in humans. Furthermore, recent reports revealed that the statins were associated with a reduced risk of HCC (8) and lower portal pressure in patients with cirrhosis (9).

Statins targeted the mevalonate pathway. This pathway is branched after farnesyl pyrophosphate (FPP) into cholesterol and geranylgeranyl pyrophosphate (GGPP) biosynthesis pathways. The inhibition of GGPP but not of cholesterol is essential for HCV RNA replication in the inhibitory activity of statins (3, 10, 11). To date, one of the proteins, FBL2, was reported as the host protein essential for HCV RNA replication. HCV RNA replication requires geranylgeranylation of FBL2 by geranylgeranyltransferase with GGPP (12).

We have attempted to examine the effects of geranyl compounds [geranylgeraniol (GGOH), geranylgeranoic acid, vitamin K_2 (VK2) and teprenone] on HCV RNA replication using the OR6 assay system and the JFH-1 infection cell culture system, because their chemical formulas are similar to that of the GGPP, a substrate for geranylgeranyltransferase in geranylgeranylation (13–15). The anti-ulcer agent teprenone (also called geranylgeranylacetone) is reported to block the function of GGPP by the competitive inhibition of the mevalonate pathway (16). Teprenone is the major component of the clinically used anti-ulcer reagent, Selbex.

Here, we reported the inhibitory activity of teprenone on HCV RNA replication and the effect of teprenone in combination with statins on their inhibitory action against geranylgeranylation.

Materials and methods

Reagents and antibodies

Teprenone (Selbex), geranylgeranoic acid, ecabet sodium and sofalcon, gefarnate were purchased from Eisai Co. Ltd (Tokyo, Japan), BIOMOL (Plymouth Meeting, PA, USA), Mitsubishi Tanabe Pharma (Osaka, Japan), Taisho Pharmaceutical Co. (Tokyo, Japan) and Dainippon Sumitomo Pharma Co. Ltd (Osaka, Japan) respectively. GGPP, GGOH, VK2, IFN-α, vitamin E, linoleic acid and mevalonate were purchased from Sigma (St Louis, MO, USA). Cyclosporine A, FLV, LOV and PRV were purchased from Calbiochem (Los Angeles, CA, USA). ATV, SIV and pitavastatin (PTV) were purchased from Astellas Pharma Inc. (Tokyo, Japan), Banyu Pharmaceutical Co. Ltd (Tokyo, Japan), and Kowa Co. Ltd (Nagoya, Japan) respectively.

The antibodies used in this study were those specific to the Core (CP11, Institute of Immunology, Tokyo), NS5A (a generous gift from Dr A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (a generous gift from Dr M. Kohara, Tokyo Metropolitan Institute of Medical Science) and β-actin (Sigma). Anti-heat shock protein (HSP) 90 and anti-HSP70 antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell cultures

OR6 is a cell line cloned from ORN/C-5B/KE RNA-replicating HuH-7 cells as described previously (2) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA, USA). ORN/C-5B/KE RNA is derived from HCV-O, and OR6c cells are cured OR6 cells from which HCV RNA was eliminated by IFN-α treatment as described previously (2). HCV-O/RLGE is the authentic HCV RNA containing adaptive mutations of Q1112R, P1115L, E1203G and K1609E in the NS3 region and replicates efficiently in OR6c cells.

OR6 reporter assay

For the *Renilla* luciferase (RL) assay, $1.0–1.5 \times 10^4$ OR6 cells were plated onto 24-well plates in triplicate and precultured for 24 h. The cells were treated with each compound for 72 h. Then, the cells were harvested and subjected to an RL assay according to the manufacturer's protocol (2).

Western blot analysis

For western blot analysis, $4–4.5 \times 10^4$ OR6 or OR6c cells harbouring HCV-O/RLGE RNA were plated onto six-well plates and cultured for 24 h, and were then treated with each compound for 72 h. Preparation of the cell lysates, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting were then performed as described previously (17).

Cell growth assay

To examine the effect of each reagent on OR6 cell growth, $6.0–6.5 \times 10^4$ OR6 cells were plated onto six-well plates in triplicate and were precultured for 24 h. The cells were treated with or without each compound for 72 h, and then the viable cells were counted after trypan blue dye treatment as described previously (18).

WST-1 cell proliferation assay

The OR6 cells (2×10^3 cells) were plated onto a 96-well plate in triplicate at 24 h before treatment with each reagent. The cells at 24, 48 and 72 h after treatment were subjected to a WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) for HMG-CoA reductase and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a method described previously (19). Briefly, using cellular total RNAs (2 μg), cDNA was synthesized using Superscript II with the oligo dT primer. One-tenth of the synthesized cDNA was subjected to PCR with the

following primer pairs: HMG-CoA reductase, 5′-ATGCC ATCCCTGTTGGAGTG-3′ and 5′-TGTTCATCCCCATG GCATCCC-3′; and GAPDH, 5′-GACTCATGACCACAG TCCATGC-3′ and 5′-GAGGAGACCACCTGGTGCTCA G-3′.

Hepatitis C virus infection experiment

For the infection experiment with the JFH-1 virus, HuH-7-derived RSc cells (1×10^5 cells) were plated onto six-well plates and cultured for 24 h (20). Then, the cells were infected with 100 μl (equivalent to a multiple of infection of 0.1–0.2) of inoculum and cultured for 24 h. The cells were treated with each reagent for 72 h. The culture supernatants and cells were collected for quantification of the Core by an enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan) and for western blot analysis respectively.

Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's *t*-test. *P* values of < 0.05 were considered statistically significant. The mean ± standard deviation is determined from at least three independent experiments.

Results

Anti-hepatitis C virus activity of teprenone is a unique feature not only among geranyl compounds but also among anti-ulcer agents

The mevalonate pathway is divided into two branches: cholesterol synthesis and GGPP synthesis pathways (Fig. 1). The statins exhibited anti-HCV activity via inhibition of geranylgeranylation of host proteins essential for HCV RNA replication. Therefore, we examined the effects of geranyl compounds [GGOH, geranylgeranoic acid, VK2 and teprenone (Selbex)] on HCV RNA replication using the OR6 assay system, because their chemical structures are similar to that of the GGPP (Fig. 2A) (16). Teprenone inhibited HCV RNA replication in a dose-dependent manner without affecting OR6 cell growth up to a concentration of 20 μg/ml (Fig. 2B). The 50% effective concentration (EC_{50}) of teprenone is 5.3 μg/ml. On the other hand, GGOH, geranylgeranoic acid and VK2 did not inhibit HCV RNA replication at the concentration without cytotoxicity (Fig. 2C–E). We also demonstrated that teprenone did not affect cell proliferation within this concentration (supporting information, Fig. S1A). These results suggest that anti-HCV activity of teprenone was not a common feature among geranyl compounds.

Teprenone is used for patients with gastritis and gastric ulcers. Therefore, we examined anti-ulcer agents for their inhibitory effects against HCV RNA replication. The chemical structures of three anti-ulcer agents – ecabet sodium, sofalcon and gefarnate – are shown in supporting information, Figure S1B. None of these agents exhibited inhibitory effects on HCV RNA replication (supporting information, Fig. S1C–E). These results indicate that the anti-HCV activity of teprenone may not be a common feature among anti-ulcer agents.

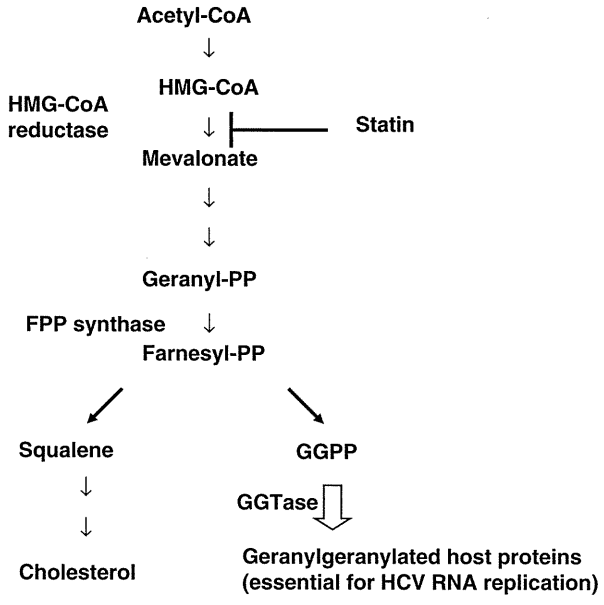

Fig. 1. Schema of the mevalonate pathway.

Teprenone inhibited authentic hepatitis C virus RNA replication

The genome-length HCV RNA replicating in the OR6 cells contained three non-natural elements – RL, neomycin phosphotransferase and encephalomyocarditis virus internal ribosomal entry site. To further confirm that the anti-HCV activity of teprenone was not because of the inhibition of these three exogenous genes or their products, we used authentic 9.6 kb HCV RNA-replicating cells. We introduced *in vitro* synthesized HCV-O/RLGE RNA into cured OR6c cells (Fig. 3A). As shown in Figure 3B, teprenone inhibited Core expression in HCV-O/RLGE-replicating OR6c cells in a dose-dependent manner. These results indicate that the anti-HCV activity of teprenone was because of the inhibition of HCV RNA itself, but not exogenous genes or their products.

Teprenone enhanced anti-hepatitis C virus activity of interferon-α

We examined whether or not teprenone would enhance the anti-HCV activity of IFN-α. We did this by studying the inhibitory effects of combinations of IFN-α (0, 2.5, 5 and 10 IU/ml) and teprenone (0, 10 and 20 μg/ml) using the OR6 assay system. Teprenone enhanced the anti-HCV activity of IFN-α in a dose-dependent manner (Fig. 4). Teprenone with IFN-α also inhibited Core expression (Fig. 4). We also demonstrated that teprenone did not

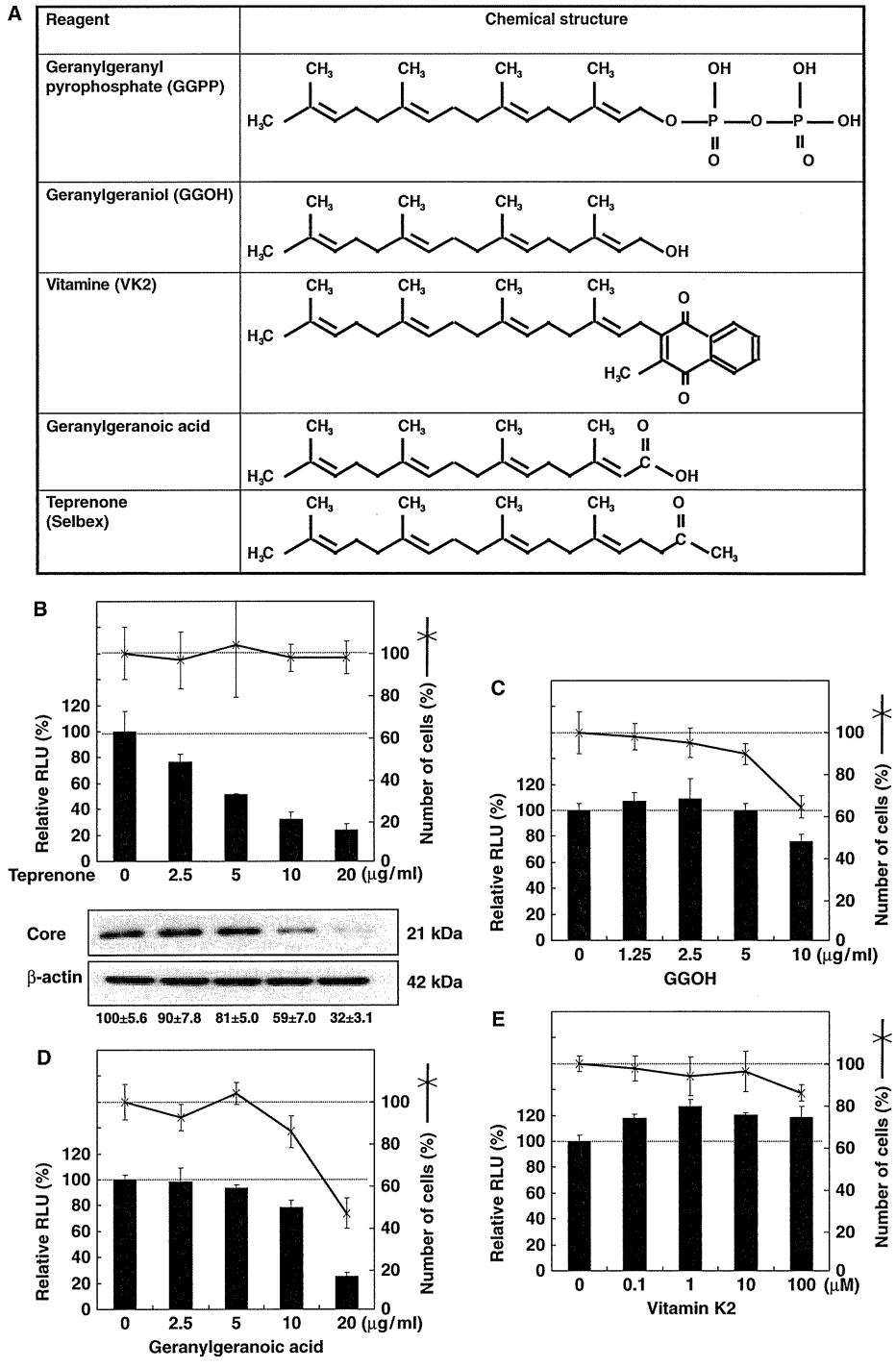

Fig. 2. The effects of geranyl compounds and anti-ulcer agents on hepatitis C virus (HCV) RNA replication. (A) Structures of geranyl compounds. (B) Anti-HCV activity of teprenone on HCV RNA replication in OR6 cells. OR6 cells were treated with teprenone (0, 2.5, 5, 10 and 20 μg/ml) for 72 h. *Renilla* luciferase (RL) activity for HCV RNA replication is shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. Cell viability was also shown as a percentage of control. After 72-h treatment, the production of the Core was analysed by immunoblotting using anti-Core antibody (lower panel). β-actin was used as a control for the amount of protein loaded per lane. The signal intensities of Core from three independent assays were quantified by densitometry and normalized by that of β-actin. Each of the mean ± standard deviation is under the lower panel. (C to E) OR6 cells were treated with geranylgeraniol (0, 1.25, 2.5, 5 and 10 μg/ml) (C), geranylgeranoic acid (0, 2.5, 5, 10 and 20 μg/ml) (D) and VK2 (0, 0.1, 1, 10 and 100 μM) (E) for 72 h. RL activity and cell viability after treatment were determined as shown in (B).

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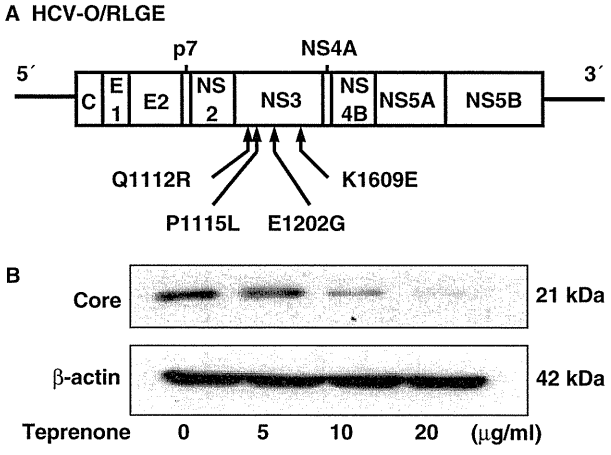

Fig. 3. Teprenone inhibited authentic hepatitis C virus (HCV) RNA replication. (A) Schematic gene organization of genome-length HCV-O/RLGE RNA. The positions of four adaptive mutations – Q1112R, P1115L, E1203G and K1609E – are indicated by arrows. (B) HCV-O/RLGE RNA was introduced into OR6c cells by electroporation as described previously (5). The cells were treated with teprenone (0, 5, 10 and 20 μg/ml) for 72 h and then the production of the Core was analysed by immunoblotting using anti-Core antibody.

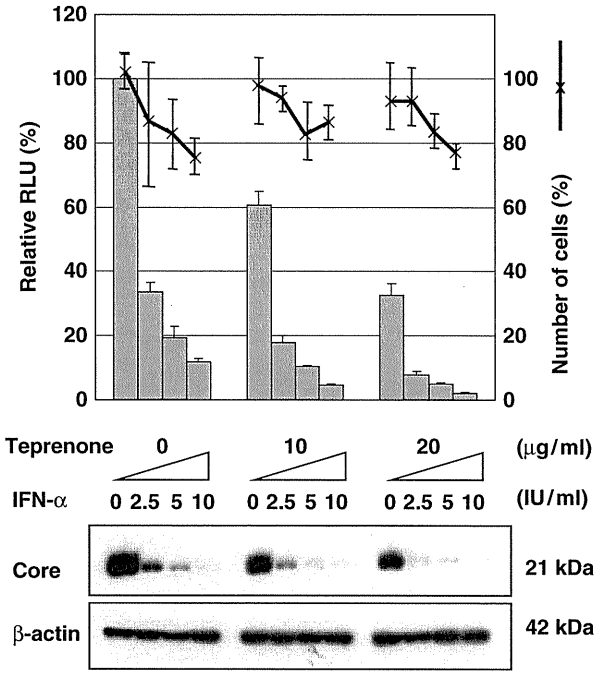

Fig. 4. Teprenone enhanced the anti-hepatitis C virus activity of interferon (IFN)-α. OR6 cells were cotreated with IFN-α (0, 2.5, 5 and 10 IU/ml) and teprenone (0, 10 and 20 μg/ml) for 72 h. *Renilla* luciferase assay was performed as described in Figure 2B. Production of the Core was analysed by immunoblotting using anti-Core antibody. The cells at 24, 48 and 72 h after treatment were subjected to a WST-1 cell proliferation assay.

affect cell proliferation within this concentration (Fig. 4). These results suggest that teprenone may be a new candidate as a complement to IFN therapy.

Teprenone exhibited anti-hepatitis C virus activity in the JFH-1 infection system

We examined the anti-HCV activity of teprenone in the JFH-1 infection system (13–15). We treated the cells with teprenone (0, 5, 10 and 20 μg/ml) at 24-h post-JFH-1 infection and cultured them for 72 h. The culture supernatants and cells were subjected to quantification of the Core by ELISA and western blot analysis respectively. Teprenone decreased the HCV Core in the supernatant (upper panel in Fig. 5A) and in the cells (lower panel in Fig. 5A) in a dose-dependent manner.

We next tested whether or not teprenone (0, 10 and 20 μg/ml) enhanced IFN-α's (0, 2.5 and 5 IU/ml) anti-HCV activity in the JFH-1 infection system. As shown in Figure 5B, teprenone enhanced the anti-HCV activity of IFN-α in a dose-dependent manner. These results suggest that teprenone also possessed anti-HCV activity in the JFH-1 infection system.

Teprenone did not inhibit geranylgeranylation

As shown in Figure 2A, the chemical structure of teprenone is similar to that of GGPP. Therefore, we examined the possibility that teprenone inhibits geranylgeranylation. Geranylgeranyl proteins possessed the C-A-A-X motif at the C-terminal of the protein: C is cystein; A is aliphatic amino acid; and X is typically leucine (or rarely isoleucine, valine or phenylalanine). Rap1A is one of the Ras-related proteins and selected to monitor the status of geranylgeranylation. We used anti-Rap1A antibody (sc-1482), which recognized only nongeranylgeranylated Rap1A (21, 22). Therefore, geranylgeranylated Rap1A is not recognized with this antibody. On the other hand, anti-Rap1 antibody (sc-65) recognizes Rap1A and Rap1B independent of the state of geranylgeranylation (22). In the following experiments, we used anti-Rap1A antibody (sc-1482) to monitor the state of geranylgeranylation.

OR6 cells were treated with PTV (1.25 μM) or teprenone (20 μg/ml) or neither. The cells were collected after treatment and subjected to luciferase assay and western blot analysis. In the untreated cells, nongeranylgeranylated Rap1A bands were not detected (Fig. 6A). PTV inhibited geranylgeranylation at 3 h and reached a plateau 12 h after treatment along with nongeranylgeranylated Rap1A bands (Fig. 6A). On the other hand, geranylgeranylation was not inhibited in the cells with teprenone treatment (Fig. 6A).

We then tested the effect of mevalonate cotreatment with PTV or teprenone. Mevalonate negated PTV's inhibitory action against geranylgeranylation and led to the loss of PTV's anti-HCV activity (Fig. 6B). However, mevalonate did not affect the anti-HCV activity of teprenone (Fig. 6B). These results indicate that teprenone

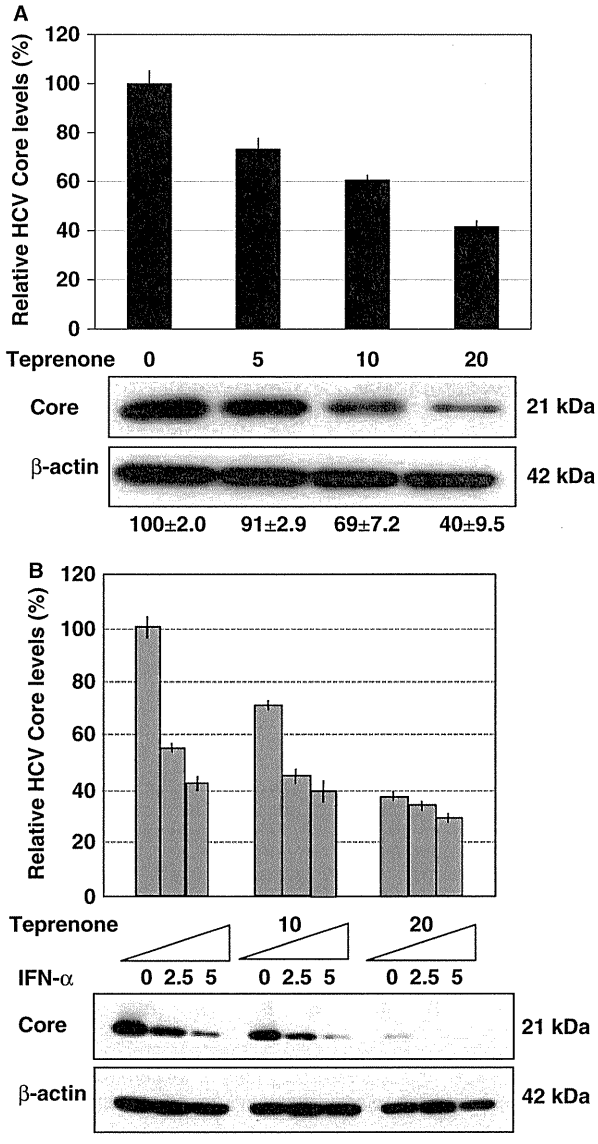

Fig. 5. Teprenone exhibited anti-hepatitis C virus (HCV) activity in the JFH-1 infection system. (A) Teprenone inhibited JFH-1 replication. HuH-7-derived RSc cells were infected with the JFH-1 virus for 24 h and were then treated with teprenone (0, 5, 10 and 20 μg/ml) for 72 h. The supernatant and the cells were subjected to quantification of the Core by ELISA and western blot analysis respectively. The signal intensities of Core were quantified by densitometry and the mean ± standard deviation is under the lower panel as shown in Figure 2B. (B) Teprenone enhanced interferon (IFN)-α's anti-HCV activity in the JFH-1 infection system. JFH-1 virus-infected cells were treated with teprenone (0, 10 and 20 μg/ml) and IFN-α (0, 2.5 and 5 IU/ml) for 72 h and then subjected to Core quantification by ELISA and western blot analysis as shown in (A).

inhibits HCV RNA replication without the inhibition of geranylgeranylation.

Statin's inhibition of HMG-CoA reductase decreased cholesterol synthesis and led to the increase of HMG-CoA reductase expression by positive feedback (3). The mRNA of HMG-CoA reductase was increased with PTV treatment but not with teprenone treatment (supporting information, Fig. S3A and B). This result suggests that teprenone, unlike PTV, did not lower the cholesterol synthesis.

The chemical structure of teprenone, which is the major component of Selbex, is similar to that of GGPP, a substrate for geranylgeranyltransferase. Therefore, we ruled out the possibility that teprenone was incorporated into host proteins instead of GGPP and led to the loss of function of the host proteins, when endogenous GGPP was depleted by PTV in OR6 cells. The nongeranylgeranylated Rap1A was detected when OR6 cells were treated with PTV (lane 3; Fig. 6C). However, exogenous GGPP decreased nongeranylgeranylated Rap1A in PTV-treated OR6 cells (lane 4; Fig. 6C). If teprenone was incorporated into Rap1A instead of GGPP and formed a psuedo-geranylgeranylation, Rap1A blotted with anti-Rap1A (sc-1482) would be decreased. Surprisingly, non-geranylgeranylated Rap1A increased in OR6 cells after treatment with PTV and teprenone (compare lanes 3 and 7 in Fig. 6C). Furthermore, it is noteworthy that the total amount of Rap1 was decreased when OR6 cells were treated with PTV and teprenone. These results suggest that teprenone was not incorporated into host protein and unexpectedly enhanced the statin's inhibitory action against geranylgeranylation.

Teprenone enhanced statins' inhibitory action against geranylgeranylation

To further investigate the unexpected results shown in Figure 6C, we tested the geranylgeranyl state and anti-HCV activity using the OR6 assay system. OR6 cells were treated with teprenone (0, 10 and 20 μg/ml) in combination with PTV (0, 0.25, 0.5 and 1.0 μM) for 72 h and subjected to western blot analysis for the geranylgeranyl state using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies, and for anti-HCV activity using anti-Core, anti-NS5A and anti-NS5B antibodies. Anti-HCV activity was also assessed by a luciferase reporter assay. Teprenone by itself did not inhibit geranylgeranylation (lanes 1–3; Fig. 7A). When teprenone was treated with PTV (0.25 μM), nongeranylgeranylated Rap1A increased in a dose-dependent manner (lanes 4–6; Fig. 7A). This result indicates that teprenone enhanced PTV's inhibitory action against geranylgeranylation in a dose-dependent manner. This effect of teprenone was also confirmed when PTV was treated at concentrations of 0.5 and 1.0 μM (lanes 7–12; Fig. 7A). HCV RNA replication and the expression of HCV proteins were decreased when nongeranylgeranylated Rap1As were increased. Next, we examined whether or not this function of teprenone is a common feature against statins. Teprenone enhanced the inhibitory action of ATV, SIV, FLV and LOV but not PRV against geranylgeranylation (lower panel in Fig. 7B). Teprenone also enhanced anti-HCV activity in combination with statins (upper panel in Fig. 7B). These results

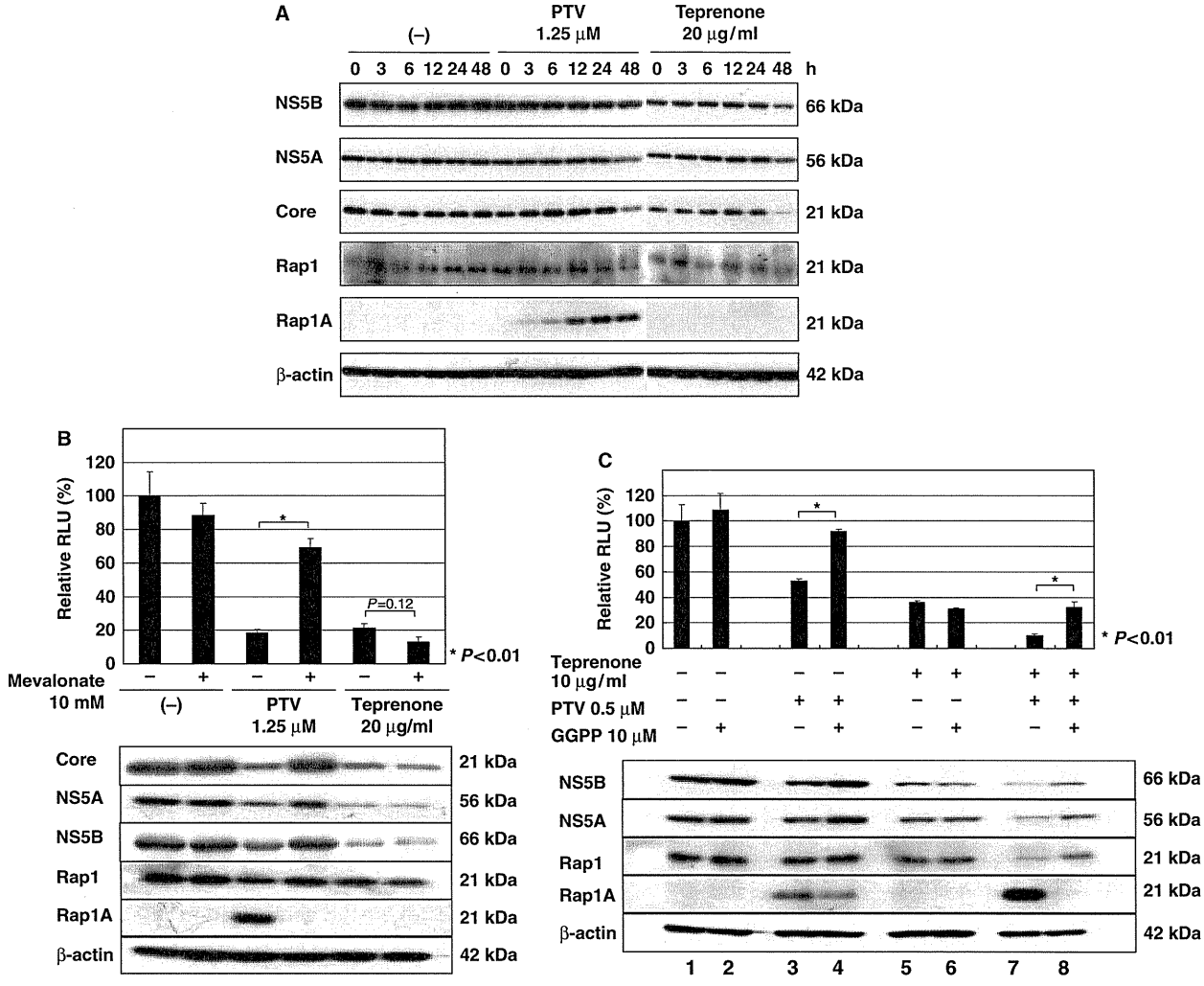

Fig. 6. Teprenone did not inhibit geranylgeranylation. (A) Teprenone did not inhibit geranylgeranylation. OR6 cells were treated with pitavastatin (PTV) (1.25 μM) or teprenone (20 μg/ml), or neither for 0, 3, 6, 12, 24 and 48 h. The cells were subjected to western blot analysis for HCV proteins using anti-NS5B, anti-NS5A and anti-Core antibodies, and for geranylgeranylation assay using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies. (B) Mevalonate did not affect the anti-HCV activity of teprenone. OR6 cells were treated with PTV (1.25 μM), teprenone (20 μg/ml) or neither in the absence or in the presence of mevalonate (10 mM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-Core, anti-NS5A, anti-NS5B, anti-Rap1A (sc-1482), anti-Rap1 (sc-65) and anti-β-actin antibodies (lower panel), as shown in (A). (C) Teprenone was not used as a substrate for GGT after the depletion of geranylgeranyl pyrophosphate (GGPP) by statin. OR6 cells were treated with teprenone (0 and 10 μg/ml), PTV (0 and 0.5 μM) and GGPP (0 and 10 μM) in the indicated combination for 72 h. Then the cells were subjected to luciferase assay (upper panel) and geranylgeranyl assay using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies (lower panel) as shown in (A).

suggest that teprenone enhances statins' inhibitory action against geranylgeranylation, except for PRV.

Discussion

In this study, we demonstrated that teprenone inhibited HCV RNA replication. Furthermore, teprenone exhibited anti-HCV activity in the genotype-2a JFH-1 infection system. Teprenone belongs to the geranyl compounds from its chemical structure and anti-ulcer agent from its clinical application. Therefore, we tested other geranyl compounds (GGOH and VK2, as well as geranylgeranoic acid) and other anti-ulcer agents (ecabet sodium, sofalcone and gefarnate) for their effect on HCV RNA replication. However, only teprenone exhibited anti-HCV activity among the reagents tested. Therefore, the anti-HCV activity of teprenone is a unique feature among these reagents.

The interview form from Selbex providing company Eisai reported the plasma concentration of teprenone. When 150 mg of Selbex was administered orally, its maximum plasma concentration reached 2.2 μg/ml. This is similar to the EC_{50} (5.3 μg/ml) of Selbex *in vitro*.

Ichikawa *et al.* (23) reported that teprenone induced the 2′,5′-oligoadenylate synthetases (2′5′-OAS) in

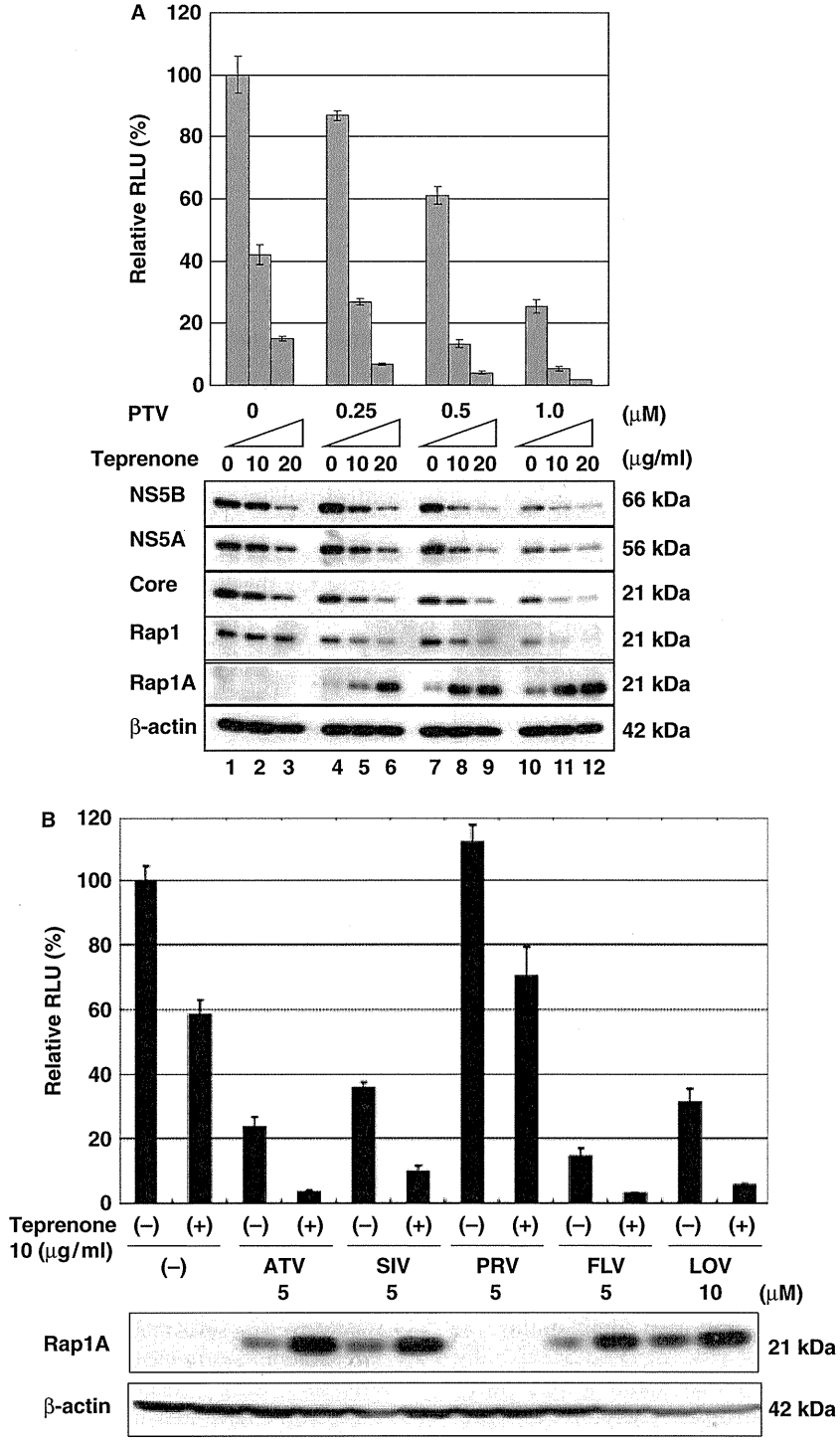

Fig. 7. Teprenone enhanced statins' inhibitory action against geranylgeranylation. (A) Teprenone enhanced pitavastatin (PTV)'s inhibitory action against geranylgeranylation. OR6 cells were treated with teprenone (0, 10 and 20 μg/ml) and PTV (0, 0.25, 0.5 and 1.0 μM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-NS5A, anti-Rap1A (sc-1482) and anti-Rap1 (sc-65), and anti-β-actin antibodies (lower panel), as shown in Figure 6A. (B) Teprenone enhanced statins' [except for pravastatin (PRV)] inhibitory action against geranylgeranylation. OR6 cells were treated with teprenone (0, 10 μg/ml) and atorvastatin (0, 5 μM), simvastatin (0, 5 μM), PRV (0, 5 μM), fluvastatin (0, 5 μM) and lovastatin (0, 10 μM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-Rap1A (sc-1482), and anti-β-actin antibodies (lower panel), as shown in Figure 6A.

human hepatoma cells. We demonstrated the activation of 2′5′-OAS and IFN-stimulated response element (ISRE) by IFN-α using the reporter assay system in our HuH-7-derived OR6 cells. However, we could not obtain evidence that teprenone activated both 2′5′-OAS and ISRE promoters (supporting information, Fig. S2A and B). Signal transducer and activator of transcription (STAT)1 and STAT2 were not phosphorylated after treatment with teprenone (supporting information, Fig. S2C). This discrepancy may have been caused by the heterogeneity of HuH-7 cells, because OR6 was selected as the clonal cell line and is highly susceptible to HCV RNA replication. Further study is needed to clarify the mechanism underlying teprenone's effect on IFN signalling.

Teprenone reportedly protects the gastric mucosa by inducing HSP (24). From this standpoint, the anti-HCV activity of teprenone was an unexpected result, because recently, it was reported that HSP90 is essential for HCV RNA replication and that an HSP90 inhibitor, geldanamycin, inhibits HCV RNA replication (25, 26). We examined whether or not teprenone induced HSP90 in hepatoma cells and found that it did not (supporting information, Fig. S4).

In this study, we monitored the geranylgeranylated state of Rap1A as a marker using nongeranylgeranylated Rap1A-detectable anti-Rap1A antibody (sc-1482). The least expected result of this sensitive geranylgeranylation assay is that teprenone enhanced statins' inhibitory action against geranylgeranylation. It is not clear in this study as to why teprenone enhanced statins' inhibitory action on geranylgeranylation. One possibility is that teprenone may cause biosynthesis from FPP to cholesterol rather than to GGPP by an unknown mechanism. To clarify this point, further study will be needed. This new function of teprenone may contribute to not only the antiviral field but also other fields, including studies on osteoporosis and on various kinds of antitumours, because geranylgeranylation and farnesylation are targets of the reagent in these fields. For example, statins interfere with the production of GGPP and FPP, which is important in the activation of small G proteins, such as K-ras and the Rho family, and disrupt the growth of malignant cells.

Recently, two important findings have been reported. Firstly, El-Serag *et al.* (8) reported that statins are associated with a reduced risk of HCC. Secondly, Abraldes *et al.* (9) reported that statin lowers portal pressure in patients with cirrhosis. Therefore, as teprenone is a strong adjuvant to statin's inhibitory action against geranylgeranylation, it may further improve portal hypertension in cirrhosis and reduce the risk of HCC in combination with statins. Although teprenone alone possesses modest anti-HCV activity, it will play a significant role in combination with IFN and/or statins in the therapy to HCV-associated liver diseases as an adjuvant like ribavirin. As teprenone is available in clinical use with a low side effect, a clinical study using teprenone in combination with IFN-α and/or statins is now underway in our institution.

In conclusion, we have shown that the anti-ulcer agent teprenone inhibited HCV RNA replication and enhanced statins' inhibitory action against geranylgeranylation. This newly discovered function of teprenone may contribute to improve the treatment of HCV-associated liver diseases (CH C, cirrhosis and HCC) as an adjuvant to statins.

Acknowledgements

The authors would like to thank Atsumi Morishita, Takashi Nakamura and Midori Takeda for their technical assistance. This work was supported by grants-in-aid for a third-term comprehensive 10-year strategy for cancer control and for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan. K. A. and K. M. were supported by a Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. The effects of anti-ulcer agents on HCV RNA replication. (A) Cell proliferation assay. OR6 cells were treated with teprenone (0, 2.5, 5, 10, and 20 μg/ml), and the cells at 24, 48, and 72 hours after treatment were subjected to WST-1 cell proliferation assay. (B) Structures of ant-ulcer agents. (C-E) OR6 cells were treated with ecabet sodium (0, 2.5, 5, 10, 20 μg/ml) (C), sofalcon (0, 2.5, 5, 10, 20 μg/ml) (D), and gefarnate (0, 2.5, 5, 10, 20 μg/ml) (E) for 72 hours. Then the cells were subjected to luciferase assay (upper panel) and Western blot analysis using anti-core, and anti-β-actin antibodies (lower panel) as shown in Figure 1B.

Fig. S2. Teprenone didn't activate IFN signaling pathway. (A and B) Luciferase assays for 2'5'OAS and ISRE promoters. p2'5'OAS-luc (A) and pISRE-luc (B) transfected OR6c cells were treated with teprenone (0, 2.5, 5, and 10 μg/ml) or IFN-α (0, 2.5, 5, and 10 IU/ml) for 6 hours and then subjected to luciferase reporter assay. (C) Teprenone didn't activate STATs in OR6 cells. OR6 cells were treated with IFN-α (500 IU/ml), PTV (1.25 μM), and teprenone (20 μg/ml) for 0, 3, 6, and 12 hours. Then the cells were subjected to Western blot analysis using anti-pSTAT1 (Tyr701), anti-STAT1, anti-pSTAT2 (Tyr689), anti-core, and anti-β-actin antibodies.

Fig. S3. Teprenone treatment didn't cause positive feedback of HMG-CoA reductase (HMGCR). OR6c cells were treated with teprenone (20 μg/ml), PTV (10 μmol/L), or neither for 24 hours. The cells were subjected to RT-PCR (A) and real-time RT-quantitative PCR (B) using HMG-CoA reductase-specific primer set. H_2O was used as a negative control. GAPDH was used as an internal control.

Fig. S4. Teprenone didn't induce HSP90 or HSP70 in HuH-7 cells. OR6 cells were treated with teprenone (20 μg/ml) for 0, 3, 6, 12, 24, and 48 hours. Then the cells were subjected to Western blot analysis using anti-HSP90, anti-HSP70 anti-core, and anti-β-actin antibodies.

Biochemical and Biophysical Research Communications 409 (2011) 663–668

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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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ARTICLE INFO

Article history:
Received 8 April 2011
Available online 17 May 2011

Keywords:
HCV
HCV RNA replication system
Li23 cells
Reporter assay for anti-HCV reagents

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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doi:10.1016/j.bbrc.2011.05.061

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

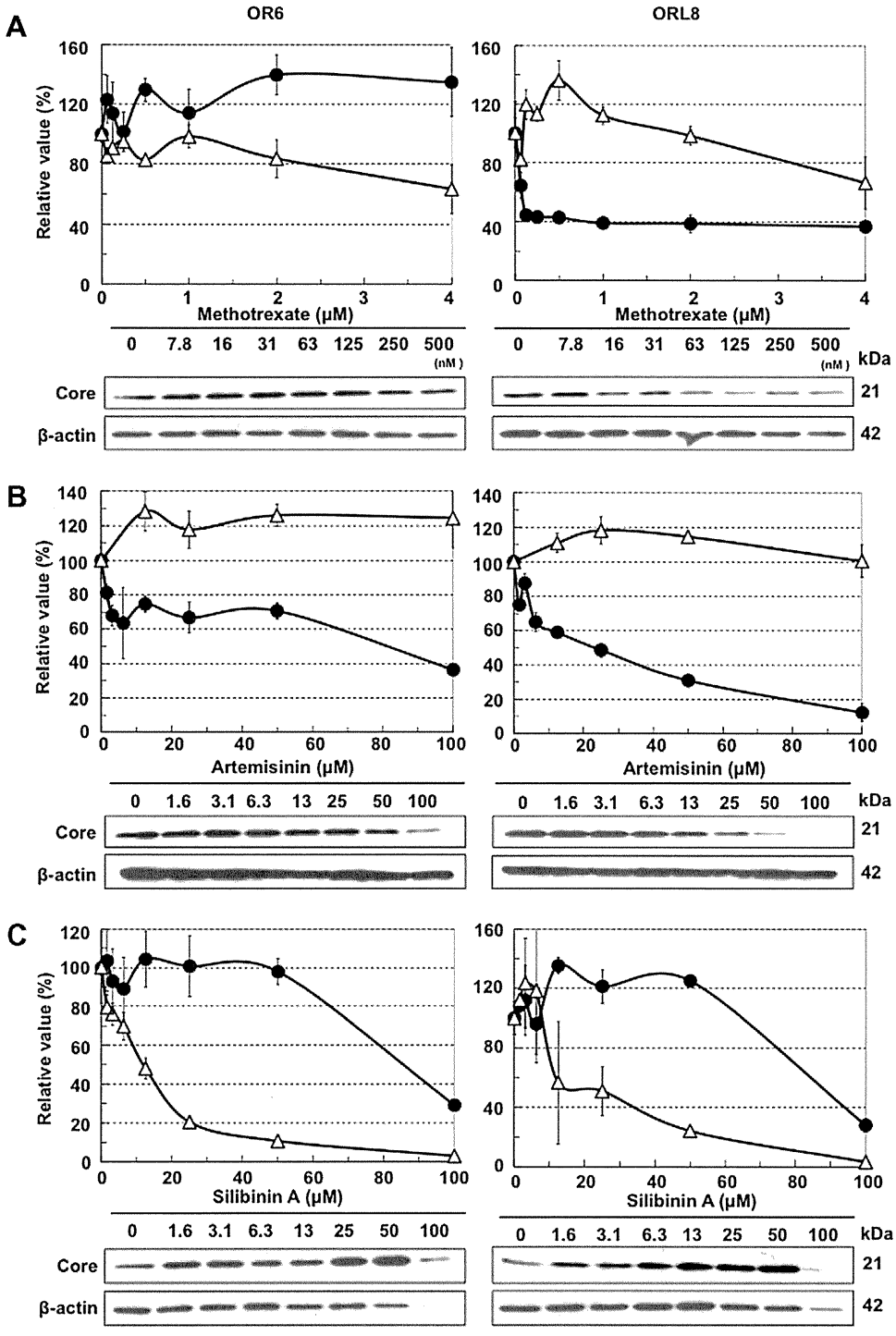

Fig. 1. Anti-HCV profiles of representative reagents in the OR6 and ORL8 assay systems. (A) Methotrexate sensitivities on genome-length HCV RNA replication in the OR6 and ORL8 assay systems. OR6 and ORL8 cells were treated with methotrexate for 72 h, followed by RL assay (black circle in the upper panel) 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.

Acknowledgments

We thank Yusuke Wataya and Hye-Sook Kim for their helpful discussions. This work was supported by grants-in-aid for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan. K. M. was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science.

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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J Gastroenterol
DOI 10.1007/s00535-011-0481-z

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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Received: 27 July 2010 / Accepted: 29 August 2011

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

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

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Published online: 25 October 2011

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).

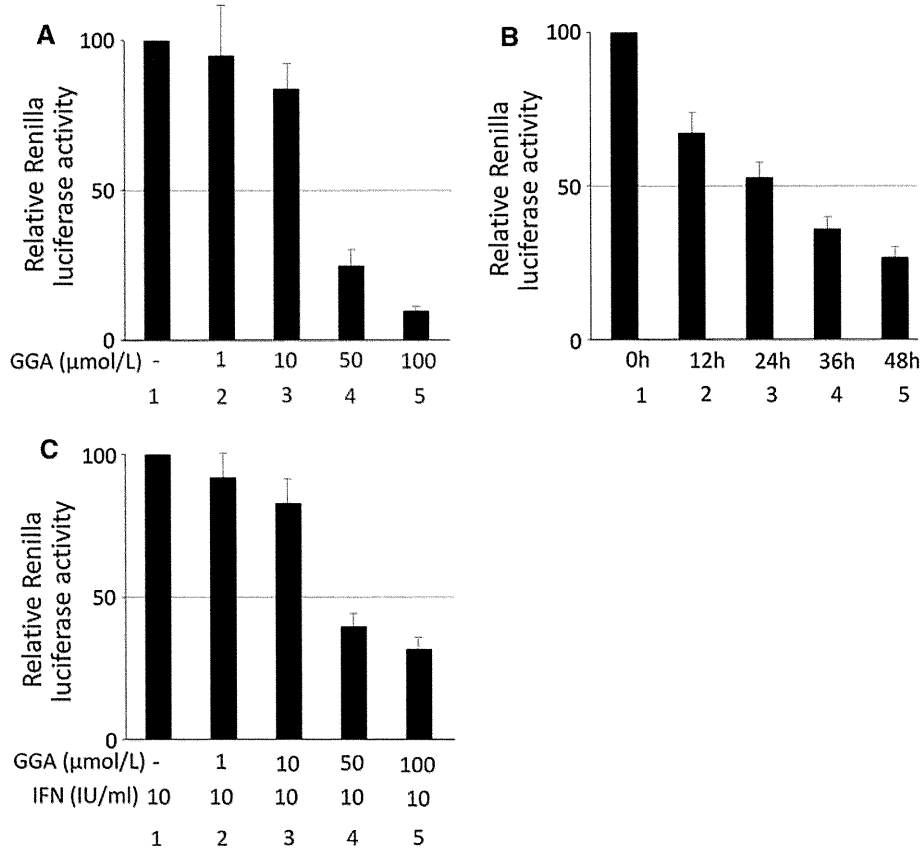

Fig. 1 The effect of GGA on the genome-length HCV RNA replication system. **a** Dose dependent effect of GGA. **b** Time course of GGA suppressed HCV replication. **c** The additive effect of GGA with IFN-α suppressed HCV replication. **a** The OR6 cells were treated with 1–100 μmol/L of GGA (*lanes 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

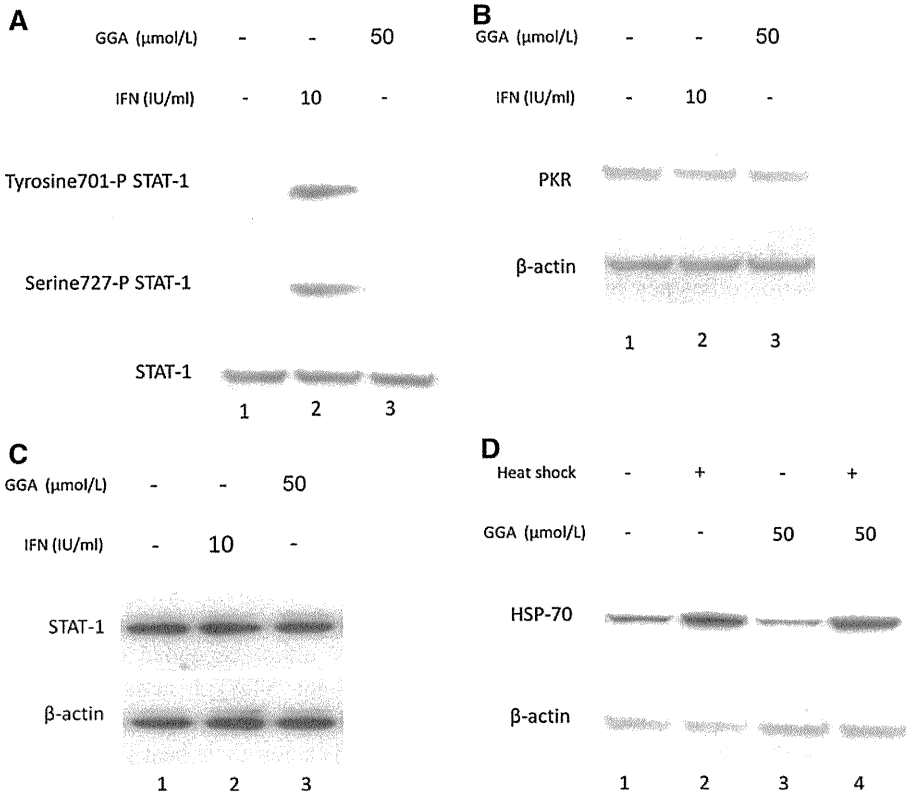

Fig. 2 Effect of GGA on STAT-1 (**a**), PKR (**b**) and HSP-70 (**c**). **a** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*) and then were phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and 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

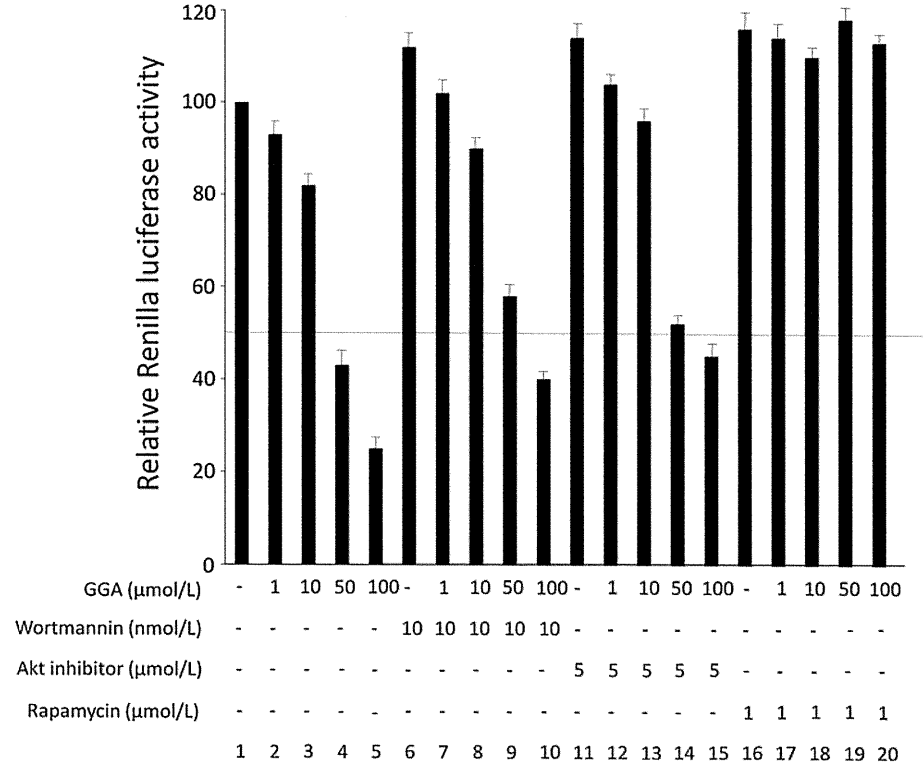

Fig. 3 Changes in GGA suppressed HCV replication by rapamycin, but not PI3-K inhibitor and Akt inhibitor. OR6 cells were treated with 1–100 μmol/L of GGA in the absence (*lanes 2–5*) or presence of pretreatment (*lanes 7–10, 12–15, 17–20*) for 3 h. *Lanes 1, 6, 11* and *16* were not treated with GGA. *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