay, WST-1 assay, and Western blot analysis were performed as described in (A).

representative reagent, pro-HCV profiles of silibinin A are shown in the upper panel of Fig. 1C. These pro-HCV profiles were confirmed by Western blot analysis (lower panels in Fig. 1C for silibinin A and data not shown for the other reagents). Since the anti-HCV activity of silibinin A was detected by the HCV replicon assay system using the Con-1 strain [14], the converse effects obtained by our assay systems using the O strain may be due to the difference in HCV strains. In summary, the differences in anti-HCV activities observed among HuH-7- and Li23-derived assay systems used in this study and the other HuH-7-derived assay systems used in the previous studies suggest that the activities of anti-HCV reagents differ depending on which HCV strains and cell lines are used in the evaluation assays.

3.2. Evaluation of 18 reagents for anti-HCV activity using AH1R assay system

We previously established a HuH-7-derived cell line (AH1), which harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis [16]. To further examine the effect of the HCV strain on anti-HCV reagent activity, we developed an AH1R assay system that is based on the AH1 cell line and that corresponds to the OR6 assay system (Mori et al., in preparation).

Using the AH1R assay system, we further evaluated the anti-HCV activities of 18 reagents, which showed differential anti-HCV activity between the OR6 and ORL8 assays, or showed either no anti-HCV activity or pro-HCV activity in both the OR6 and ORL8 assays. The results of the evaluation are shown in Table 1. The comparisons of the data obtained by the OR6 and AH1R assays revealed that the difference in the EC_{50} value from reagent to reagent was held within the range of one-third to threefold. However, we noticed that the EC_{50} value (5.3 μM) of artemisinin in the AH1R assay was remarkably lower than that (81 μM) in the OR6 assay (Supplementary Fig. 2 and Table 1), suggesting that artemisinin's anti-HCV activity differs depending on the HCV strain. Furthermore, the results of the AH1R assay revealed that cephalotaxine, belonging to class B, would be recategorized into class D. In summary, some reagents showed differential anti-HCV activities between the HuH-7-derived OR6 (O strain) and AH1R (AH1 strain) assay systems, although most of the reagents showed similar levels of anti-HCV activity in both assays. Taking together the results of the previous and present studies, we conclude that plural assay systems derived from different cell lines and HCV strains are needed for the objective evaluation of anti-HCV reagents.

4. Discussion

In the present study, we demonstrated for the first time that a Li23-cell-derived drug assay system, not a HuH-7-derived system, was important to use for the objective evaluation of anti-HCV reagents. In addition, we demonstrated that assay systems derived from different HCV strains were also necessary for the objective evaluation of anti-HCV reagents.

Among the 26 reagents evaluated by our assay systems, methotrexate showed the most drastic differences between the HuH-7- and Li23-derived assay systems in terms of anti-HCV activity. Although methotrexate showed very weak anti-HCV activity in the HuH-7-derived assay (Con-1 strain) used in a previous study [13] as well as in our OR6 and AH1R assays (O and AH1 strains), the ORL8 assay revealed very strong anti-HCV activity (SI > 2000). Such drastic differences in both assays suggest that some host factor or factors required for HCV RNA replication are different between these two cell lines, although the anti-HCV target of methotrexate is unclear. Since methotrexate is currently used as an anti-cancer drug or anti-rheumatic drug and its EC_{50} value for HCV RNA replication is 0.1 μM, it may be a potential candidate for enhancing the effects of the current combination therapy of PEG-IFN and ribavirin.

The anti-HCV activities of two antimalarial drugs, artemisinin and its derivative artesunate, are interesting. Although Paeshuyse et al. [14] showed that artemisinin possessed weak or moderate anti-HCV activity using a HuH-7- or HuH-6-derived subgenomic HCV replicon system, artemisinin's anti-HCV mechanism was unclear. On the other hand, Efferth et al. [15] reported that artesunate, the most studied artemisinin-derivative for the treatment of severe malaria, possessed antiviral activity against Epstein–Barr virus, human cytomegalovirus, human herpesvirus 6A, herpes simplex virus 1, and so on, except for HCV with the low micromolar range, although artesunate's precise antiviral mechanism was ambiguous. Therefore, we supposed, and our assay systems clearly detected, that both artemisinin and artesunate possess anti-HCV activity. Especially, the AH1R assay was the most sensitive to artemisinin (EC_{50} 5.3 μM), and the ORL8 assay was the most sensitive to artesunate (EC_{50} 0.22 μM). Preliminary experiments for the anti-HCV mechanisms of these reagents showed that they did not activate the IFN-signaling pathway (data not shown), and that they did not induce the oxidative stress (data not shown) as observed in the treatment with a broad range of anti-HCV reagents, including cyclosporine A [8,17]. Further studies are needed to clarify the anti-HCV mechanisms of these reagents. Since the largest SI value of artemisinin was 58 in the AH1R assay and that of artesunate was 16 in the ORL8 assay, these reagents may be also useful for the treatment of patients with chronic hepatitis.

In this study, we demonstrated that many anti-HCV reagents showed differential anti-HCV activities among different assay systems (OR6, ORL8, and AH1R) on HCV RNA replication. These results suggest that reliance on only a single assay system may lead to an incorrect evaluation of anti-HCV candidates. Therefore, we propose that plural assay systems derived from different cell lines and HCV strains should be used in order to evaluate anti-HCV candidates. Furthermore, plural assay systems derived from at least two different cell origins would be also useful for the screening of anti-HCV candidates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.061.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells

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Abstract
Background Geranylgeranylacetone (GGA), an isoprenoid compound which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating ISGF3 formation in human hepatoma cells. This drug has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.
Methods OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. After treatment, the cells were harvested with Renilla lysis reagent and then subjected to a luciferase assay according to the manufacturer's protocol.
Result The results showed that GGA had anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1, and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.
Conclusions GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

Keywords mTOR · STAT-1 · Interferon · HCV · GGA

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
GGA	Geranylgeranylacetone
siRNA	Small interfering RNA

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Introduction

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are

used in combination with IFN-α to ameliorate the salvage rate of HCV infection [2]. It is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment since IFN-α is the most basic agent for HCV treatment. Any agents that can support IFN activity will improve the therapeutic effect for HCV infected patients.

Geranylgeranylacetone (GGA), an isoprenoid compound, which includes retinoids, has been used orally as an anti-ulcer drug developed in Japan [3]. GGA protects the gastric mucosa from various types of stress without affecting gastric acid secretion [4, 5]. Moreover, GGA suppresses cell growth and induces differentiation or apoptosis in several human leukemia cells [6, 7]. Another isoprenoid compound, 3,7,11,15-tetramethyl-2,4,6,-10,14-hexadecapentaenoic acid, which is designated as an acyclic retinoid because it has the ability to interact with nuclear retinoid receptors [8], causes apoptosis in certain human hepatoma cells [9]. GGA acts as a potent inducer of antiviral gene expression by stimulating the ISGF3 formation in human hepatoma cells [10]. GGA induces the expression of antiviral proteins such as 2′5′-oligoadenylate synthetase (2′5′-OAS) and double-stranded RNA-dependent protein kinase (PKR) in hepatoma cell lines. GGA stimulates 2′5′-OAS and PKR gene expression at the transcriptional level through the formation of interferon-stimulated gene factor 3 (ISGF-3), which regulates the transcription of both genes. GGA induces the expression of signal transducers and activators of transcription 1, 2 (STAT-1, STAT-2) and p48 proteins, components of ISGF3, together with the phosphorylation of STAT1 [10]. However, no anti-HCV activity was observed.

A cell culture HCV replicon system has been developed as a useful tool for the study of HCV replication and mass screening for anti-HCV reagents. OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE [11], were used to examine the influence of the anti-HCV effect of IFN. The luciferase activity in cell lysate of OR6 was correlated with the HCV-RNA concentration, and the IC50 of IFN-α was less than 10 IU/mL [11]. The OR6 system is a useful and sensitive cell culture replicon system.

This study verified the anti-HCV activity of GGA in the OR6 system. In addition, the mechanisms of anti-HCV activity were examined in OR6 cells.

Materials and methods

Reagents

GGA was a generous gift from Eisai Co. (Tokyo, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). Wortmannin, LY294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fatal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication.

Reporter gene assay

The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and GGA. After treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI, USA) and luciferase activity in the cells was determined using a luciferase reporter assay system and a TD-20/20 luminometer. The data were expressed as the relative luciferase activity.

Western blotting and antibodies

Western blotting with anti-STAT-1, anti-PKR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-serine-2448 phosphorylated mTOR, anti-mTOR, anti-threonine-389 phosphorylated p70S6K, anti-p70S6K (Cell Signaling, Beverly, MA, USA) and anti-HSP70 (Stressmarq Biosciences Inc, Victoria, Canada) was performed as described previously [10]. Briefly, OR6 cells were lysed by the addition of a lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium *o*-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

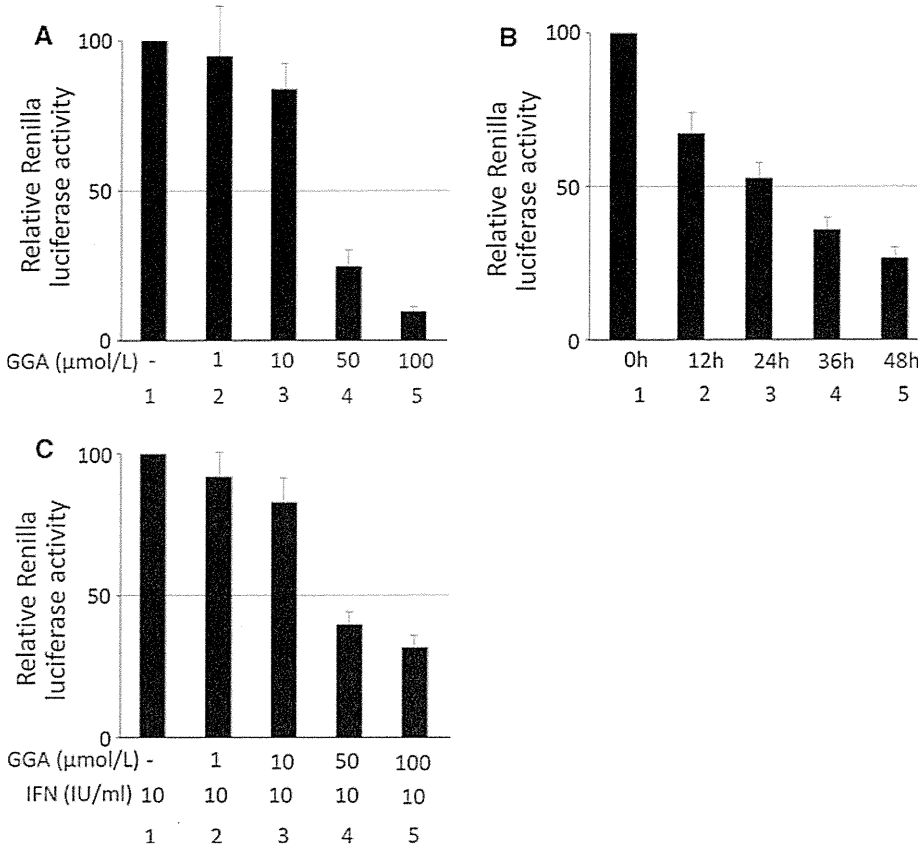

Fig. 1 The effect of GGA on the genome-length HCV RNA replication system. **a** Dose dependent effect of GGA. **b** Time course of GGA suppressed HCV replication. **c** The additive effect of GGA with IFN-α suppressed HCV replication. **a** The OR6 cells were treated with 1–100 μmol/L of GGA (*lanes 2–5*) and *lane 1* was not treated. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments. The differences between *lane 3* versus *4*, *lane 3* versus *5* and *lane 3* versus *5* were statistically significant. **b** The OR6 cells were treated 50 μmol/L of GGA and at the indicated time, HCV replicon assay was done ($n = 4$). The differences between *lane 1* versus *3–5* and *lane 2* versus *4*, *5* were statistically significant. **c** The OR6 cells were treated with 10 IU/mL of IFN-α in the absence (*lane 1*) or presence of treatment with 1–100 μmol/L of GGA (*lanes 2–5*). Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The differences between *lane 1* versus *4*, *5* were statistically significant. Statistical significance was accepted as a P value of <0.05. The data are expressed as the mean ± SD and are representative of four similar experiments

siRNA transfection assay

mTOR gene knockdown was performed using siRNA (Cell Signaling, Beverly, MA, USA). OR6 cells were transfected with100 nmol/L mTOR specific and non-targeted siRNA as a control in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 50 μmol/L GGA.

mTOR kinase activity assay

The cells were washed two times with TBS and lysed by addition of lysis buffer [50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol (w/v), 1% Tween-20 detergent (w/v), 1 mM EDTA, 20 nM microcystin-LR, 25 mM NaF, and a cocktail of protease inhibitors]. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity using a commercially available kit (Calbiochem, San Diego, USA) according to the manufacturer's instructions.

Results

GGA with or without IFN had anti-HCV activity

OR6 cells, the full-length HCV replication system, were used to examine the effect of GGA. The cells were treated with 1–100 μmol/L of GGA for 24 h and the amount of HCV replicon was measured by the *Renilla* luciferase assay (Fig. 1a). The relative *Renilla* luciferase activity decreased in a dose-dependent manner. Furthermore, GGA

induced anti-HCV replicon activity was time dependent (Fig. 1b). GGA was combined with IFN-α to examine the additive effect (Fig. 1c). One or 10 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity slightly (Fig. 1c). However, 50 or 100 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity with statistical difference. GGA treatment did not have any statistically significant effect on cell viability from 1 to 100 μmol/L of GGA for 24 h (data not shown).

GGA did not activate the tyrosine-701 and serine-727 on STAT-1, and did not induce PKR and HSP-70 in OR6 cells

GGA mediated phosphorylation of STAT-1 at the tyrosine-701 and serine-727 residues was investigated using antibodies to phospho-specific STAT-1 on OR6 cells. No phosphorylation of tyrosine-701 and serine-727 on STAT-1 was detected in OR6 cells (Fig. 2a). IFN induce anti-viral protein, PKR, and STAT-1 has an interferon stimulating responsive element (ISRE) in the promoter region [12]. The expression levels of both proteins did not change throughout this study, as indicated by a Western blotting analysis (Fig. 2b, c). Next, the role of HSP in the mechanism of GGA activity was examined because GGA is an inducer of HSP. The HSP-70 expression was increased by pre-exposure to heat shock (Fig. 2d, lanes 2, 4), but it did not increase due to the effects of GGA (Fig. 2d, lanes 3, 4).

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor, were able to cancel the GGA induced anti-HCV activity

The role of the PI3-K-Akt-mTOR pathway the anti-HCV activity of GGA was examined in OR6 cells. The cells were treated with GGA after 3 h in the presence or absence of rapamycin as an mTOR inhibitor, Akt inhibitor, or wortmannin as a PI3-K inhibitor (Fig. 3). Pretreatment with rapamycin attenuated the anti-HCV replication effect

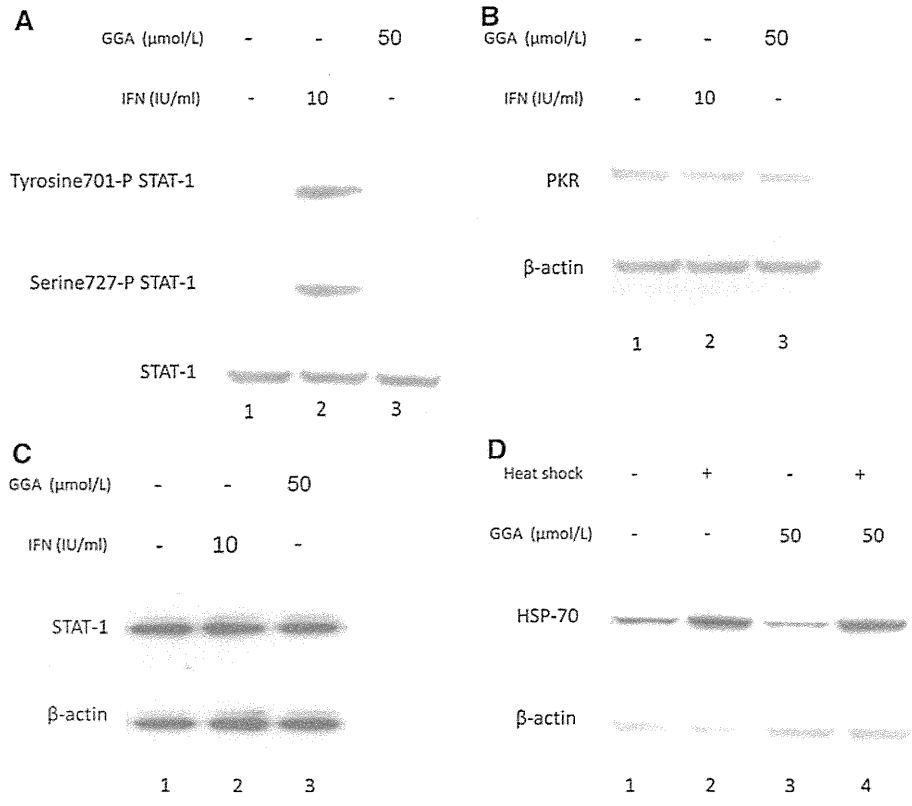

Fig. 2 Effect of GGA on STAT-1 (**a**), PKR (**b**) and HSP-70 (**c**). **a** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*) and then were phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and at serine-727 residue (*middle panel*), the expression STAT-1 (*lower panel*) was analyzed by Western blotting. **b** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*), and then the expression of PKR (*upper panel*) was analyzed by a Western blotting analysis. The β-actin (*lower panel*) protein expression was used as an internal control. **c** The OR6 cells were either untreated (*lane 1*) or given heat shock (at 42°C 15 min, overnight recovery at 37°C) (*lanes 2, 4*) or treated with 50 μmol/L of GGA (*lanes 3, 4*) and then the expression HSP-70 (*upper panel*) was analyzed by Western blotting. β-Actin (*lower panel*) protein is the internal control

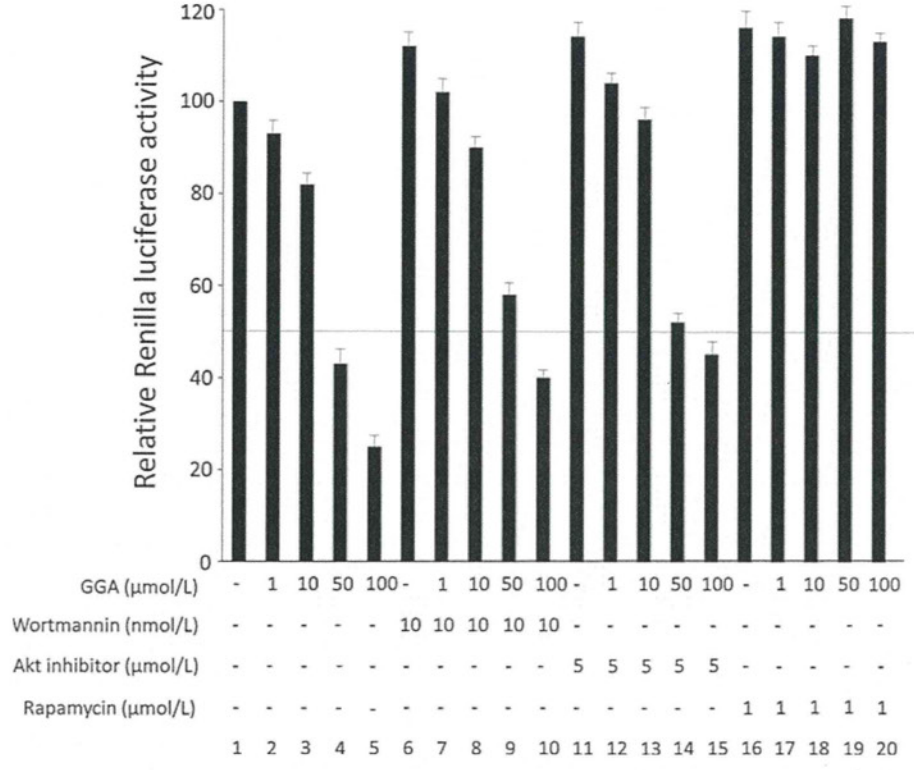

Fig. 3 Changes in GGA suppressed HCV replication by rapamycin, but not PI3-K inhibitor and Akt inhibitor. OR6 cells were treated with 1–100 μmol/L of GGA in the absence (*lanes 2–5*) or presence of pretreatment (*lanes 7–10, 12–15, 17–20*) for 3 h. *Lanes 1, 6, 11* and *16* were not treated with GGA. *Lanes 6, 11* and *16* were treated with wortmannin, an Akt inhibitor, and rapamycin, respectively. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments

in comparison to GGA alone (Fig. 3, lanes 17–20), whereas pretreatment with wortmannin and Akt inhibitor did not increase the *Renilla* luciferase activity (Fig. 3, lanes 7–10, 12–15). siRNA transfection was used for mTOR knockdown to explore role of mTOR in the anti-HCV activity (Fig. 4). The transfection efficiency of the siRNA was confirmed by a Western blotting analysis. In this experiment, the detectable band intensities were quantified by the National Institutes of Health image software program. Although the transfection efficiency of siRNA was barely 46% (Fig. 4a), GGA-induced anti-HCV activity was clearly inhibited in mTOR-siRNA transfected cells (Fig. 4b, lane 4, 6) in comparison to the control cells (Fig. 4b, lanes 3, 5).

GGA induced mTOR activity, mTOR phosphorylation and p70S6K phosphorylation in OR6 cells

The phosphorylation of the serine-2448 residues of mTOR by 50 μmol/L of GGA was detected 30 min after GGA treatment. The band intensity of serine-2448 phosphorylated mTOR decreased by pretreatment with rapamycin but was almost same as with GGA alone following pretreatment with LY294002 (Fig. 5a). Furthermore, an mTOR activity assay was conducted to confirm the activity mechanism of GGA (Fig. 5b). The mTOR activity was increased by treatment with GGA alone (Fig. 5b, lane 4) and was inhibited by pretreatment with rapamycin (Fig. 5b, lane 6), whereas pretreatment with LY94002 did not suppress the mTOR activity (Fig. 5b, lane 5). Furthermore, to evaluate the mTOR activity, we investigated the level of phospholylated-p70S6K by a Western blotting analysis (Fig. 5c). The phosphorylation of the threonine-389 residue of p70S6K by 50 μmol/L of GGA was detected. Similar to mTOR, the band intensity of phospho-threonine-389 of p70S6K decreased after pretreatment with rapamycin, but the intensity was almost the same as that seen following treatment with GGA alone after pretreatment with LY294002 (Fig. 5c).

Discussion

GGA demonstrated the anti-HCV activity in this study. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity. An additive effect was observed with the combination of IFN and GGA.

GGA is a non-toxic heat shock protein (HSP) 70 inducer [13]. Various GGA activities outside of the stomach are also related to HSP induction [14–16]. GGA induced HSP-70 exerts an anti-ischemic stress activity in the heart and liver [16, 17], an anti-inflammatory activity in various cell types [18] and promotes liver regeneration [19]. GGA induces thioredoxin as well as HSP-70 in hepatocytes and other cells [20]. Thioredoxin anti-virus activity, is induced by AP-1 and NF-κB but not HSP-70 [21]. GGA has potent

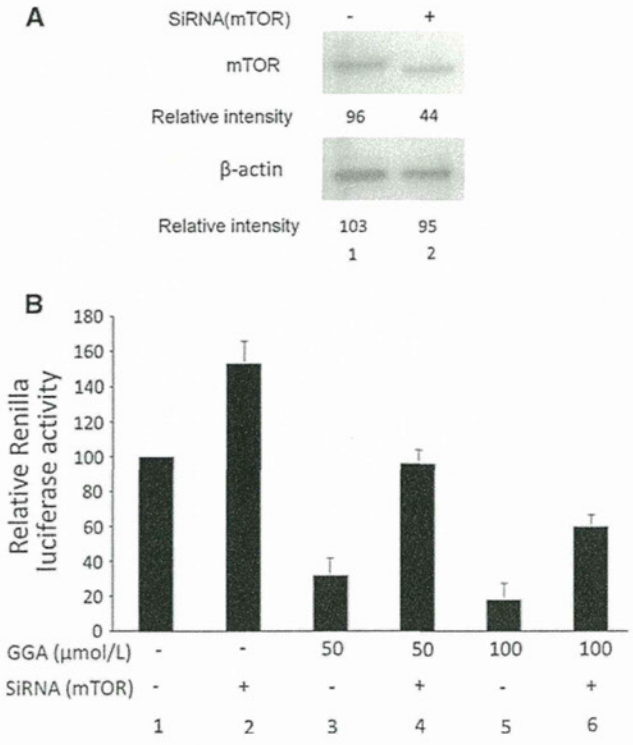

Fig. 4 Changes in GGA suppressed HCV replication by mTOR-siRNA. **a** OR6 cells were transfected with mTOR-siRNA (*lane 1*) or the non-targeted siRNA (*lane 2*). The expression of mTOR was evaluated by a Western blotting analysis. **b** The OR6 cells were transfected with mTOR-siRNA (*lanes 2*, *4* and *6*) and the non-targeted siRNA (*lanes 1*, *3* and *5*). One day later, the cells were treated with GGA (*lanes 3–6*). The HCV replicon assay is the same as Fig. 3. Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The *Renilla* luciferase activity increased in the OR6 cells transfected with mTOR-siRNA (*lane 2*) in comparison to the non-targeted siRNA (*lane 1*). However, in OR6 cells treated with GGA, there was a greater elevation of *Renilla* luciferase activity in OR6 cells transfected with mTOR-siRNA (*lanes 4* and *6*) as compared to that with the non-targeted siRNA (*lanes 3* and *5*). The data are expressed as the mean ± SD and are representative example of four similar experiments

antiviral activity via the enhancement of antiviral factors and can clinically provide protection from influenza virus infection [22]. GGA significantly inhibits the synthesis of influenza virus-associated proteins and prominently enhances the expression of human myxovirus resistance 1 (MxA) followed by increased HSP-70 transcription [22]. Moreover, GGA augments the expression of an interferon-inducible double-strand RNA-activated protein kinase (PKR) gene and promotes PKR autophosphorylation and concomitantly alpha subunit of eukaryotic initiation factor 2 phosphorylation during influenza virus infection [22]. These anti-virus activities are related to GGA induced HSP-70. But, HSP-70 protein and PKR were not induced by GGA in OR6 cells in the current study. There is apparently no relationship between the GGA induced anti-HCV activity and HSP, PKR in OR6 cells. Therefore, we thought that HSP and PKR-independent anti-HCV activity induced by GGA was present in this hepatoma-derived cell line.

GGA induction of anti-viral protein is dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 [10]. However, GGA did not induce STAT-1 tyrosine phosphorylation and anti-virus protein, PKR, in OR6 cells in this study. Moreover, the GGA induced anti-HCV activity depended on mTOR activity, not STAT-1. OR6 cells are full length HCV replicon transfected HuH-7 cells [11]. HCV virus products inhibit the Jak-STAT pathway [23–25]. The mechanism of inhibition of the Jak-STAT pathway is multi-factorial including the suppressor of cytokine signaling 3 (SOCS-3) expression [26], protein phosphatase 2A (PP2A) induction [27], STAT-3 expression [28] and IL-8 expression [29]. GGA induced STAT-1 tyrosine phosphorylation and inducible PKR protein levels are also minor. Generally, the replicon transfection induces the intrinsic IFN [30], but STAT-1 tyrosine phosphorylation was not detected in combined OR6 cells. HCV replicon produced viral product might be inhibiting GGA-induced STAT-1 tyrosine phosphorylation.

mTOR is associated with the IFN induced anti-HCV signal [31]. The IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalomyocarditis virus effect [32]. IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. A relationship has been observed between the replication of the hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV [33]. The IFN induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity and Jak independent TOR activity involves STAT-1 phosphorylation and nuclear localization, and then PKR is expressed in hepatocytes [31]. No relationship between GGA and mTOR has been reported. However, GGA induced anti-HCV activity depended on mTOR activity independent of PI3-K-Akt, as observed with IFN induced mTOR activity.

When 150 mg of GGA was administered orally, the serum concentration of GGA was approximately 7 μmol/L [34]. The concentration of GGA in the portal blood would be several-fold higher than the serum concentration of GGA; therefore, we speculated that the pharmacological action that would be obtained in clinical practice would be the same as that observed in this study.

GGA, a drug that can be safely administered orally, has mTOR dependent anti-HCV activity. The combination of IFN and GGA has an additive effect on anti-HCV activity. The current results suggest that combination therapy with

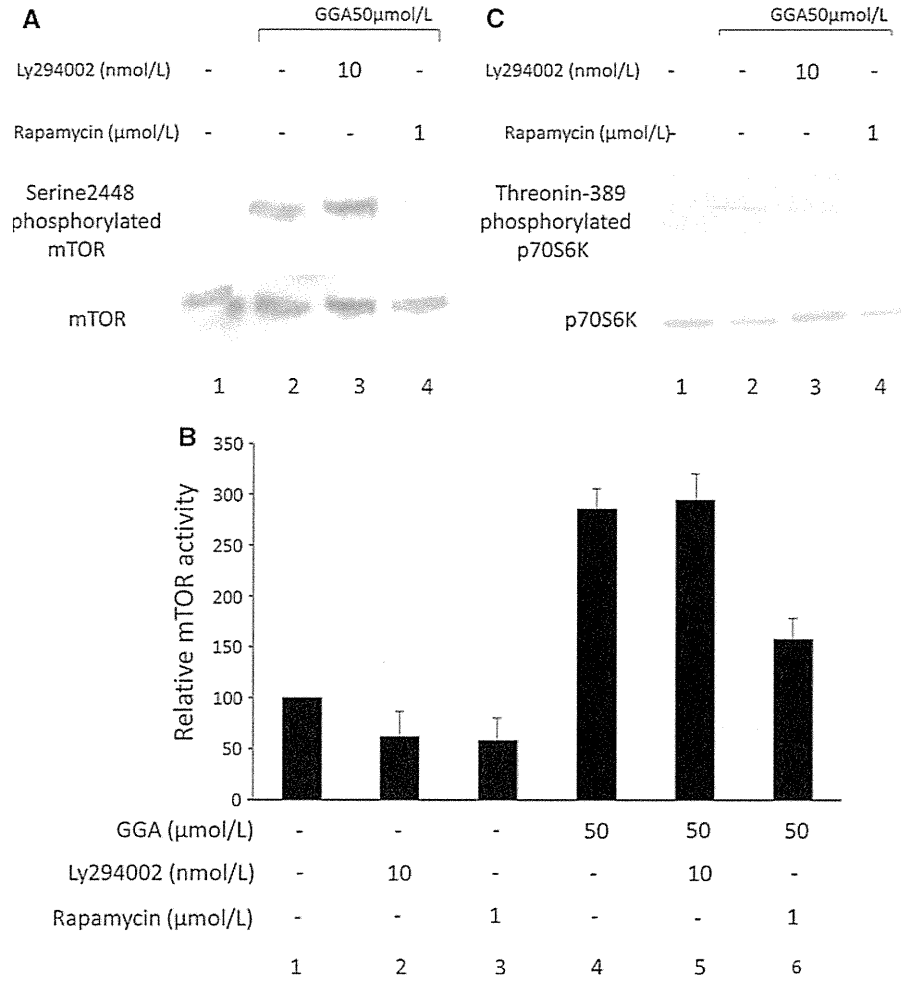

Fig. 5 Effect of GGA on mTOR and effect of LY294002 and rapamycin on GGA-induced serine phosphorylated mTOR and threonine phosphorylated p70S6K. **a** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min and then were phosphorylated mTOR at serine-2448 residue (*upper panel*), the expression of mTOR (*lower panel*) was analyzed by Western blotting. **b** After pretreatment with 10 nmol/L LY294002 (*lanes 2* and *5*) and 1 μmol/L rapamycin (*lanes 3* and *6*) for 3 h, the OR6 cells were either untreated (*lanes 1–3*) or treated with 50 μmol/L GGA (*lanes 4–6*) for 30 min. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit ($n = 4$). The differences between *lanes 1* and *4*, *lanes 4* and *6*, and *lanes 5* and *6* were statistically significant. The data are expressed as the mean ± SD and are representative of four similar experiments. **c** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L, and with rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min, and then were examined for phosphorylated p70S6K at the threonine-389 residue (*upper panel*), or the expression of p70S6K (*lower panel*) by a Western blotting analysis

GGA and IFN is, therefore, expected to improve the anti-HCV activity. It will, therefore, be necessary to examine the clinical effectiveness of the combination with GGA and IFN for HCV patients in the future.

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Development of a drug assay system with hepatitis C virus genome derived from a patient with acute hepatitis C

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Abstract We developed a new cell culture drug assay system (AH1R), in which genome-length hepatitis C virus (HCV) RNA (AH1 strain of genotype 1b derived from a patient with acute hepatitis C) efficiently replicates. By comparing the AH1R system with the OR6 assay system that we developed previously (O strain of genotype 1b derived from an HCV-positive blood donor), we demonstrated that the anti-HCV profiles of reagents including interferon-γ and cyclosporine A significantly differed between these assay systems. Furthermore, we found unexpectedly that rolipram, an anti-inflammatory drug, showed anti-HCV activity in the AH1R assay but not in the OR6 assay, suggesting that the anti-HCV activity of rolipram differs depending on the HCV strain. Taken together, these results suggest that the AH1R assay system is useful for the objective evaluation of anti-HCV reagents and for the discovery of different classes of anti-HCV reagents.

Keywords HCV · Acute hepatitis C · Anti-HCV drug assay system · Anti-HCV activity of rolipram

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [1, 2]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1].

Human hepatoma HuH-7 cell culture-based HCV replicon systems derived from a number of HCV strains have been widely used for various studies on HCV RNA replication [3, 4] since the first replicon system (based on the Con1 strain of genotype 1b) was developed in 1999 [5]. Genome-length HCV RNA replication systems (see Fig. 2 for details) derived from a limited number of HCV strains (H77, N, Con1, O, and JFH-1) are also sometimes used for such studies, as they are more useful than the replicon systems lacking the structural region of HCV, although the production of infectious HCV from the genome-length HCV RNA has not been demonstrated to date [3, 4]. Furthermore, these RNA replication systems have been improved enough to be suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [3, 4, 6]. We also developed an HuH-7-derived cell culture assay system (OR6) in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [7]. Such reporter assay systems could save time and facilitate the mass screening of anti-HCV reagents, since the values of luciferase correlated well with the level of HCV RNA after treatment with anti-HCV reagents. Furthermore, OR6 assay system became more useful as a drug assay system than the HCV subgenomic replicon-based reporter assay systems developed to date [3, 4], because the older systems lack the Core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

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Indeed, by the screening of preexisting drugs using the OR6 assay system, we have identified mizoribine [8], statins [9], hydroxyurea [10], and teprenone [11] as new anti-HCV drug candidates, indicating that the OR6 assay system is useful for the discovery of anti-HCV reagents.

On the other hand, we previously established for the first time an HuH-7-derived cell line (AH1) that harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis C [12]. In that study, we noticed different anti-HCV profiles of interferon (IFN)-γ or cyclosporine A (CsA) between AH1 and O cells supporting genome-length HCV RNA (O strain) replication [7]. From these results, we supposed that the diverse effects of IFN-γ or CsA were attributable to the difference in HCV strains [12].

To test this assumption in detail, we first developed an AH1 strain-derived assay system (AH1R) corresponding to the OR6 assay system, and then performed a comparative analysis using AH1R and OR6 assay systems. In this article, we report that the difference in HCV strains causes the diverse effects of anti-HCV reagents, and we found unexpectedly by AH1R assay that rolipram, an anti-inflammatory drug, is an anti-HCV drug candidate.

Materials and methods

Reagents

IFN-α, IFN-γ, and CsA were purchased from Sigma-Aldrich (St. Louis, MO). Rolipram was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plasmid construction

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ was constructed from pAH1 N/C-5B/PL,LS,TA,$(VA)_3$ encoding genome-length HCV RNA clone 2 (See Fig. 2) obtained from AH1 cells [12], by introducing a fragment of the RL gene from pORN/C-5B into the *Asc*I site before the neomycin phosphotransferase (Neo^R) gene as previously described [7].

RNA synthesis

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ DNA was linearized by *Xba*I, and used for RNA synthesis with T7 MEGAscript (Ambion, Austin TX) as previously described [7].

Cell cultures

AH1R and OR6 cells supporting genome-length HCV RNAs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.3 mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA). AH1c-cured cells, which were created by eliminating HCV RNA from AH1 cells [12] by IFN-γ treatment, were also cultured in DMEM supplemented with 10% FBS.

RNA transfection and selection of G418-resistant cells

Genome-length HCV (AH1RN/C-5B/PL,LS,TA,$(VA)_3$) RNA synthesized in vitro was transfected into AH1c cells by electroporation, and the cells were selected in the presence of G418 (0.3 mg/mL) for 3 weeks as described previously [13].

RL assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, RL assay was performed as described previously [14]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and cultured with the medium in the absence of G418 for 24 h. The cells were then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to a luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Applied Science, Indianapolis, IN, USA) as described previously [7]. The experiments were done in triplicate.

IFN-α treatment to evaluate the assay systems

To monitor the anti-HCV effect of IFN-α on AH1R cells, 2×10^4 cells and 5×10^5 cells were plated onto 24-well plates (for luciferase assay) and 10 cm plates (for quantitative RT-PCR assay) in triplicate, respectively, and cultured for 24 h. The cells were then treated with IFN-α at final concentrations of 0, 1, 10, and 100 IU/mL for 24 h, and subjected to luciferase and quantitative RT-PCR assays as described above.

Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as described previously [13]. The antibodies used in this study were those against HCV Core (CP11 monoclonal antibody;