individual. However, treatments were terminated irrespective of serum ferritin levels when blood hemoglobin concentrations decreased to less than 10 g/dL.

Statistical analysis

The relationships between variables were analyzed using the Spearman's correlation coefficient by rank, and a partial correlation coefficient was calculated to remove the influence of confounding variables. Values after phlebotomy were compared with baseline values using a Wilcoxon rank sum test. All analyses were performed using SPSS software for Windows, version 14.0 (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 was considered significant.

RESULTS

General characteristics of and histological findings in patients

OF THE 309 HCV-infected patients who underwent liver biopsies, 203 patients met the criteria. Because many excessive drinkers among the male patients were excluded from the analysis, the number of females exceeded that of males in the study population. A summary of the clinical data for the liver biopsy findings in these patients is shown in Tables 1 and 2. Of the 203 patients that qualified, body mass index (BMI) was greater than 25 (kg/m^2) in 57 patients (28%), and 124 patients (61%) had a varying degree of hepatic steatosis, as shown in Table 2. Iron staining was performed in only 198 patients; a varying degree of iron loading was observed in 71 patients. Fourteen patients (7%) suffered from type 2 diabetes mellitus. The fibrosis scores of these patients were F1 in two patients, F2 in six, F3 in five, and F4 in only one. Seventy patients had received IFN-based antiviral therapy before the study and this treatment had failed to eradicate HCV.

Table 1 Baseline characteristics of patients

	Mean values of clinical data
Age	56.0 ± 11.9
Male/Female	73/146
BMI (kg/m^2)	22.9 ± 3.1
IFN: yes/no	70/133
ALT (IU/L)	75.5 ± 59.3
γ-GTP (U/L)	56.0 ± 51.0
FPG (mg/dl)	96.8 ± 13.1
HOMA-IR	2.3 ± 1.4
Ferritin (ng/ml)	174.2 ± 161.0
TG (mg/dl)	99.5 ± 50.5
Plt (×10^4/ml)	17.4 ± 5.3
HCV-RNA (KIU/ml)	1516 ± 1484.7
Serogroup 1/2	162/41
Trx (ng/ml)	30.4 ± 15.4

Data are expressed as mean ± standard deviation.
ALT, alanine aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; γ-GTP, γ-glutamylcysteine transpeptidases; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance; IFN, interferon; Plt, platelet; TG, triglyceride; Trx, thioredoxin.

Table 2 Histological findings on liver biopsy

	No. patients
F0/F1/F2/F3/F4	3/72/71/51/6
A0/A1/A2/A3	2/79/89/33
Steatosis:	
None	79
<10%	54
<30%	53
<60%	17
Iron load:	
Grade 0/1/2/3	127/33/25/13

Data are expressed as number of patients.

Predictors of the fibrosis score

The stage was significantly correlated with age, BMI, grade, grades of steatosis, iron score, ALT levels, platelet counts, ferritin levels, HOMA-IR, and serum Trx levels (Table 3). In a multiple regression analysis, grade, HOMA-IR, and serum Trx levels were shown to be

Table 3 Variables correlated with fibrosis scores

	Coefficient	Univariate	Multivariate
Age	$r = 0.163$	$P = 0.019$	$P = 0.931$
BMI	$r = 0.199$	$P = 0.004$	$P = 0.920$
Grade	$r = 0.869$	$P < 0.00001$	$P < 0.00001$
Steatosis	$r = 0.412$	$P < 0.00001$	$P = 0.761$
Iron score	$r = 0.155$	$P = 0.030$	$P = 0.437$
ALT	$r = 0.416$	$P < 0.00001$	$P = 0.259$
Plt	$r = -0.376$	$P < 0.00001$	$P = 0.119$
Ferritin	$r = 0.189$	$P = 0.010$	$P = 0.227$
HOMA-IR	$r = 0.406$	$P < 0.00001$	$P = 0.043$
Trx	$r = 0.365$	$P = 0.00006$	$P = 0.003$

Multiple regression analysis was used to analyze variables independently correlated with fibrosis scores.
ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; Plt, platelet; Trx, thioredoxin.

independently correlated with stage (Table 3). Although the grade of steatosis is reported to predict rapid fibrosis progression,[5] it was not an independent variable in the multivariate analysis. Considering that IR is a major cause of hepatic steatosis,[11] HOMA–IR should be more significant than steatosis in this model.

Relationship between grades of steatosis and HOMA–IR or serum Trx levels

Steatosis has been considered to independently contribute to the progression of fibrosis in patients with chronic hepatitis C.[5] Therefore, we focused on the relationships between steatosis and either IR or oxidative stress. We found that grades of steatosis were significantly correlated not only with HOMA–IR, but also with serum Trx levels (HOMA–IR; $r = 0.344$, $P = 0.00002$: Trx; $r = 0.3$, $P < 0.001$). These findings suggested that oxidative stress could have a significant role in fibrosis progression through steatogenesis. We then focused on the relationship between IR and oxidative stress.

Relationship between HOMA–IR and serum Trx levels

HOMA–IR was significantly correlated with serum Trx levels (Fig. 1a: $r = 0.262$, $P = 0.012$) and BMI (Fig. 1b: $r = 0.302$, $P = 0.0002$). After adjustment for the effect of each variable using a corrected correlation coefficient, a significant relationship with HOMA–IR still remained for both serum Trx levels ($r = 0.244$, $P = 0.02$) and BMI ($r = 0.284$, $P = 0.006$). These results indicated that IR was attributable to oxidative stress, irrespective of obesity.

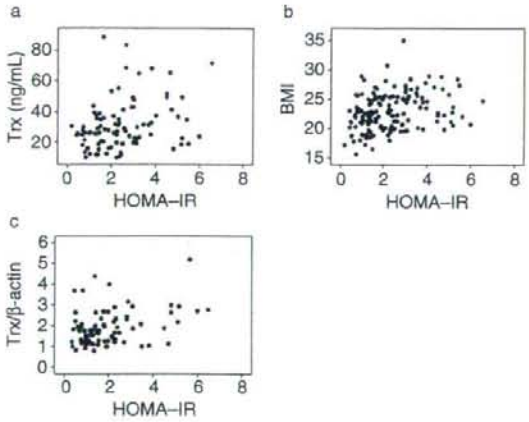

Figure 1 Correlation between homeostasis model assessment–insulin resistance (HOMA–IR) and serum levels of thioredoxin (Trx) (a), body mass index (BMI) (b), and mRNA levels of Trx (c). Both serum Trx levels and BMI were significantly correlated with HOMA–IR (serum Trx levels; $r = 0.262$, $P = 0.012$: BMI; $r = 0.302$, $P = 0.0002$). HOMA–IR was also significantly correlated with hepatic Trx levels ($r = 0.273$, $P = 0.014$).

Table 4 Factors correlated with HOMA–IR in subgroup patients ($n = 101$)

	Coefficient	Univariate	Multivariate
Hepatic Trx	$r = 0.273$	$P = 0.014$	$P = 0.011$
Grade	$r = 0.233$	$P = 0.038$	$P = 0.170$
Steatosis	$r = 0.286$	$P = 0.010$	$P = 0.251$
ALT	$r = 0.287$	$r = 0.010$	$r = 0.517$

Multiple regression analysis was used to analyze variables independently correlated with HOMA-IR.
ALT, alanine aminotransferase; HOMA–IR, homeostasis model assessment–insulin resistance; Trx, thioredoxin.

Relationship between HOMA–IR and hepatic Trx levels

Since Trx is known to be ubiquitously expressed,[17] we compared the mRNA levels of hepatic Trx with HOMA–IR in 101 patients whose liver biopsy specimens were available. The mRNA levels of Trx were significantly correlated with HOMA–IR (Fig. 1c: $r = 0.273$, $P = 0.014$). Among these patients, HOMA–IR also significantly correlated with grade, steatosis, and ALT levels (Table 4). In a multiple regression analysis, only the level of hepatic Trx was independently correlated with HOMA–IR (Table 4).

Effects of phlebotomy on ALT and serum Trx levels and HOMA–IR

All patients completed treatment without a significant change in body weight (age; 60.8 ± 10.8 kg, male/female; 15/8, BMI; 25.3 ± 2.6 kg/m^2, F0/F1/F2/F3/F4; 3/8/8/4, serogroup 1/2; 20/3). Nine patients had experienced IFN therapy before phlebotomy, whereas 14 patients had not experienced IFN therapy because of either old age or personal reasons. Changes in the serum levels of ALT, Trx, ferritin, HOMA–IR in the 23 patients that received phlebotomy are summarized in Table 5. Overall, the serum levels of ALT, Trx, and HOMA–IR were significantly decreased after phlebotomy compared with baseline values ($P < 0.00001$, $P = 0.023$, $P = 0.022$, respectively). These results indicated the efficacy of phlebotomy on insulin sensitivity as well as on liver function

Table 5 Changes in ALT, Trx, ferritin, and HOMA-IR after phlebotomy ($n = 23$)

	Before	After	Difference
BMI (kg/m^2)	23.6 (19.1–29.4)	24.0 (19.1–29.4)	NS
AST (IU/L)	67.0 (21–527)	51.0 (32–129)	$P < 0.005$
ALT (IU/L)	42.5 (27–121)	29.5 (24–53)	$P < 0.00001$
γ-GTP (IU/L)	89.0 (29–287)	60.5 (23–218)	$P < 0.0005$
Trx (ng/ml)	36.1 (20.2–79.4)	26.7 (18.1–32.7)	$P = 0.023$
Ferritin (ng/ml)	409.5 (125–1028)	20 (20–53)	$P < 0.00001$
HOMA-IR	3.5 (0.9–4.6)	2.4 (0.8–3.7)	$P = 0.022$

Data are expressed as medians (±range), Wilcoxon signed-ranks test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, glutamylcysteine transpeptidases; HOMA-IR, homeostasis model assessment–insulin resistance; Trx, thioredoxin.

tests in chronic hepatitis C patients. Furthermore, we analyzed the effects of phlebotomy on HOMA-IR in patients with a history of past IFN therapy and found that there were significant decreases in HOMA-IR (from 4.2 [3.7–4.6] to 2.9 [2.3–3.7], $P = 0.043$).

DISCUSSION

THE PRESENT STUDY shows that oxidative stress is an independent factor in the development of IR in patients with chronic hepatitis C, and validates the beneficial effect of phlebotomy on insulin sensitivity. To our knowledge, our report is the first to show a direct relationship between IR and oxidative stress in patients with HCV. We excluded alcohol drinkers, patients treated with steatosis-inducing drugs, and patients infected with HCV genotype 3a[18] from our analysis, as these are confounding factors affecting steatosis.

In general, the development of IR and steatosis is due to host-associated factors (e.g. obesity). The molecular mechanism underlying IR involves dysregulation of insulin-stimulated tyrosine phosphorylation of insulin receptor substrates (IRS).[19] This is achieved by phosphorylation of serine/threonine residues in IRS by either increased or decreased levels of adipokines associated with obesity (such as tumor necrosis factor [TNF]-α and adiponectin), thereby inhibiting tyrosine phosphorylation.[19] However, a high prevalence (61%) of steatosis, despite a low prevalence (28%) of obesity (BMI >25 kg/m^2) or diabetes (7%), indicated that there are mechanisms regulating insulin sensitivity other than obesity. In our study, HOMA-IR was significantly correlated with serum Trx levels, independent of BMI. Furthermore, the hepatic Trx levels independently predicted HOMA-IR in subgroup patients. Thus, hepatic oxidative stress directly contributes to IR in chronic hepatitis C patients.

Our hypothesis is supported by the following findings. First, chronic hepatitis C is characterized by oxidative stress-induced liver injury.[10,14,20] The overproduction of ROS could result from inflammatory cells,[10] iron overload,[20] and presumably the direct association of HCV core protein with mitochondria in hepatocytes.[21] In addition, steatosis, a prominent feature of chronic hepatitis C,[2–5] could result in oxidative stress.[11] Second, the increased abundance of ROS inhibits tyrosine phosphorylation of IRS in hepatocytes via the activation of stress-sensitive pathways, such as the c-Jun N-terminal kinase (JNK)[12] and nuclear factor (NF)-κB[22] pathways. JNK directly phosphorylates serine/threonine residues in IRS,[12] while NF-κB inhibits tyrosine phosphorylation via the induction of TNF-α.[22] The failure of hepatic insulin signaling subsequently leads to systemic IR.[12]

The question arising from this correlation between IR and oxidative stress is how metabolic disorders and liver injury can develop simultaneously in patients with HCV infection. One possible mechanism could be an interaction between IR and oxidative stress. IR results in hepatic steatosis,[11] which leads to increased ROS production concomitant with an increase in the number of inflammatory cells[10] and/or iron overload.[23] Conversely, ROS could exacerbate insulin sensitivity to promote steatosis,[11,12] and could promote the recruitment of inflammatory cells and fibrosis through lipid peroxidation products.[24,25] Thus, IR, steatosis, and oxidative stress could be involved in a feedback loop that exacerbates liver injury. This hypothesis is supported by the findings that HOMA-IR was significantly correlated with the serum and hepatic Trx levels, and both the HOMA-IR and serum Trx levels were significantly correlated with grades of steatosis.

Finally, we employed phlebotomy to validate the interaction between IR and oxidative stress, because

phlebotomy is useful for reducing hepatic oxidative stress.[20] Although phlebotomy is known to improve liver function tests in patients with HCV infection, its efficacy on insulin metabolism has not been well documented. Therefore, our findings provide new insight into the efficacy of phlebotomy. Notably, phlebotomy significantly improved HOMA–IR, even in patients who had been refractory to IFN. However, the long-term outcome of phlebotomy was unclear in this study, and a follow-up study should be performed.

In conclusion, we demonstrated an association between oxidative stress and IR in patients infected with HCV genotype 1 or 2. Our findings will contribute to our understanding of how metabolic disorders can develop in patients with chronic hepatitis C. Antioxidative therapy is a promising treatment to improve the pathogenesis of HCV.

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Hepatology Research 2008; **38**: 52–59 doi: 10.1111/j.1872-034X.2007.00205.x

Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

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Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naïve. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum Hb ≤ 8.5 g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

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Received 29 December 2006; revision 11 April 2007; accepted 25 May 2007.

of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm^3 and platelets ≥10^5/mm^3 within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Crnn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm^3)	5317 ± 1207
Neutrocytes (/mm^3)	2778 ± 902
Platelets (×10^4/mm^3)	17.4 ± 4.0
RBC (×10^4/mm^3)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 μg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = 32.3 × BW × (1 − 0.0094 × age) × (1 − 0.42 × sex)/Scr (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel–Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

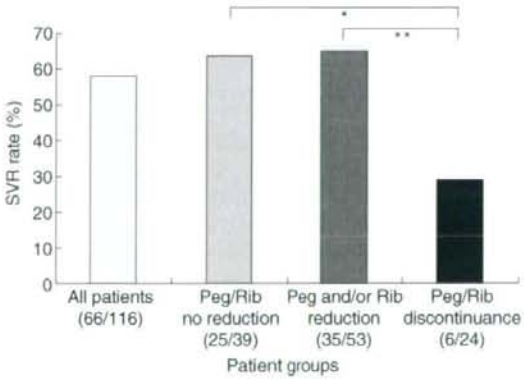

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (▨), patients without dose reduction; (■), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: *$P = 0.003$; **$P = 0.001$.

Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
$20 \leq$ CL/F ($n = 12$)	67% (8/12)	25% (3/12)	8% (1/12)	0
$15 \leq$ CL/F < 20 ($n = 23$)	57% (13/23)	30% (7/23)	13% (3/23)	0
$10 \leq$ CL/F < 15 ($n = 39$)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
CL/F < 10 ($n = 42$)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

$P = 0.031$ (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
$20 \leq$ CL/F ($n = 12$)	92% (11/12)	12% (1/12)	0
$15 \leq$ CL/F < 20 ($n = 23$)	83% (19/23)	17% (4/23)	0
$10 \leq$ CL/F < 15 ($n = 39$)	72% (28/39)	23% (9/39)	5% (2/39)
CL/F < 10 ($n = 42$)	50% (21/42)	43% (18/42)	7% (3/42)

$P = 0.009$ (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs ($P = 0.003$), and the group with reduction of PegIFN and/or ribavirin ($P = 0.001$).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the CL/F ≥ 20, 13% (3/23) for the $15 \leq$ CL/F < 20, 23% (9/39) for the $10 \leq$ CL/F < 15, and 26% (11/42) for the CL/F < 10 group. Ribavirin did not have to be discontinued due to severe anemia among patients with $15 \leq$ CL/F, but did for the 18% (2/11) of those with CL/F < 10 and 22% (2/9) of those with $10 \leq$ CL/F < 15. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of CL/F < 10, and 5% (2/39) of the group of $10 \leq$ CL/F < 15. No patients of the group of CL/F ≥15 showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin ($n = 53$), 1.6 ± 1.2 g/dL among those with dose reduction ($n = 39$), and 1.8 ± 1.0 g/dL among

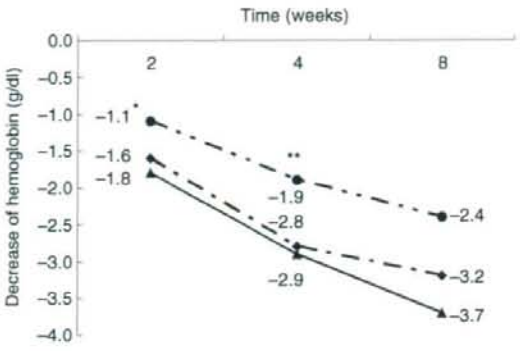

Figure 2 Course of ΔHb in the initial phase. (—···—), No reduction; (—·—·—), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).

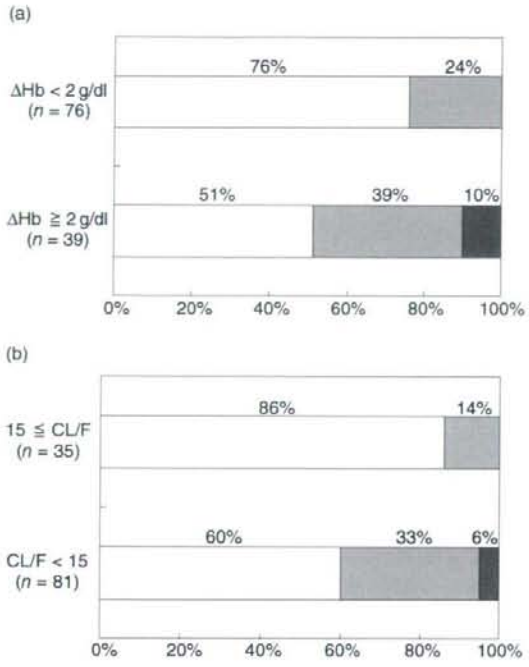

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (▩), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dl. decrease at two weeks from the baseline). $P = 0.009$ (Mantel–Haenszel χ^2-test). (b) according to CL/F levels. $P = 0.001$ (Mantel–Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL ($n = 76$)	ΔHb ≥ 2 g/dL ($n = 39$)
CL/F ≥ 15 ($n = 35$)	0/25	0/10
CL/F < 15 ($n = 80$)	0/51	4/29 (14%)

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients ($n = 5$) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

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Hepatitis C Virus Replication Is Inhibited by 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738) Through Enhancing Interferon-β

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A derivative of soyasapogenol, 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738), ameliorates liver injury induced by Concanavalin A in mice. We examined whether ME3738 has independent antiviral effects against hepatitis C virus (HCV) using an established HCV replication model that expresses the full-length genotype 1a HCV complementary DNA plasmid (pT7-flHCV-Rz) under the control of a replication-defective adenoviral vector expressing T7 polymerase. Hepatocellular carcinoma (HepG2) cells, human hepatoma (Huh7) cells, or monkey kidney (CV-1) cells were transfected with pT7-flHCV-Rz, and infected with adenoviral vector expressing T7 polymerase. ME3738 or interferon-α (IFN-α) was added thereafter and then protein and RNA were harvested from the cells at 9 days after infection. HCV-positive and HCV-negative strands were measured by real-time reverse-transcription polymerase chain reaction and HCV core protein expression was measured using an enzyme-linked immunosorbent assay. The messenger RNA levels of innate antiviral response-related genes were assessed using real-time reverse-transcription polymerase chain reaction. ME3738 dose-dependently reduced HCV-RNA and core protein in hepatocyte-derived cell lines. The antiviral effect was more pronounced in HepG2 than in Huh7 cells. ME3738 increased messenger RNA levels of interferon-β (IFN-β) and of IFN-stimulated genes (2′-5′ oligoadenylate synthetase, myxovirus resistance protein A [MxA]). Interferon-β knockdown by small interfering RNA abrogated the anti-HCV effect of ME3738. Moreover, the anti-HCV effects were synergistic when ME3738 was combined with IFN-α. *Conclusion:* ME3738 has antiviral effects against HCV. The enhancement of autocrine IFN-β suggests that ME3738 exerts antiviral action along the type I IFN pathway. This anti-HCV action by ME3738 was synergistically enhanced when combined with IFN-α. ME3738 might be a useful anti-HCV drug either with or without IFN-α. (HEPATOLOGY 2008;48:59-70.)

Abbreviations: Ad-T7pol, adenoviral vector expressing T7 polymerase; cDNA, complementary DNA; CV-1, African monkey kidney cell line; dsRNA, double-stranded RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HepG2, human hepatocellular carcinoma cell line; Huh7, human hepatoma cell line; IC_{90}, 90% inhibition concentration; IFN, interferon; IPS-1, interferon-β promoter stimulator 1; ISG, interferon stimulated gene; IRF-3, interferon regulatory factor 3; LacZ, β-galactosidase; ME3738, 22β-methoxyolean-12-ene-3β, 24(4β)-diol; mRNA, messenger RNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; MxA, myxovirus resistance protein A; nt, nucleotide(s); OAS, oligoadenylate synthetase; PCR, polymerase chain reaction; PKR, protein kinase R; RIG-I, retinoic acid-inducible gene; RT, reverse-transcription; rTth, recombinant thermostable reverse transcriptase; siRNA, small interfering RNA; UTR, untranslated region.

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Received September 30, 2007; accepted February 11, 2008.

Supported by a Grant-in-Aid for Scientific Research (JSPS KAKENHI 17590650, 19590771) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (to Y.H.).

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22289

Potential conflict of interest: Nothing to report.

Supplementary material for this article can be found on the HEPATOLOGY Web site (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, including hepatitis, cirrhosis, and hepatocellular carcinoma.[1] Interferon (IFN) combined with ribavirin is the standard treatment for chronic HCV infection, although this combination more effectively eliminates genotype 2 rather than genotype 1 HCV.[2] Thus, an anti-HCV drug is required, particularly to combat infection with genotype 1.

The full-length HCV tissue culture model still has some limitations, not only for screening novel antiviral agents but also with respect to the ability to precisely characterize the antiviral effect of IFN, particularly against genotype 1 HCV infection. An infectious HCV production system has been established *in vitro* using genotype 2a in human hepatoma (Huh7) cells.[3,4] However, genotype 2a is not representative of the genotype 1 strains of HCV that are principally associated with liver disease worldwide. Although Yi et al.[5] established an infectious genotype 1a HCV production system, it produces small quantities of virus. The basis for HCV permissiveness in Huh7 cells has not been fully explained, but might relate to defects in endogenous IFN-β induction triggered by viral double-stranded RNA (dsRNA), and insufficient expression of Toll-like receptor 3 that recognizes dsRNA.[6] Therefore, interactions between host cellular proteins and HCV using systems based strictly on Huh7 cells might be confounded by the relative IFN-β deficiency in these cells.

We previously described cell-based HCV replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV complementary DNA (cDNA) plasmid and infected with a recombinant adenoviral vector encoding T7 polymerase.[7] This replication system is based on the genotype 1a isolate, H77, and it allows the evaluation of HCV replication in various cell lines, including Huh7. We used this model to identify new anti-HCV therapies with better profiles.

Triterpenoid saponins are natural sugar conjugates of triterpenes that possess various biological effects. Soyasaponins I and II extracted from Glycine max L. Merrill prevent liver injury and hyperlipidemia.[8] Furthermore, soyasaponin II has antiviral effects against herpes simplex virus, human cytomegalovirus, influenza virus, and human immunodeficiency virus.[9]

We demonstrated that soyasapogenol A, an aglycon of soyasaponin, ameliorates liver failure in a mouse model of Concanavalin A-induced liver injury.[10] We identified 22β-methoxyolean-12-ene-3β, 24(4β)-diol (ME3738), a derivative of soyasapogenol that ameliorates liver injury in several animal models of acute and chronic liver injury. If ME3738 exerts antiviral effects, especially against HCV, in addition to ameliorating liver damage, patients with HCV would derive more benefit. Therefore, the present study investigates the inhibitory effect of ME3738 against HCV replication, analyzes participating host factors, and measures the anti-HCV effect of ME3738 combined with IFN-α.

Materials and Methods

Reagents. The synthesis of ME3738 (Fig. 1) has been described.[11] We obtained ME3738 and IFN-α 2b from Meiji Seika (Yokohama, Japan) and from Schering-Plough (Kenilworth, NJ), respectively.

Cell Lines. We maintained hepatocellular carcinoma (HepG2) cells (American Type Culture Collection, Manassas, VA), human hepatoma (Huh7) cells (Dr. Robert Lanford, Southwest Foundation for Biomedical Research), and African green monkey kidney (CV-1) cells (American Type Culture Collection) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Plasmids and Transfection-Infection. The binary replication system can synthesize positive-strand and negative-strand HCV RNA, produce HCV protein, and generate quasispecies.[7,12,13] Briefly, we adapted a plasmid containing the infectious full-length genotype 1 cDNA sequence corresponding to the H77 prototype strain[14] at the 5′ and 3′ termini with the T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz. We transfected HepG2, Huh7, or CV-1 cells at 70% confluence on six-well plates with pT7-fl-HCV-Rz (3, 3, and 1 μg/well, respectively) using Lipofectamine (Invitrogen, Carlsbad, CA). To assess ME3738-induced inhibition of T7 polymerase activity, we transfected the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase (LacZ) gene, into the cells. We delivered T7 polymerase to the HepG2, Huh7, and CV-1 cells using a replication-defective adenovirus vec-

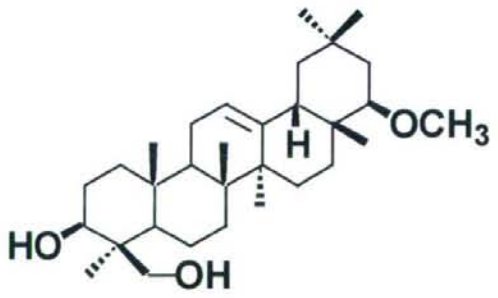


Fig. 1. Structure of ME3738. ME3738 is a derivative of soyasapogenol that ameliorates liver failure in mouse models of Concanavalin A (Con A)-induced liver injury.

tor (adenoviral vector expressing T7 polymerase [Ad-T7pol]) 24 hours after pT7-flHCV-Rz transfection. Control experiments included a replication-defective adenovirus vector lacking the T7 polymerase gene (Ad-Psi5; Harvard Gene Therapy Initiative Viral Vector Core Services, Boston, MA). We infected cells with the adenovirus vector at a multiplicity of infection of 10.

ME3738 and IFN-α Evaluation. We added ME3738 (0.1 to 10 μM) 3 hours after infection with Ad-T7pol. We also added IFN-α 2b (100 IU/mL) with or without ME3738 (0.1 μM) 3 hours after Ad-T7pol infection. We changed all media at day 1 after infection and every 2 days thereafter.

Cellular RNA Extraction. RNA extracted using TRIzol (Invitrogen) was digested twice for 4 hours each with deoxyribonuclease I using the DNA-free kit (Ambion, Austin, TX) following the manufacturer's protocol. Plasmid DNA was completely digested and removed from the RNA samples with deoxyribonuclease I because we did not detect any polymerase chain reaction (PCR) products. We quantified RNA by ultraviolet spectrum analysis.

Ribonuclease Protection Assay. We detected the HCV RNA-negative strand as described.[12] In brief, we generated antigenomic RNA by *in vitro* transcription using a sense-oriented [α-^{32}P] uridine triphosphate-labeled probe (corresponding to 98 nucleotides of the 3′ terminal HCV genome) and T7 polymerase from the vector pHCV-3′ T.[15] We generated transcripts using the RPA III kit (Ambion) according to the manufacturer's instructions.

Quantitative Real-Time Reverse-Transcription PCR. We quantified positive-strand and negative-strand HCV RNAs by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) and SYBR green I dye (Roche Diagnostics) as described.[16] We synthesized complementary DNA from RNA (1 μg) in a mixture containing 5 U of recombinant thermostable reverse transcriptase (rTth) and 10 pM of the appropriate primer for reverse-transcription.[17,18] We detected positive-strand HCV RNA using the HCV-I antisense primer (5′-TGG ATG CAC GGT CTA CGA GAC CTC-3′, nucleotides [nt] 342-320 of the 5′ untranslated region [UTR]).[19] We detected negative-strand HCV RNA using the HCV-II sense primer (5′-CAC TCC CCT GTG AGG AAC T-3′, nt 38-56 of the 5′UTR)[19]. Positive-strand and negative-strand HCV PCR amplification proceeded using 2 μL of purified cDNA in a reaction mixture containing 1 μL of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of $MgCl_2$, 5 pM of the antisense primer KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′; nt 311-288 of the 5′UTR), and 5 pM of the sense primer KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′; nt 68-91 of the 5′UTR). The PCR amplification consisted of initial denaturation for 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 5 seconds at 70°C, and 15 seconds at 72°C.

We quantified cellular messenger RNA (mRNA) by reverse-transcription with the same amount of RNA that was used for HCV-positive and HCV-negative strand analysis, and the oligonucleotide $d(T)_{16}$ primer under standard conditions.[20] For real-time PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-β, IFN-α, 2′-5′ oligoadenylate synthetase (OAS), myxovirus resistance protein A (MxA), and IFN regulatory factor 3 (IRF-3), we included commercial primer sets for each (Roche Search LC, Mannheim, Germany). For real-time PCR amplification of protein kinase R (PKR), we applied the forward (5′-AGC ACA CTC GCT TCT GAA TC-3′) and reverse (5′-CTG GTC TCA GGA TCA TAA TC-3′) primers under the following conditions: 10 seconds at 95°C, 10 seconds at 58°C, and 15 seconds at 72°C.[20] We included specific primers for retinoic acid-inducible gene I (RIG-I: forward primer, 5′-GTG CAA AGC CTT GGC ATG T-3′ and reverse primer, 5′-TGG CTT GGG ATG TGG TCT ACT C-3′), and IFN-β promoter stimulator 1 (IPS-1: forward primer, 5′-GCA ATG TGG ATG TTG TAG AG-3′ and reverse primer, 5′-CTG AAG GGT ATT GAA GAG ATG-3′) under the following conditions: 10 seconds at 95°C, 10 seconds at 60°C, and 6 seconds at 72°C. For real-time PCR amplification of LacZ, we included sense (5′-GCC TGC GAT GTC GGT TTC CGC GAG G-3′) and antisense (5′-GCC AGC GCG GAT CAT CGG TCA GAC G-3′) primers under the following conditions: 10 seconds at 95°C, 10 seconds at 68°C, and 16 seconds at 72°C.[21]

We quantified DNA by measuring SYBR green I dye incorporation into PCR products at 530 nm according to the manufacturer's instructions. We generated a standard curve for HCV using a PCR product corresponding to nt 38-342 of the 5′UTR. At the end of each run, we generated a DNA melting curve to control for sample homogeneity and quality. We electroporated a subset of samples and sequenced them to confirm the identity of the amplified PCR product. Data are expressed as the copy numbers of HCV RNA or cellular mRNA per molecule of GAPDH mRNA.

Enzyme-Linked Immunosorbent Assay for HCV Core Antigen. We adjusted cell culture lysates to 0.2 mg/mL. We measured concentrations of HCV core antigen using the HCV core protein enzyme-linked immunosorbent assay (ELISA) kit (Ortho-Clinical Diagnostics, Raritan, NJ) following the manufacturer's instructions.[22]

Core ELISA data are expressed as femtomoles of HCV core antigen per microgram of total protein. The lower level of detection for this assay was below 1.5 pg/mL.

MTS Assay. We assessed reagent cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay with the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI), according to the manufacturer's instructions.

RNA Interference and Neutralizing IFN-β by Antibody. We transfected cells with three small interfering RNAs (siRNAs) targeting IFN-β using Lipofectamine RNAiMax (Invitrogen). The final siRNA concentration was 20 nM at 1 day before transfection with pT7-flHCV-Rz. The target sequences of the three IFN-β siRNAs were AAU CCA AGC AAG UUG UAG CUC AUG G (HSS105232), AAG CCU CCC AUU CAA UUG CCA GG (HSS105233), and AUU UGG AGG AGA CAC UUG UUG GUC A (HSS105234), respectively. We purchased the IFN-β siRNAs and negative control siRNA from Invitrogen.

We used mouse antihuman IFN-β antibody (R&D Systems, Minneapolis, MN) or mouse isotype control antibody (R&D Systems) to neutralize IFN-β in cell culture supernatants. We added 1 μg/mL of each antibody to the supernatant after infection with Ad-T7pol, and then incubated it until day 3.

Statistical Analysis. We statistically analyzed values using SPSS 14.0 software (SPSS, Tokyo, Japan). We compared differences in mean values using the Mann-Whitney U-test.

Results

Replication-Defective Adenoviral Vectors Replicated HCV RNA in HepG2 Cells. Previously, we refined a full-length HCV replication system in the hepatocyte-derived and non-hepatocyte-derived cell lines, Huh7 and CV-1, respectively.[7] The Huh7 cell line or its derivatives are used in many HCV replication systems, such as replicon models. However, because of the relative IFN-β deficiency of Huh7 cell lines,[6] interactions between the host cellular proteins and HCV in the Huh7 cell line might differ from those *in vivo*. Therefore, we examined whether or not HCV replicates in another hepatocyte-derived cell line. HepG2 cells were transfected with pT7-flHCV-Rz and then infected with Ad-T7pol. The ribonuclease protection assay detected HCV-negative strands in HepG2 (Fig. 2A), Huh7, and CV-1 (Fig. 2B) cells. The parental replication-incompetent vector Ad-Psi5 served as an adenoviral vector control. Negative-strand HCV RNA was undetectable in HepG2, Huh7,

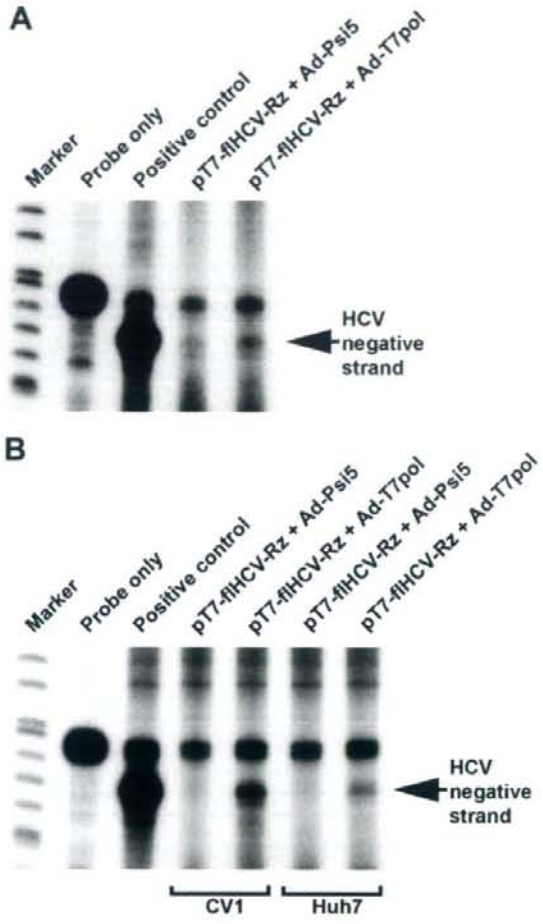
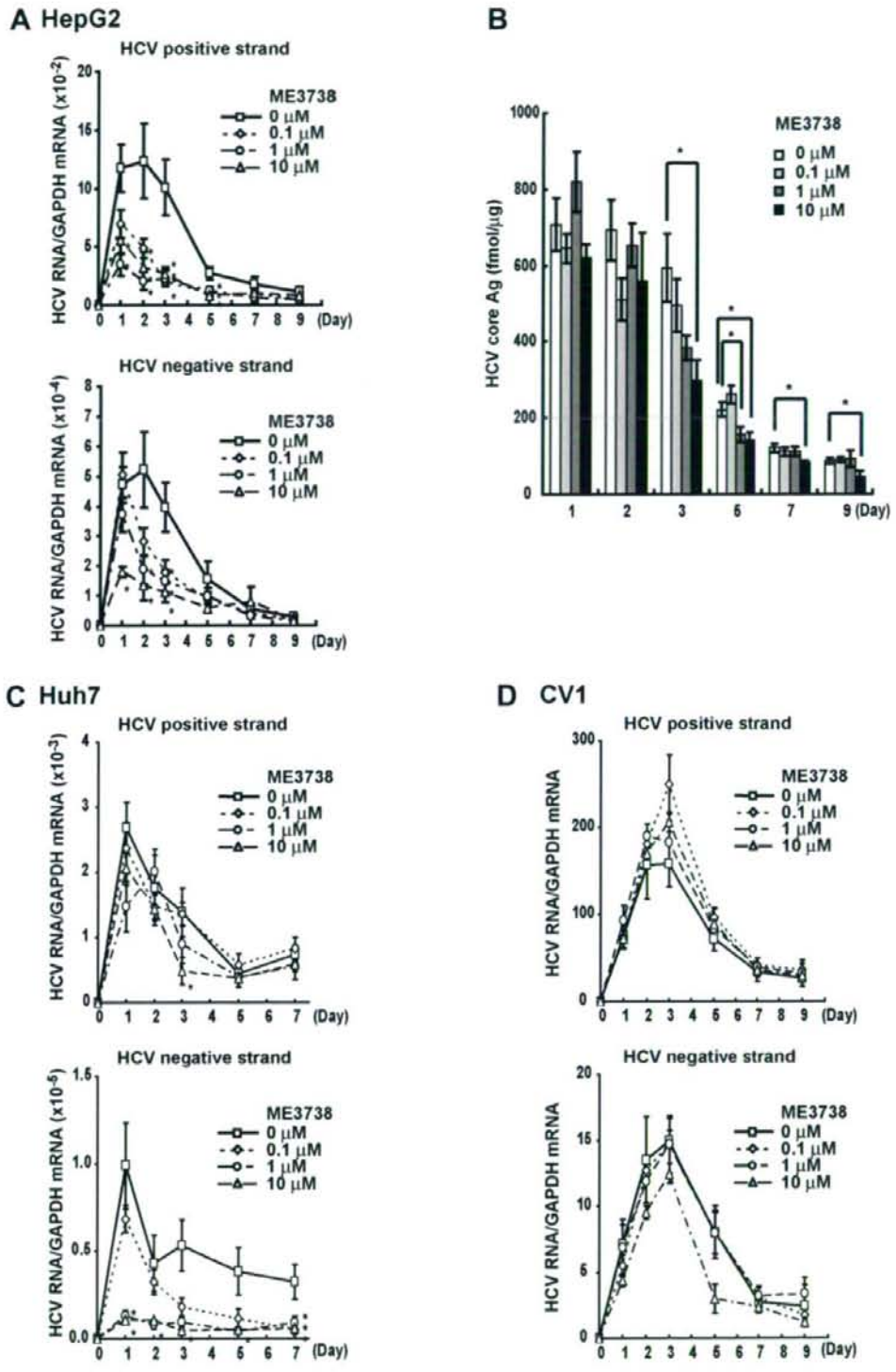


Fig. 2. Synthesis of HCV RNA-negative strand. After transfection with pT7-flHCV-Rz plasmid, cells were infected with adenoviral vectors expressing recombinant adeno-T7 polymerase (Ad-T7pol) or with adenoviral vectors lacking T7 polymerase gene (Ad-Psi5) at multiplicity of infection (MOI) of 10. Ribonuclease protection assays detected HCV-negative strands in (A) HepG2, and (B) Huh7 and CV-1cells.

Fig. 3. Inhibitory effects of ME3738 on HCV replication in hepatocyte-derived cell lines. (A) Real-time reverse-transcription PCR (RT-PCR) quantified HCV-positive and HCV-negative strands over time in cells cultured with or without ME3738. Levels of both HCV RNA strands were significantly and dose-dependently decreased by ME3738 in HepG2 cells. Data indicate means ± standard error (SE) for six replicates ($^*P < 0.05$). (B) Decrease of HCV core protein after HCV RNA decline determined by enzyme-linked immunosorbent assay (ELISA). Data indicate means ± SE for four replicates ($^*P < 0.05$). (C) ME3738 significantly decreased HCV-positive strand only at day 3 in Huh7 cells, but not to the extent found in HepG2 cells. ME3738 more significantly reduced HCV RNA-negative, than HCV RNA-positive strands in Huh7 cells. Data indicate means ± SE for six replicates ($^*P < 0.05$). (D) Levels of neither HCV RNA strand significantly decreased in CV-1 cells, suggesting that anti-HCV effects of ME3738 are specific to hepatocyte-derived cell lines. Data indicate means ± SE for three replicates.


A HepG2
HCV positive strand
HCV RNA/GAPDH mRNA (x10-2)
ME3738
0 μM
0.1 μM
1 μM
10 μM
(Day)
HCV negative strand
HCV RNA/GAPDH mRNA (x10-4)
B
HCV core Ag (fmol/μg)
C Huh7
HCV positive strand
HCV RNA/GAPDH mRNA (x10-3)
HCV negative strand
HCV RNA/GAPDH mRNA (x10-5)
D CV1
HCV positive strand
HCV RNA/GAPDH mRNA
HCV negative strand

and CV-1 cells transfected with the control Ad-Psi5 vector (Fig. 2A,B).

ME3738 Inhibited HCV Expression. To assess the inhibitory effects of ME3738 on HCV replication, we cultured cells with ME3738 (0, 0.1, 1, and 10 μM) after Ad-T7pol infection.

Levels of HCV RNA-positive (Fig. 3A) and HCV RNA-negative strands in HepG2 cells were significantly and dose-dependently decreased by ME3738 from days 1 to 5 and from days 1 to 3, respectively. The concentration of ME3738 required for 50% inhibition (IC_{50}) of HCV RNA-positive strands calculated from the dose-response data obtained on day 2 with ME3738 was 0.03 μM. ME3738 significantly and dose-dependently decreased levels of HCV core protein in HepG2 cells on days 3 to 9; that is, after the HCV RNA level decreased (Fig. 3B).

ME3738 significantly and dose-dependently decreased HCV RNA-positive strands in Huh7 cells (Fig. 3C) at day 3. The dose-dependent effect of ME3738 was even more pronounced on the level of HCV RNA-negative, than HCV RNA-positive strands in Huh7 cells, and was detectable from days 1 to 9. Whereas a significant decrease in HCV RNA levels was associated with ME3738 in HepG2 and Huh7 cells, no such decrease was evident in CV-1 cells (Fig. 3D). These findings suggest that ME3738 has antiviral effects against HCV in hepatocyte-derived cells and that the effect is more pronounced in HepG2, than in Huh7 cells.

To confirm that the reduction in HCV RNA levels induced by ME3738 in HepG2 cells was not due to a nonspecific response such as inhibited T7 polymerase activity, we transfected the plasmid OS8 into HepG2 cells and then measured LacZ mRNA levels in the presence of various doses of ME3738. ME3738 did not significantly reduce LacZ mRNA expression in HepG2 cells (Supplementary Fig. 1A). In addition, the MTS assay showed that ME3738 was not significantly cytotoxic to HepG2 cells (Supplementary Fig. 1B).

Huh7 cells have impaired Toll-like receptor 3 and dsRNA signaling, and thus, limited induction of IFN-stimulated genes (ISGs).[6,23,24] Because ME3738 exerts more powerful antiviral effects against HCV in HepG2 than in Huh7 cells, we examined the signaling pathway of IFN and ISGs.

IFN-β and ISG mRNA Expression in HepG2 and Huh7 Cells. The induction of type I IFNs represents an early protective response to many viral infections in mammalian cells. The induction of IFN-β in particular represents the immediate response of cells to viral infection, and precedes the transcription of most IFN-α species.[25] ME3738 inhibited HCV replication soon after Ad-T7pol infection in hepatocyte-derived cell lines. Because the inhibitory effects against HCV replication by ME3738 were the most rapid and robust in HepG2 cells, we examined IFN-β mRNA expression in our replication model using these cells. After expression with HCV without ME3738, high levels of IFN-β mRNA persisted for 3 days and diminished thereafter in HepG2 cells (Fig. 4A). The IFN-β mRNA level was increased up to 340-fold compared with the basal level. At day 1, which is the earliest time point after Ad-T7pol infection, ME3738 dose-dependently increased IFN-β mRNA expression in HepG2 cells. To further determine whether the IFN-β mRNA expression enhanced by ME3738 depends on an antiviral response against HCV replication, we transfected the plasmid OS8 into HepG2 cells with or without ME3738, and we measured IFN-β mRNA levels (Fig. 4B). Although the IFN-β mRNA level in the cells cultured without ME3738 was increased up to only 4.5-fold compared with the basal level, it was 1.3% of that in cells expressing HCV. Moreover, ME3738 did not enhance IFN-β mRNA expression in cells transfected with pOS8 (Fig. 4B). The IFN-β enhancement was more remarkable in HepG2 than in Huh7 cells (Fig. 4B). The IFN-β mRNA levels in Huh7 cells without ME3738 were increased by only up to three-fold compared with the 340-fold increase in HepG2 cells. Moreover, the level of IFN-α mRNA was not altered in HepG2 and Huh7 cell lines cultured with ME3738 (Supplementary Fig. 2).

Because ME3738 significantly enhanced the IFN-β expression activated by HCV replication at the earliest time point, we considered whether ISGs are also increased following IFN-β enhancement. In HepG2 cells treated without ME3738, 2′-5′ OAS, MxA, and PKR mRNA were also induced by HCV RNA replication at day 2. The expression of both 2′-5′ OAS and MxA mRNA in HepG2 cells was also significantly and dose-dependently enhanced by ME3738 at day 2, but not that of PKR mRNA (Fig. 4C). These data demonstrated that ME3738 enhanced the induction of endogenous IFN-β and ISGs triggered by the dsRNA of HCV replication. Like the unremarkable enhancement of IFN-β mRNA expression by ME3738 (Fig. 4B), the mRNA expression of the ISGs, 2′-5′ OAS, MxA, and PKR, was not enhanced in Huh7 cells (Fig. 4D) compared with that in HepG2 cells. These findings suggested that the differences in the anti-HCV effects in HepG2 and Huh7 cells correlate with cell-specific variations in IFN-β mRNA expression.

Intracellular dsRNA produced during viral replication is recognized by RIG-I,[26,27] then IPS-1 activates IRF-3, which subsequently induces IFN-β transcription.[28,29] We therefore examined these molecules, and measured each mRNA level by quantitative real-time RT (reverse-transcription)-PCR. However, ME3738 had no effect on

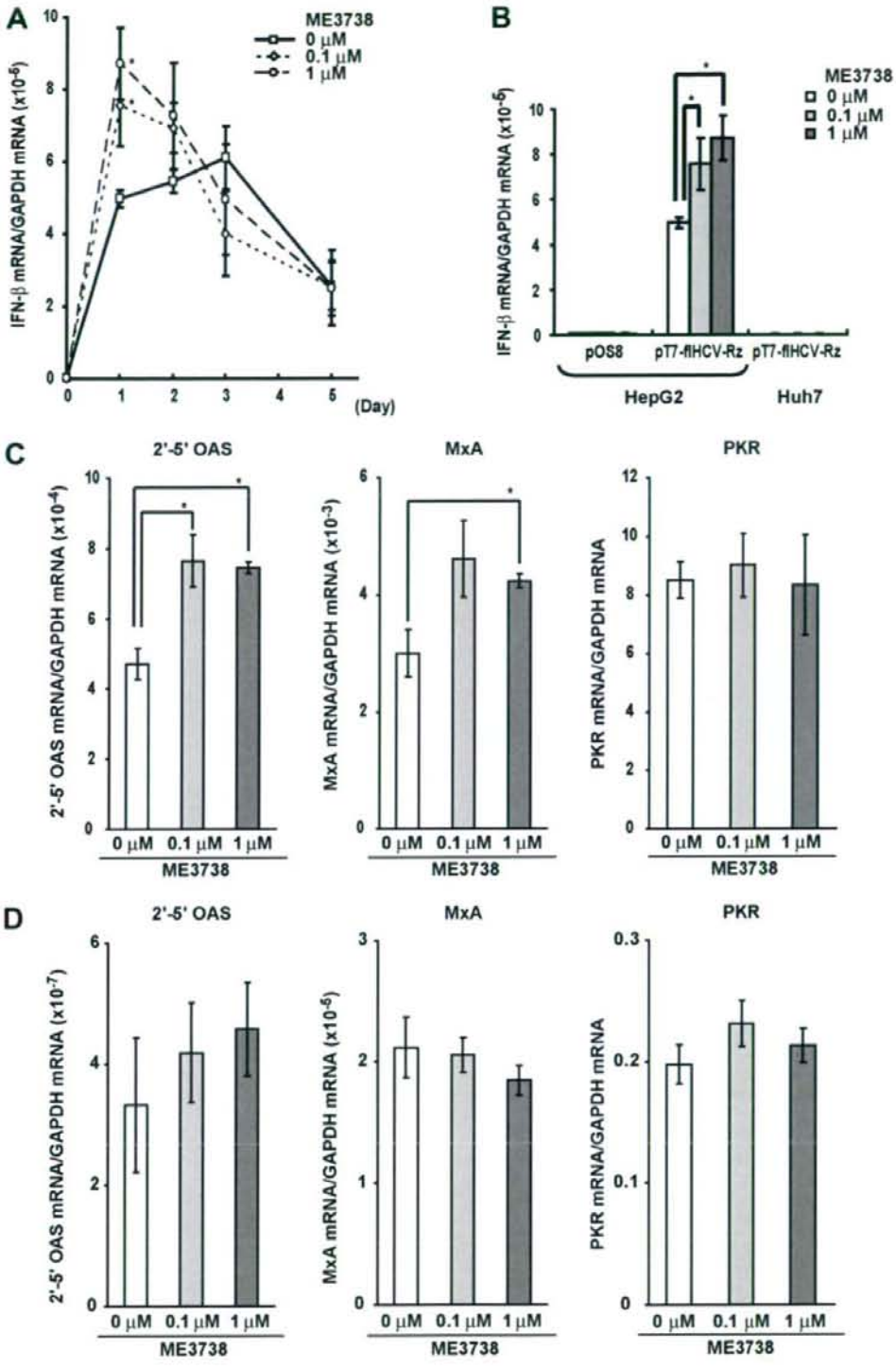


Fig. 4. ME3738 enhances IFN-β and ISG expression in HepG2 cells. (A) Time course of IFN-β mRNA assessed by real-time reverse-transcription PCR (RT-PCR). ME3738 more significantly and dose-dependently increased IFN-β mRNA expression in HepG2 cells at day 1 (earliest time point after Ad-T7pol infection). (B) ME3738 induced little or no IFN-β mRNA in HepG2 cells transfected with plasmid OS8 instead of pT7-flHCV-Rz, or in Huh7 cells transfected with pT7-flHCV-Rz, respectively. (C) ME3738 upregulated 2'-5' OAS and MxA, but not PKR mRNA in HepG2 cells. (D) ME3738 did not enhance either 2'-5' OAS, MxA, or PKR mRNA expression in Huh7 cells. Data from HepG2 or Huh7 cells indicate means $\pm$ standard error (SE) for four and six replicates, respectively ($^*P < 0.05$).

RIG-I, IPS-1, and IRF-3 mRNA expression at day 1, or at the earliest time point (data not shown). ME3738 seemed to enhance IFN-β expression triggered by HCV replication without augmentation by receptor, adaptor protein, and transcription factor mRNA expression.

Enhanced IFN-β Participates in Anti-HCV Effects of ME3738 in HepG2 Cells. We applied RNA interference to knock down IFN-β expression and determine whether ME3738 inhibits HCV replication through IFN-β enhancement. Cells transfected with IFN-β-specific siRNA before pT7-flHCV-Rz, but not with scrambled control siRNA, caused an 85% reduction in the IFN-β mRNA level triggered by HCV replication in the presence or absence of ME3738 (Fig. 5A). Furthermore, ME3738 exerted anti-HCV effects in HepG2 cells transfected with scrambled control siRNA, but not with IFN-β siRNA (Fig. 5B). We also included a neutralizing antibody to IFN-β in cell supernatants. The anti-HCV effect of ME3738 was also diminished on day 3 in cells incubated with the neutralizing antibody compared with the control antibody (Fig. 5C). These data indicate that ME3738 requires enhanced IFN-β expression to exert anti-HCV effects in HepG2 cells.

ME3738 and IFN-α Synergistically Inhibit HCV Replication. The combination of IFN and ribavirin is the current standard therapy for infection with chronic hepatitis C.[2] Because we found that ME3738 inhibited HCV replication through IFN-β enhancement, we postulated that ME3738 could be combined with IFN like ribavirin.

Our previous study showed that 1,000 IU/mL of IFN-α inhibits HCV replication from day 2.[7] However, a low dose of IFN (100 IU/mL) alone is insufficient to inhibit HCV replication. To evaluate whether ME3738 augments the anti-HCV activity of IFN-α, HepG2 cells were infected with Ad-T7pol and then incubated with suboptimal doses of ME3738 (0.1 μM) and IFN-α (100 IU/mL) (Fig. 6A). The results showed that HCV replication was considerably reduced by the combination compared with that by either ME3738 or IFN alone. We examined whether the effect of this combination was synergistic or additive using the isobologram method.[30,31] The combination of ME3738 (0.1 μM) and IFN-α (100 IU/mL) inhibited about 90% of HCV RNA replication 2 days after Ad-T7pol infection. The amounts of IFN-α and ME3738 to induce 90% inhibition (IC_{90}) were 1,792 IU/mL and 1.06 μM, respectively. These data were used to generate isoboles, which demonstrated 90% inhibition of HCV RNA replication, and the 90% isobole revealed that the anti-HCV action of ME3738 and IFN-α was synergistic (Fig. 6B).

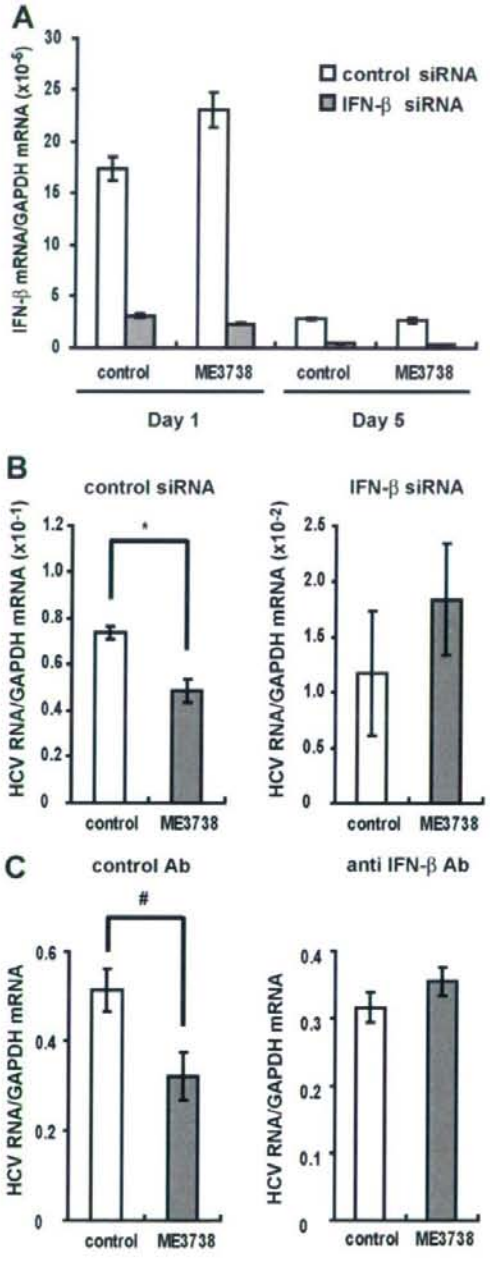


Fig. 5. Anti-HCV effects of ME3738 abrogated in HepG2 cells transfected with IFN-β siRNA. HepG2 cells were transfected with siRNAs targeting IFN-β before HCV expression. Data indicate means $\pm$ standard error (SE) for four replicates. (A) Transfection with IFN-β-specific siRNA caused reproducible 85% reductions in IFN-β mRNA levels with or without ME3738. (B) Transfection with IFN-β siRNA reduces anti-HCV effects of ME3738, indicating that enhanced IFN-β expression correlates with anti-HCV effects of ME3738 ($^*P < 0.05$). (C) Neutralizing antibody to IFN-β (anti IFN-β Ab) or control antibody (control Ab) added to cell culture supernatants after infection with Ad-T7pol diminished anti-HCV effects of ME3738 compared with control Ab ($\#P = 0.061$).