A

SiRNA(mTOR)	-	+
mTOR		
Relative intensity	96	44
β-actin		
Relative intensity	103	95
	1	2

B

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-
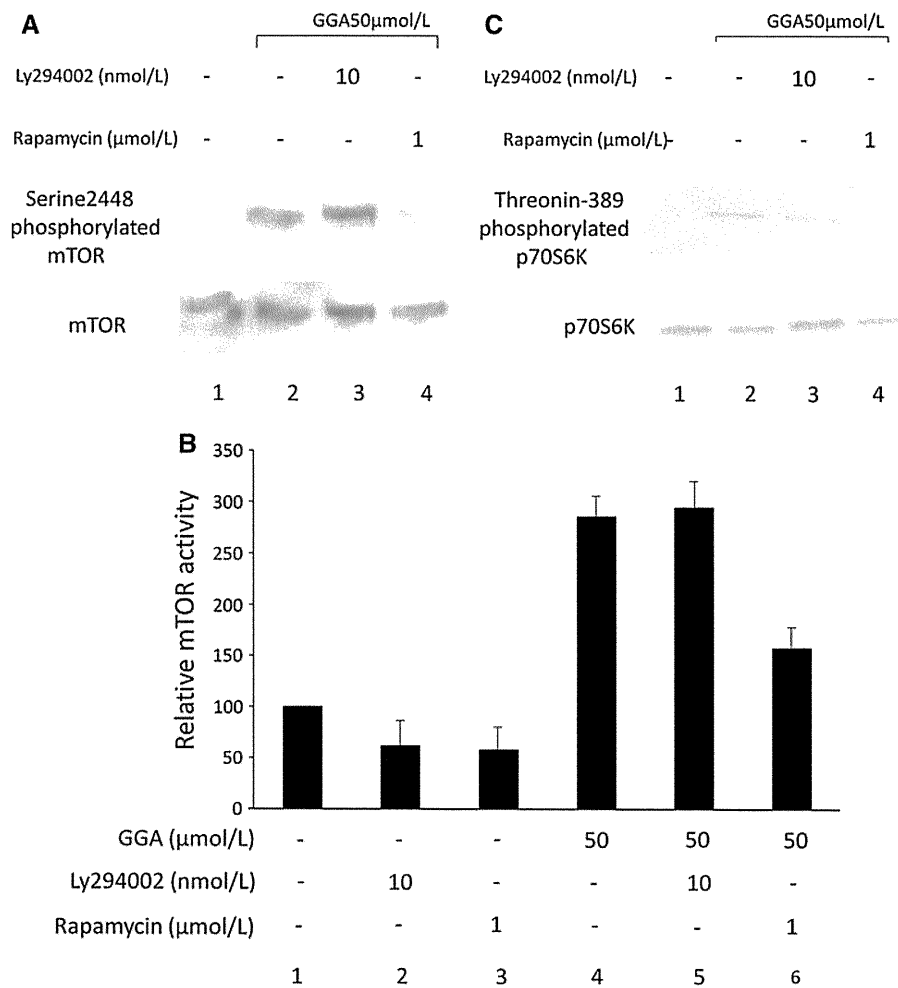
HCV activity and HSP, PKR in OR6 cells. Therefore, we thought that HSP and PKR-independent anti-HCV activity induced by GGA was present in this hepatoma-derived cell line.

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

A

		GGA50µmol/L	
Ly294002 (nmol/L)	-	-	10	-
Rapamycin (µmol/L)	-	-	-	1

Serine2448 phosphorylated mTOR

mTOR

    1   2   3   4

C

		GGA50µmol/L	
Ly294002 (nmol/L)	-	-	10	-
Rapamycin (µmol/L)	-	-	-	1

Threonin-389 phosphorylated p70S6K

p70S6K

    1   2   3   4

B

Relative mTOR activity (y-axis: 0, 50, 100, 150, 200, 250, 300, 350)

GGA (µmol/L)	-	-	-	50	50	50
Ly294002 (nmol/L)	-	10	-	-	10	-
Rapamycin (µmol/L)	-	-	1	-	-	1
	1	2	3	4	5	6

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;

Institute of Immunology, Tokyo), NS5B, and E2 (generous gifts from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma, St. Louis, MO, USA) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

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 rolipram 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 rolipram was estimated. The selective index (SI) value of rolipram was also estimated by dividing the CC_{50} value by the EC_{50} value.

RT-PCR and sequencing

To amplify the genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [7, 15]. Briefly, one fragment covered from 5'-untranslated region to NS3, with a final product of approximately 6.2 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC. PrimScript (Takara Bio) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. The nucleotide sequences of each of the three independent clones obtained were determined using the Big Dye terminator cycle sequencing kit on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Differences between AH1R and OR6 cell lines were tested using Student's t test. P values <0.05 were considered statistically significant.

Results

Development of a luciferase reporter assay system that facilitates the quantitative monitoring of genome-length HCV-AH1 RNA replication

To develop an HCV AH1 strain-derived assay system corresponding to the OR6 assay system [7], a genome-length HCV RNA encoding RL (AH1RN/C-5B/PL,LS,TA,(VA)$_3$)

was transfected into AH1c cells. Following 3 weeks of culturing in the presence of G418, more than 10 colonies were obtained, and then 8 colonies (#2, #3, #4, #5, #6, #8, #13, and #14) were successfully proliferated. We initially selected colonies #2, #3, and #14 because they had high levels of RL activity ($>4 \times 10^6$ U/1.6×10^5 cells) (Fig. 1a). However, RT-PCR and the sequencing analyses revealed that the genome-length HCV-AH1 RNAs obtained from these colonies each had an approximately 1 kb deletion in the E2 region (data not shown). In this regard, we previously observed similar phenomenon and described the difficulty of the development of a luciferase reporter assay system using the genome-length HCV RNA of more than 12 kb [7], suggesting that the NS5B polymerase possesses the limited elongation ability (probably up to a total length of 12 kb). Indeed, in that study, we could overcome this obstacle by the selection of the colony harboring a complete genome-length HCV RNA among the obtained G418-resistant colonies [7]. Therefore, we next carried out the selection among the other colonies. Fortunately, we found that colony #4, showing a rather high level of RL activity (2×10^6 U/1.6×10^5 cells), possessed a complete genome-length HCV-AH1 RNA without any deleted forms, although most of the other colonies possessed some amounts of a deleted form in addition to a complete genome-length HCV-AH1 RNA (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in colony #4 (data not shown). From these results, we finally selected colony #4, and it was thereafter referred to as AH1R and used for the following studies.

We first demonstrated that AH1R cells expressed sufficient levels of HCV proteins (Core, E2, and NS5B) by Western blot analysis for the evaluation of anti-HCV reagents, and the expression levels were almost equivalent to those in OR6 cells (Fig. 1b). In this analysis, we confirmed that the size of the E2 protein in AH1R cells was 7 kDa larger than that in OR6 cells (Fig. 1b), as observed previously [12]. This result indicates that AH1R cells express AH1 strain-derived E2 protein possessing two extra N-glycosylation sites [12]. We next demonstrated good correlations between the levels of RL activity and HCV RNA in AH1R cells (Fig. 1c), as we previously demonstrated in OR6 cells treated with IFN-α for 24 h [7]. These correlations indicate that AH1R cells were as useful as OR6 cells as a luciferase assay system.

Aa substitutions detected in genome-length HCV RNA in AH1R cells

To examine whether or not genome-length HCV RNA in AH1R cells possesses additional conserved mutations such as adaptive mutations, we performed a sequence analysis of HCV RNA in AH1R cells. The results (Fig. 2) revealed that
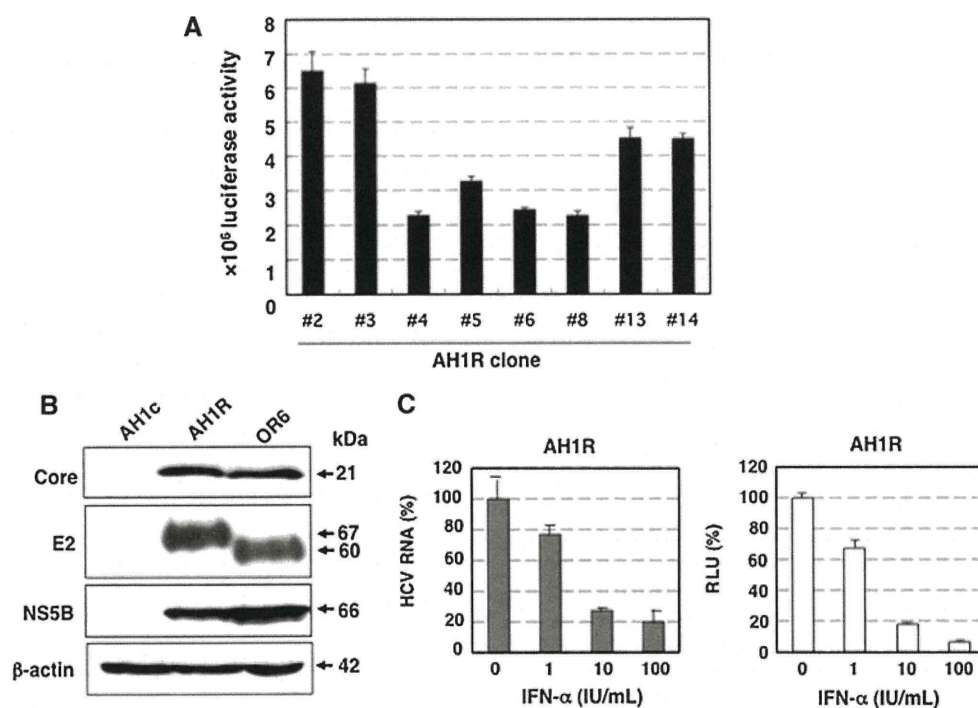
Fig. 1 Characterization of AH1R cells harboring genome-length HCV RNA. **a** Selection of G418-resistant cell clones. The levels of HCV RNA in G418-resistant cells were monitored by RL assay. **b** Western blot analysis. AH1c, AH1R, and OR6 cells were used for the comparison. *Core, E2,* and *NS5B* were detected by Western blot analysis. β-actin was used as a control for the amount of protein loaded per lane. **c** RL activity is correlated with HCV RNA level.

The AH1R cells were treated with IFN-α (0, 1, 10, and 100 IU/mL) for 24 h, and then a luciferase reporter assay (*right panel*) and quantitative RT-PCR (*left panel*) were performed. The relative luciferase activity (RLU) (%) or HCV RNA (%) calculated at each point, when the level of luciferase activity or HCV RNA in non-treated cells was assigned to be 100%, is presented here

two additional mutations accompanying aa substitutions (W860R (NS2) and A1218E (NS3)) were detected commonly among the three independent clones sequenced, suggesting that these additional mutations are required for the efficient replication or stability of genome-length HCV RNA. The P1115L (NS3), L1262S (NS3), V1897A (NS4B), and V2360A (NS5A) mutations derived from the sAH1 replicon [12] were conserved in AH1R cell-derived clones. However, AH1-clone-2-specific mutations (T1338A and V1880A) were almost reverted to the consensus sequences of AH1 RNA [12] except for V1880A in AH1R clone 2 (Fig. 2). In addition, the Q63R (Core) mutation was observed in two of three clones (Fig. 2).

Comparison between the AH1R and OR6 assay systems regarding the sensitivities to IFN-α, IFN-γ, and CsA

Using quantitative RT-PCR analysis, we previously examined the anti-HCV activities of IFN-α, IFN-γ, and CsA in AH1 and O cells, and noticed different anti-HCV profiles of IFN-γ and CsA between AH1 and O cells [12]. In that study, AH1 cells seemed to be more sensitive than the O cells to CsA (significant difference was observed

when 0.063, 0.12, or 0.25 μg/mL of CsA was used). Conversely, AH1 cells seemed to be less sensitive than the O cells to IFN-γ (significant difference was observed when 1 or 10 IU/mL of IFN-γ was used). However, we were not able to determine precisely the EC$_{50}$ values of these reagents, because of the unevenness of the data obtained by RT-PCR.

After developing the AH1R assay system in this study, we determined the EC$_{50}$ values of IFN-α, IFN-γ, and CsA using the AH1R assay and compared the values with those obtained by the OR6 assay. The results revealed that AH1R assay was more sensitive than OR6 assay to IFN-α (EC$_{50}$; 0.31 IU/mL for AH1R, 0.45 IU/mL for OR6) (Fig. 3a) and CsA (EC$_{50}$; 0.11 μg/mL for AH1R, 0.42 μg/mL for OR6) (Fig. 3b), and that the OR6 assay was more sensitive than the AH1R assay to IFN-γ (EC$_{50}$; 0.69 IU/mL for AH1R, 0.28 IU/mL for OR6) (Fig. 3c). Regarding these anti-HCV reagents, the anti-HCV activities observed between the AH1R and OR6 assays differed significantly in all of the concentrations examined (Fig. 3). In addition, regarding these anti-HCV reagents, cell growth was not suppressed within the concentrations used. Regarding IFN-γ and CsA, the present results clearly support those of our previous
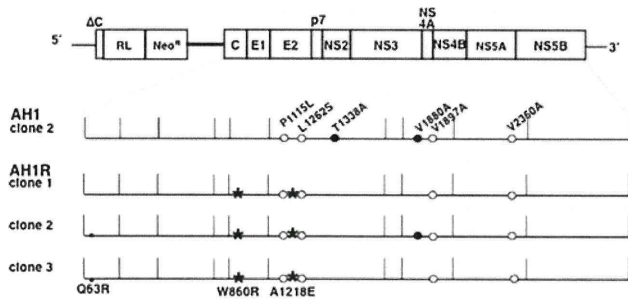
Fig. 2 Aa substitutions detected in intracellular AH1R genome-length HCV RNA. The *upper portion* shows schematic gene organization of genome-length HCV RNA encoding the *RL* gene developed in this study. Genome-length HCV RNA consists of 2 cistrons. In the first cistron, RL is translated as a fusion protein with Neo^R by HCV-IRES, and in the second cistron, all of HCV proteins (C-NS5B) are translated by encephalomyocarditis virus (EMCV)-IRES introduced in the region upstream of C-NS5B regions. Genome-length HCV RNA-replicating cells possess the G418-resistant phenotype because Neo^R is produced by the efficient replication of genome-length HCV RNA. Therefore, when genome-length HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418. In this system, anti-HCV activity is able to evaluate the value of the reporter (RL activity) instead of the quantification of HCV RNA or HCV proteins. In addition, it has been known that the infectious HCV is not produced from this RNA replication system [3, 4, 6]. Core to NS5B regions of three independent clones (*AH1R clones 1–3*) sequenced are presented. W860R and A1218E conserved substitutions are indicated by *asterisks*. Q63R substitutions detected in two of three clones are each indicated by a *small dot*. Core to NS5B regions of *AH1 clone 2*, used to establish the AH1R cell line, are also presented. AH1-specific conserved substitutions and *AH1-clone-2*-specific substitutions are indicated by *open circles* and *black circles*, respectively

study [12]. Therefore, we suggest that the diverse effects of these anti-HCV reagents are due to the difference in HCV strains, although we are not able to completely exclude the possibility that AH1R cells are compromised cells causing the different responses against anti-HCV reagents. In summary, the previous and present findings suggest that the AH1R assay system is also useful for the evaluation of anti-HCV reagents as an independent assay system.
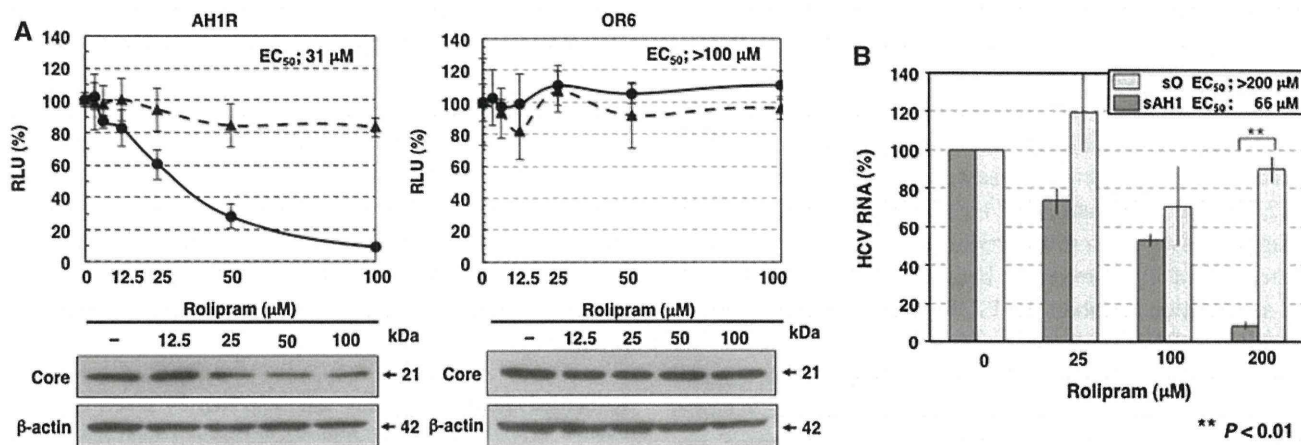
Anti-HCV activity of rolipram was clearly observed in the AH1R assay, but not in the OR6 assay

From the above findings, we supposed that the anti-HCV reagents reported to date might show diverse effects between the drug assay systems derived from the different HCV strains. To test this assumption, we used the AH1R and OR6 assay systems to evaluate the anti-HCV activity of more than 10 pre-existing drugs (6-Azauridine, bisindoly maleimide 1, carvedilol, cephalotaxine, clemizole, 2′-deoxy-5-fluorouridine, esomeprazole, guanazole, hemin, homoharringtonine, methotrexate, nitazoxanide, resveratrol, rolipram, silibinin A, Y27632, etc.), which other groups had evaluated using an assay system derived from the Con1 strain (genotype 1b) or JFH-1 strain (genotype 2a). The results revealed that most of these reagents in the AH1R assay showed similar levels of anti-HCV activities compared with those in the OR6 assay or those of the previous studies (data not shown). However, we found that only rolipram, a selective phosphodiesterase 4 (PDE4) inhibitor [16] that is used as an anti-inflammatory drug, showed moderate anti-HCV activity (EC_{50} 31 μM; $CC_{50} > 200$ μM; SI > 6) in the AH1R assay, but no such activity in the OR6 assay (upper panel in Fig. 4a). This remarkable difference was confirmed by Western blot analysis (lower panel in Fig. 4a). It is unlikely that rolipram's anti-HCV activity is due to the inhibition of exogenous RL, Neo^R or encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), all of which are encoded in the genome-length HCV RNA, because the AH1R and OR6 assay systems possess the same structure of genome-length HCV RNA except for HCV ORF. To demonstrate that rolipram's anti-HCV activity is not due to the clonal specificity of the cells or the specificity of genome-length HCV RNA, we examined the anti-HCV activity of rolipram using the monoclonal HCV replicon RNA-replicating cells (sAH1 cells for AH1 strain [12], and sO cells for O strain [13]). The results

Fig. 3 The diverse effects of anti-HCV reagents on AH1R and OR6 assay systems. AH1R and OR6 cells were treated with anti-HCV reagents for 72 h, and then the RL assay was performed as described in Fig. 1c. **a** Effect of IFN-α. **b** Effect of CsA. **c** Effect of IFN-γ

* $P < 0.05$
** $P < 0.01$
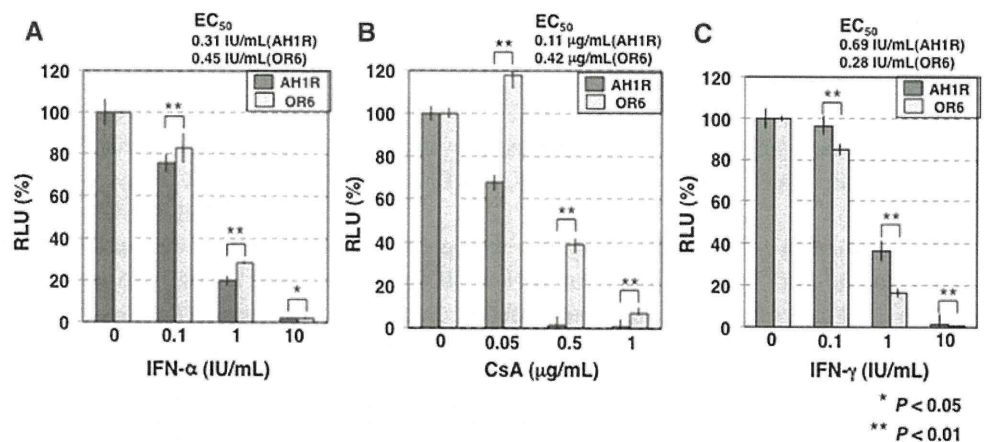
Fig. 4 Anti-HCV activity of rolipram. **a** Rolipram sensitivities on genome-length HCV RNA replication in AH1R and OR6 assay systems. AH1R and OR6 cells were treated with rolipram for 72 h, followed by RL assay (*black circle* with *linear line* in the *upper panels*) and WST-1 assay (*black triangle* with *broken line* in the *upper panels*). The relative value (%) calculated at each point, when the level in non-treated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (*lower panels*). **b** Rolipram sensitivities on HCV replicon RNA replication in sAH1 and sO cells. sAH1 and sO cells were treated with rolipram for 72 h, and extracted total RNAs were subjected to quantitative RT-PCR for HCV 5′ untranslated region as described previously [7]. The HCV RNA (%) calculated at each point, when the level of HCV RNA in non-treated cells was assigned to be 100%, is presented here

revealed by quantitative RT-PCR that rolipram showed moderate anti-HCV activity (EC$_{50}$ 66 μM) in sAH1 cells, but no such activity in sO cells (Fig. 4b). Anti-HCV activity of rolipram in sAH1 cells was a little weaker than that in AH1R cells (Fig. 4b). The similar phenomenon that the anti-HCV activity in genome-length HCV RNA-based reporter assay is stronger than that in HCV subgenomic replicon-based reporter assay was observed regarding other anti-HCV reagents in our previous studies [14, 17, 18]. This result suggests that the anti-HCV activity of rolipram is not either a clone-specific or genome-length HCV RNA-specific phenomenon. In our previous studies also [14, 18], we demonstrated that anti-HCV activities of several reagents including ribavirin and statins were not due to the clonal specificity of the cells. On the other hand, it was recently reported that rolipram did not show anti-HCV activity in the JFH-1 strain-derived assay [19]. Taken together, the previous and present results suggest that rolipram's anti-HCV activity differs depending on the HCV strain. In summary, rolipram was identified as a new anti-HCV candidate using the AH1R assay system.

Discussion

In the present study, we developed for the first time a drug assay system (AH1R), derived from the HCV-AH1 strain (from a patient with acute hepatitis C), in which HCV-AH1

RNA is efficiently replicated. Using this system, we found that rolipram, an anti-inflammatory drug, had potential anti-HCV activity. This potential had not been detected by preexisting assay systems such as OR6, in which HCV-O RNA was derived from an HCV-positive blood donor. Since an HCV replicon harboring the sAH1 cell line, the parent of the AH1R cell line, was obtained from OR6-cured cells [12], the divergence in rolipram's effects between AH1R and OR6 cells is probably attributable to the difference in HCV strains rather than to the difference in cell clones. Indeed, rolipram's anti-HCV activity was not observed in another ORL8 assay system (O strain), which was recently developed using a new hepatoma Li23 cell line (data not shown) [15]. Therefore, we propose that multiple assay systems derived from different HCV strains are required for the discovery of anti-HCV reagents such as rolipram or for the objective evaluation of anti-HCV activity.

Comparative evaluation analysis of anti-HCV activities of IFN-α, IFN-γ, and CsA using AH1-strain-derived AH1R and O-strain-derived OR6 assay systems demonstrated that each of these anti-HCV reagents showed significantly diverse antiviral effects between the two systems. Regarding IFN-γ and CsA, the present results obtained using a luciferase reporter assay fully supported our previous findings [12] using quantitative RT-PCR analysis. However, in the present analysis, we noticed that IFN-α also showed significantly diverse effects (especially at less than 1 IU/mL) between the AH1R and OR6 assays.

The differences in IFN-α sensitivity may be attributable to the difference in aa sequences in the IFN sensitivity-determining region (ISDR; aa 2209-2248 in the HCV-1b genotype), in which aa substitutions correlate well with IFN sensitivity in patients with chronic hepatitis C [20], because the AH1 strain possesses three aa substitutions (T2217A, H2218R, and A2224 V) in ISDR, whereas the O strain possesses no aa substitutions. However, no report has demonstrated the correlation between IFN sensitivity and the substitution numbers in ISDR using the cell culture-based HCV RNA replication system.

Alternatively, Akuta et al. [21] reported that aa substitutions at position 70 and/or position 91 in the HCV Core region of patients infected with the HCV-1b genotype are pretreatment predictors of null virological response (NVR) to pegylated IFN/ribavirin combination therapy. In particular, substitutions of arginine (R) by glutamine (Q) at position 70, and/or leucine (L) by methionine (M) at position 91, were common in NVR. The patients with position-70 substitutions often showed little or no decrease in HCV RNA levels during the early phase of IFN-α treatment [21]. Regarding this point, it is interesting that position 70 in the AH1 strain is R (wild type) and that in the O strain is Q (mutant type), whereas position 91 is L (wild type) in both strains. Therefore, wild-type R in position 70 of the AH1 strain may contribute to the high sensitivity to IFN-α in the AH1R assay. Regarding positions 70 and 91 of the HCV Core, it is noteworthy that, among all of the HCV strains used thus far to develop HCV replicon systems, only the AH1 strain possesses double wild-type aa (data not shown). Therefore, the AH1R assay system may be useful for further study of sensitivity to IFN/ribavirin treatment.

The anti-HCV activity of rolipram, which is currently used as an anti-inflammatory drug, is interesting, although its anti-HCV mechanism is unclear. As a selective PDE4 inhibitor [16], rolipram may attenuate fibroblast activities that can lead to fibrosis and may be particularly effective in the presence of transforming growth factor (TGF)-β1-induced fibroblast stimulation [22]. On the other hand, HCV enhances hepatic fibrosis progression through the generation of reactive oxygen species and the induction of TGF-β1 [23]. Taken together, the previous and present results suggest that rolipram may inhibit both HCV RNA replication and HCV-enhanced hepatic fibrosis. However, it is unclear that rolipram shows anti-HCV activity against the majority of HCV strains, because rolipram has been effective for AH1 strain, but not for O strain. Although rolipram's anti-HCV activity would be HCV-strain-specific, it is not clear which HCV strain is the major type regarding the sensitivity to rolipram. Since developed assay systems using genome-length HCV RNA-replicating cells are limited to several HCV strains including O and AH1

strains to date, further analysis using the assay systems of other HCV strains will be needed to clarify this point.

In this study, we demonstrated that the AH1R assay system, which was for the first time developed using an HCV strain derived from a patient with acute hepatitis C, showed different sensitivities against anti-HCV reagents in comparison with assay systems in current use, such as OR6 assay. Therefore, AH1R assay system would be useful for various HCV studies including the evaluation of anti-HCV reagents and the identification of antiviral targets.

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