Table 1 Characteristics of study patients

		PEG IFN-α-2a§ + ME3738 50 mg/day ($n = 46$)	PEG IFN-α-2a§ + ME3738 200 mg/day ($n = 45$)	PEG IFN-α-2a§ + ME3738 800 mg/day ($n = 44$)	Total ($n = 135$)	Test result
Sex	Male	17 (37.0%)	16 (35.6%)	16 (36.4%)	49 (36.3%)	$P = 1.0000$ (Fisher's exact test)
	Female	29 (63.0%)	29 (64.4%)	28 (63.6%)	86 (63.7%)	
Age	Mean	56.5	59.8	56.6	57.6	$P = 0.3166$ (1-way ANOVA)
	Standard deviation	12.6	9.8	11.9	11.5	
	Median	61	61	60	60	
	Range	23–73	40–75	26–76	23–76	
Bodyweight	Mean	55.9	55.9	57.6	56.5	$P = 0.7033$ (1-way ANOVA)
	Standard deviation	12.1	11.3	9.8	11.1	
	Median	54.5	54.0	55.6	54.9	
	Range	34.9–88.6	39.2–82.5	43.0–84.0	34.9–88.6	
Latest liver biopsy result	F0–F2	21 (45.7%)	19 (42.2%)	14 (31.8%)	54 (40.0%)	$P = 0.4524$ (Fisher's exact test)
	F3	0 (0.0%)	1 (2.2%)	2 (4.5%)	3 (2.2%)	
	No test result	25 (54.3%)	25 (55.6%)	28 (63.6%)	78 (57.8%)	
Presence/absence of complication	Absence	2 (4.3%)	6 (13.3%)	5 (11.4%)	13 (9.6%)	$P = 0.3167$ (Fisher's exact test)
	Presence	44 (95.7%)	39 (86.7%)	39 (88.6%)	122 (90.4%)	
Presence/absence of diabetes	Absence	43 (93.5%)	45 (100.0%)	42 (95.5%)	130 (96.3%)	$P = 0.2843$ (Fisher's exact test)
	Presence	3 (6.5%)	0 (0.0%)	2 (4.5%)	5 (3.7%)	
Presence/absence of concomitant drug	Absence	0 (0.0%)	0 (0.0%)	1 (2.3%)	1 (0.7%)	$P = 0.3259$ (Fisher's exact test)
	Presence	46 (100.0%)	45 (100.0%)	43 (97.7%)	134 (99.3%)	
Presence/absence of concomitant therapy	Absence	29 (63.0%)	32 (71.1%)	35 (79.5%)	96 (71.1%)	$P = 0.2356$ (Fisher's exact test)
	Presence	17 (37.0%)	13 (28.9%)	9 (20.5%)	39 (28.9%)	
Baseline hemoglobin	Mean	13.9	14.2	14	14	$P = 0.6521$ (1-way ANOVA)
Pegasys dose change	No	26 (56.5%)	29 (64.4%)	25 (56.8%)	80 (59.3%)	$P = 0.8599$ (Fisher's exact test)
	Dose reduction	13 (28.3%)	12 (26.7%)	12 (27.3%)	37 (27.4%)	
	Dose withdrawal	7 (15.2%)	4 (8.9%)	7 (15.9%)	18 (13.3%)	
HCV RNA-1b† (NS5A)	0 or 1	45 (97.8%)	39 (86.7%)	40 (90.9%)	124 (91.9%)	$P = 0.1333$ (Fisher's exact test)
	Not less than 2	1 (2.2%)	6 (13.3%)	4 (9.1%)	11 (8.1%)	
HCV RNA-1b† IFN/RBV mutation at 70	Wild type	37 (80.4%)	26 (57.8%)	29 (65.9%)	92 (68.1%)	$P = 0.0611$ (Fisher's exact test)
	Mutant type/competitive type	9 (19.6%)	19 (42.2%)	14 (31.8%)	42 (31.1%)	
	Other	0 (0.0%)	0 (0.0%)	1 (2.3%)	1 (0.7%)	
HCV RNA-1b† IFN/RBV‡ mutation at 91	Wild type	34 (73.9%)	23 (51.1%)	31 (70.5%)	88 (65.2%)	$P = 0.0529$ (Fisher's exact test)
	Mutant type/competitive type	12 (26.1%)	22 (48.9%)	13 (29.5%)	47 (34.8%)	
Baseline HCV RNA†	Mean	6.7	6.6	6.7	6.6	$P = 0.4157$ (1-way ANOVA)
FibroIndex	Mean	1.43	1.5	1.41	1.45	$P = 0.5513$ (1-way ANOVA)
IL-28B	Not determined	5 (10.9%)	1 (2.2%)	3 (6.8%)	9 (6.7%)	$P = 0.8000$ (Fisher's exact test)
	Major homo allele	31 (67.4%)	32 (71.1%)	29 (65.9%)	92 (68.1%)	
	Minor hetero allele	9 (19.6%)	12 (26.7%)	12 (27.3%)	33 (24.4%)	
	Minor homo allele	1 (2.2%)	0 (0.0%)	0 (0.0%)	1 (0.7%)	

†Hepatitis C virus.
‡Interferon/ribavirin.
§Pegylated interferon alpha-2a.

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Table 2 Undetectable HCV RNA during the treatment period

No. of subjects (%)		Treatment period						
		4 weeks	8 weeks	12 weeks	24 weeks	48 weeks (EOT†)	Follow up‡ (12 weeks)	Follow up‡ (24 weeks)
PEG IFN-α-2a§ + ME3738 50 mg/day	$n = 46$	2	8	12	23	23	3	3
		4.3%	17.4%	26.1%	50.0%	50.0%	6.5%	6.5%
PEG IFN-α-2a§ + ME3738 200 mg/day	$n = 45$	3	4	12	20	26	5	3
		6.7%	8.9%	26.7%	44.4%	57.8%	11.1%	6.7%
PEG IFN-α-2a§ + ME3738 800 mg/day	$n = 44$	3	5	7	12	17	3	2
		6.8%	11.4%	15.9%	27.3%	38.6%	6.8%	4.5%
Total	$n = 135$	8	17	31	55	66	11	8
		5.9%	12.6%	23.0%	40.7%	48.9%	8.1%	5.9%

†End of treatment.
‡12/24 weeks after EOT.
§Pegylated interferon-α-2a.

follow-up observation in most of the subjects who showed viral reactivation.

Table 3 shows the patient background factors which might, and are considered to, have influenced the SVR in this study. With respect to sex, the effect was higher in men than in women, and with respect to age, the effect was lower in the elderly (≥60 years) than in younger subjects (<60 years). Thus, the effect was the lowest in elderly female subjects. There were no definite differences in FibroIndex showing the grade of liver fibrosis. In terms of ISDR, which is a predictive viral factor for IFN therapy in patients infected with HCV genotype 1b, the effect was high in mutants (no. of mutations,: ≥2), but no clear results were obtained in mutations of Core 70 and Core 91. Finally, in terms of *IL28B* SNP, SVR was only seen with the major homo allele.

Treatment with ME3738 showed no clear influence on ALT levels in this study (no relevant data are shown).

Safety

At least one adverse event was noted to 134 subjects among 135 subjects, excluding one subject in whom the study was discontinued after 5 weeks of treatment. Table 4 shows the adverse events noted with an incidence of at least 20% in the entire study population.

Frequently observed adverse events were fever, malaise, headache, nasopharyngitis, pruritus, retinopathy and diarrhea. Frequently observed abnormal laboratory findings were decreased white blood cell count, decreased platelet count, decreased neutrophil count, increased hyaluronic acid, decreased hemoglobin, decreased hematocrit and decreased red blood cell count.

In this study, decreased hemoglobin was observed in 45.9% (62/135) of subjects, and the hemoglobin level decreased to less than 10 g/dL in the treatment period in 19.3% (26/135) of subjects. Figure 2 shows the time-course of changes in the hemoglobin level in each ME3738 treatment group.

DISCUSSION

IN THIS STUDY conducted in patients with naïve HCV, 135 subjects were administrated ME3738 and 27 subjects were withdrawn from this study by week 12 for various reasons, including applicability to the study discontinuation criteria specified in the protocol. As a result, study treatment was continued in 108 subjects after 12 weeks of treatment. Among these, 16 subjects discontinued the study for reasons such as occurrence of an adverse event. There were 92 subjects who completed

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Table 3 Comparison of clinical characteristics and viral types between subjects with and without sustained virological response

	Virological response	
	Subjects with SVR†, *n* (%)	Subjects without SVR†, *n* (%)
SVR†		
Entire subjects	8/135 (5.9%)	127/135 (94.1%)
By sex and age		
Men	5/49 (10.2%)	44/49 (89.8%)
Age, <60 years	4/23 (17.4%)	19/23 (82.6%)
Age, ≥60 years	1/26 (3.8%)	25/26 (96.2%)
Women	3/86 (3.5%)	83/86 (96.5%)
Age, <60 years	3/36 (8.3%)	33/36 (91.7%)
Age, ≥60 years	0/50 (0%)	50/50 (100%)
FibroIndex		
>1.25	5/95 (5.3%)	90/95 (94.7%)
≤1.25	3/40 (7.5%)	37/40 (92.5%)
ISDR‡		
Wild (0–1)	5/124 (4.0%)	119/124 (96.0%)
Mutant (>2)	3/11 (27.3%)	8/11 (72.7%)
Core region amino acid substitution site		
Wild	5/92 (5.4%)	87/92 (94.6%)
70-mutant	3/43 (7.0%)	40/43 (93.0%)
Wild	2/88 (2.3%)	86/88 (97.7%)
91-mutant	6/47 (12.8%)	41/47 (87.2%)
Genotyping of *IL-28B* SNP§		
Major homo allele	8/92 (8.7%)	84/92 (91.3%)
Minor hetero/homo allele	0/34 (0%)	34/34 (100%)

†Sustained virological response.
‡Interferon sensitivity determining region.
§Single nucleotide polymorphism.

the 48-week treatment. As compared with the standard combination therapy of PEG IFN-α-2b and RBV, it appeared that the subject withdrawal rate was lower and the treatment completion rate was higher in the current study.

An antiviral effect was seen in 48.9% of subjects after 48 weeks of treatment, but SVR was judged in only 5.9% of subjects at the end of the follow-up observation period. On the other hand, in a clinical study of combination therapy with PEG IFN-α-2a plus RBV conducted in Japan in patients with naïve chronic hepatitis C (HCV genotype 1b, high viral load), the viral disappearance rate was 86.9% (86/99) and SVR was judged in 59.4% (57/96) of subjects at EOT.[12] In the combination therapy with ME3738 plus PEG IFN-α-2a, the proportion of subjects with SVR was markedly lower than that of subjects who achieved viral disappearance after 12 or 48 weeks of treatment, suggesting that suppression of viral reactivation is weaker with the combination therapy that includes ME3738 than with the combination therapy that includes RBV.

In addition, there were no differences in SVR among the three different ME3738 doses.

In terms of the influence of ME3738 treatment on ALT levels, no consistent tendency could be found in the combination therapy used in this study.

In the safety evaluation, most adverse events noted in this study were comparable in severity and frequency with the events frequently noted with PEG IFN-α-2a monotherapy,[13] and no new adverse events were noted with the ME3738 combination therapy, apart from the two events described below. This suggests that the adverse events noted with ME3738 plus PEG IFN-α-2a combination therapy were not substantially different in severity and frequency from the adverse events noted with PEG IFN-α-2a monotherapy, and that the safety of ME3738 combination therapy is high.

Increased hyaluronic acid and increased blood immunoglobulin G were adverse events that were not frequently seen with PEG IFN-α-2a monotherapy, but were frequently seen in this study. Because these parameters are seldom determined in a time-course manner in

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Table 4 Most common adverse events

n (%)	PEG IFN-α-2a† + ME3738 50 mg/day (*n* = 46)	PEG IFN-α-2a† + ME3738 200 mg/day (*n* = 45)	PEG IFN-α-2a† + ME3738 800 mg/day (*n* = 44)	Total (*n* = 135)
Eye disorders				
Retinopathy	14 (30.4%)	15 (33.3%)	13 (29.5%)	42 (31.1%)
Gastrointestinal disorders				
Diarrhea	19 (41.3%)	14 (31.1%)	9 (20.5%)	42 (31.1%)
General disorders and administration site conditions				
Malaise	21 (45.7%)	18 (40.0%)	18 (40.9%)	57 (42.2%)
Fever	27 (58.7%)	22 (48.9%)	31 (70.5%)	80 (59.3%)
Infections and infestations				
Nasopharyngitis	16 (34.8%)	15 (33.3%)	12 (27.3%)	43 (31.9%)
Metabolism and nutrition disorders				
Decreased appetite	8 (17.4%)	8 (17.8%)	13 (29.5%)	29 (21.5%)
Musculoskeletal and connective tissue disorders				
Arthralgia	14 (30.4%)	15 (33.3%)	11 (25.0%)	40 (29.6%)
Nervous system disorders				
Headache	17 (37.0%)	15 (33.3%)	16 (36.4%)	48 (35.6%)
Skin and subcutaneous tissue disorders				
Alopecia	13 (28.3%)	14 (31.1%)	8 (18.2%)	35 (25.9%)
Pruritus	11 (23.9%)	15 (33.3%)	17 (38.6%)	43 (31.9%)
Investigations				
White blood cell count decreased	40 (87.0%)	40 (88.9%)	37 (84.1%)	117 (86.7%)
Red blood cell count decreased	20 (43.5%)	14 (31.1%)	21 (47.7%)	55 (40.7%)
Hemoglobin decreased	24 (52.2%)	15 (33.3%)	23 (52.3%)	62 (45.9%)
Hematocrit decreased	18 (39.1%)	17 (7.8%)	21 (47.7%)	56 (41.5%)
Platelet count decreased	35 (76.1%)	39 (86.7%)	36 (81.8%)	110 (81.5%)
Neutrophil count decreased	32 (69.6%)	37 (82.2%)	38 (86.4%)	107 (79.3%)
Aspartate aminotransferase increased	11 (23.9%)	11 (24.4%)	10 (22.7%)	32 (23.7%)
Blood triglycerides increased	11 (23.9%)	8 (17.8%)	8 (18.2%)	27 (20.0%)
Hyaluronic acid increased	23 (50.0%)	21 (46.7%)	19 (43.2%)	63 (46.7%)
Blood immunoglobulin G increased	10 (21.7%)	8 (17.8%)	9 (20.5%)	27 (20.0%)
Total	46 (100%)	45 (100%)	43 (97.7%)	134 (99.3%)

†Pegylated interferon-α-2a.

regular clinical practice, these two events have not been previously reported, and it was unclear whether these events were caused by ME3738 plus PEG IFN-α-2a combination therapy or by the primary disease itself. Furthermore, in subjects who developed these two events, no particular symptoms or findings were reported, suggesting that these two events may not be clinically problematic.

In a double-blind clinical study conducted in Japan comparing PEG IFN-α-2a plus RBV combination therapy and PEG IFN-α-2a plus placebo (48-week treatment) combination therapy, the reported incidence of decreased hemoglobin was 89.9% and 49.5%, respectively,[12] and the incidence of decreased hemoglobin noted in the present study was 45.9%. Considering the severity of decreased hemoglobin, the proportion of subjects in whom the hemoglobin level decreased to less than 10 g/dL and that resulted in dose reduction or withdrawal of RBV was 33.0% in the group receiving the PEG IFN-α-2a plus RBV combination and 4.0% in the group receiving the PEG IFN-α-2a plus placebo combination.[12] In the present study, the proportion of subjects in whom the hemoglobin level decreased to less than 10 g/dL during the study period was 19.3%.

In terms of the incidence of decreased hemoglobin, when patient background factors were compared

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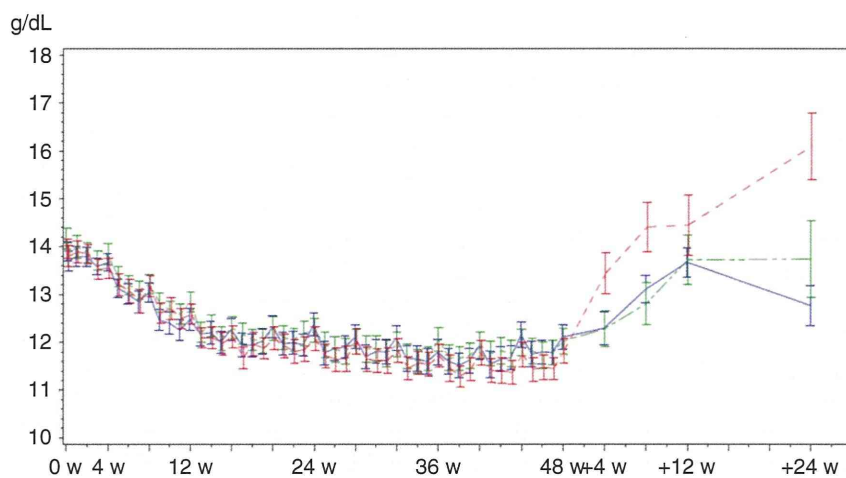

Figure 2 Time-course changes in hemoglobin levels. ----, ME3738 50 mg/day; -··-, ME3738 200 mg/day; —, ME3738 800 mg/day.

between the present study and the above double-blind study (the RBV co-administration study), the mean age was higher in the present study by approximately 5 years, and the male : female ratio was 4:6 in the present study, but 7:3 in the RBV co-administration study.[12] Furthermore, the mean baseline hemoglobin level was 14.0 g/dL in the present study, but 14.76 g/dL in the RBV co-administration study.[13] The incidence of decreased hemoglobin was lower in the present study than in the RBV co-administration study despite less favorable patient background factors in the present study.

As described, ME3738 was found to have a good safety profile and showed inflammatory suppression effects in the liver. In addition, Ogasawara *et al.* reported that ME3738 showed antiproliferative effects on liver cancer cells in an *in vitro* and *in vivo* liver tumor nude mouse model using hepatocellular carcinoma cell lines.[14] These findings suggest that long-term co-administration of ME3738 (which does not cause severe hemoglobin decrease) with PEG IFN is a therapeutic option anticipated to suppress inflammation in the liver, decrease the amount of HCV RNA, suppress proliferation of HCV, and suppress the onset of liver cancer in patients with chronic hepatitis C in whom the standard combination therapy with PEG IFN and RBV cannot be used because of a decrease in hemoglobin levels or because the patient is elderly.

ME3738 was concurrently used with PEG IFN-α-2a treatment; however, a clear additional effect on SVR was not confirmed in this trial.

ACKNOWLEDGMENTS

THE AUTHOR GRATEFULLY acknowledges the co-investigators in the affiliated hospital, and all investigators who participated in this study. In addition to the hospitals of the study authors, data were supplied by the following hospitals: Department of Hepatology, Yoshida Hospital; Department of Gastroenterology, Showa University School of Medicine; Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital; Department of Gastroenterology, National Center for Global Health and Medicine Center Hospital; Department of Hepatology, Toranomon Hospital; Department of Gastroenterology, Juntendo University School of Medicine, Shizuoka Hospital; Division of Liver and Biliary Diseases, Department of Internal Medicine, Fujita Health University School of Medicine; Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science; Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital; Department of Hepatology, Osaka City University Graduate School of Medicine; Department of Gastroenterology, National Hospital Organization(NHO), Osaka Minami Medical Center; Department of Internal Medicine, NHO, Minamiwakayama Medical Center; Department of Gastroenterology, Osaka City Juso Hospital; Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine; Department of Internal Medicine (Gastroenterology), Toyama Prefectural Central Hospital; Department of Internal Medicine, Tonami Municipal Tonami General Hospital;

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Inhibitory Effects of Caffeic Acid Phenethyl Ester Derivatives on Replication of Hepatitis C Virus

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Abstract

Caffeic acid phenethyl ester (CAPE) has been reported as a multifunctional compound. In this report, we tested the effect of CAPE and its derivatives on hepatitis C virus (HCV) replication in order to develop an effective anti-HCV compound. CAPE and CAPE derivatives exhibited anti-HCV activity against an HCV replicon cell line of genotype 1b with EC_{50} values in a range from 1.0 to 109.6 μM. Analyses of chemical structure and antiviral activity suggested that the length of the n-alkyl side chain and catechol moiety are responsible for the anti-HCV activity of these compounds. Caffeic acid n-octyl ester exhibited the highest anti-HCV activity among the tested derivatives with an EC_{50} value of 1.0 μM and an SI value of 63.1 by using the replicon cell line derived from genotype 1b strain Con1. Treatment with caffeic acid n-octyl ester inhibited HCV replication of genotype 2a at a similar level to that of genotype 1b irrespectively of interferon signaling. Caffeic acid n-octyl ester could synergistically enhance the anti-HCV activities of interferon-alpha 2b, daclatasvir, and VX-222, but neither telaprevir nor danoprevir. These results suggest that caffeic acid n-octyl ester is a potential candidate for novel anti-HCV chemotherapy drugs.

Citation: Shen H, Yamashita A, Nakakoshi M, Yokoe H, Sudo M, et al. (2013) Inhibitory Effects of Caffeic Acid Phenethyl Ester Derivatives on Replication of Hepatitis C Virus. PLoS ONE 8(12): e82299. doi:10.1371/journal.pone.0082299

Editor: Hak Hotta, Kobe University, Japan

Received September 15, 2013; **Accepted** October 31, 2013; **Published** December 17, 2013

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Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Hepatitis C virus (HCV) is well known as a major causative agent of chronic liver disease including cirrhosis and hepatocellular carcinoma and is thought to persistently infect 170 million patients worldwide [1]. HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome that is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb [2]. The single polypeptide coded by the genome is composed of 3,000 amino acids and is cleaved by host and viral proteases, resulting in 10 proteins, which are classified into structural and nonstructural proteins [3]. The viral genome is transcribed by a replication complex consisting of NS3 to NS5B and host factors [4]. NS3 forms a complex with NS4A and becomes a fully active form to cleave the C-terminal parts of the nonstructural proteins. The advanced NS3/4A protease inhibitors, telaprevir and boceprevir, have been employed in the treatment of chronic hepatitis C patients infected with genotype 1 [5]. Sustained virologic response (SVR) was reportedly 80% in patients infected with genotype 1 following triple combination therapy with pegylated interferon, ribavirin, and telaprevir [6], although the therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7]. In addition, there is the possibility of the emergence of drug-resistant viruses following treatment with those anti-HCV drugs [8] Thus, further study is required for development of safer and more effective anti-HCV compounds.

Several recent reports indicate that silbinin [9], epigallocatechin-3-gallate [10], curcumin [11], quercetin [12] and proanthocyanidins [13], which all originate from natural sources, have exhibited inhibitory activity against HCV replication in cultured cells. Caffeic acid phenethyl ester (CAPE) is an active component included in propolis prepared from honeybee hives, and has a similar structure to flavonoids (Fig. 1A). CAPE has multifunctional properties containing anti-inflammatory [14], antiviral [15], anticarcinogenic [16], and immunomodulatory activities [15]. CAPE also inhibits enzymatic activities of endogenous and viral proteins [17–19] and transcriptional activity of NF-kappaB [14,20]. In addition, CAPE could suppress HCV replication enhanced by using the NF-kappaB activation activity of morphine [21], although it has been unknown which of moieties including CAPE is responsible for anti-HCV activity. Furthermore, it is not clear whether chemical modification of CAPE could enhance anti-HCV activity or not. In this report, we examined the effect of

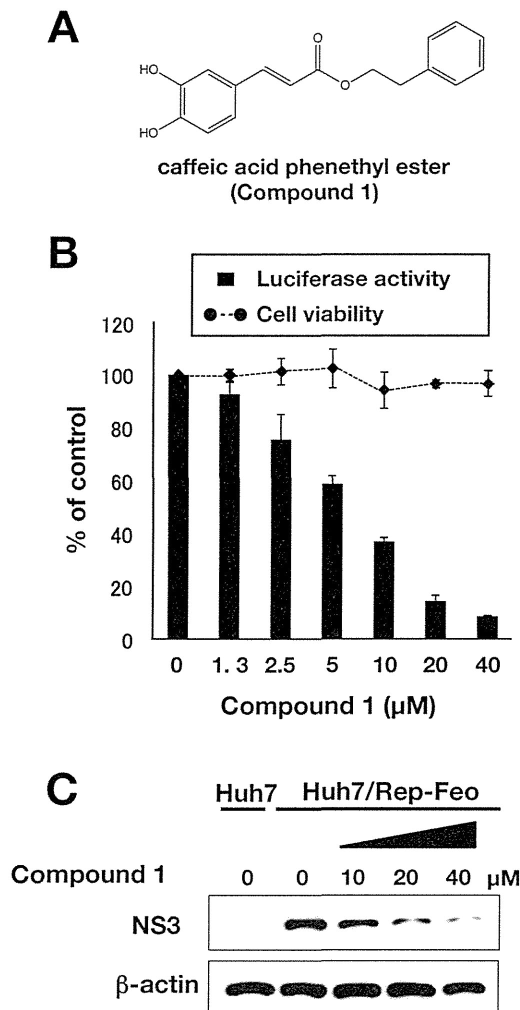


Figure 1. Effect of CAPE on viral replication in the replicon cell line of genotype 1b. (A) Molecular structure of CAPE. (B) Huh7/Rep-Feo cells were incubated for 72 h in a medium containing various concentrations of CAPE. Luciferase and cytotoxicity assays were carried out by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent results from three independent experiments. (C) Protein extract was prepared from Huh7/Rep-Feo cells treated for 72 h with the indicated concentration of CAPE and it was then was subjected to Western blotting using antibodies to NS3 and beta-actin.
doi:10.1371/journal.pone.0082299.g001

CAPE derivatives on HCV proliferation to develop more effective and safer anti-HCV compounds.

Results

Effect of CAPE on HCV RNA replication in HCV subgenomic replicon cells

CAPE is composed of ester of caffeic acid and phenethyl alcohol (Fig. 1A). We examined the effect of CAPE (compound **1**) on both viral replication and cell growth in the HCV subgenomic replicon cell line Huh7/Rep-Feo. The replicon cell line was treated with various concentrations of compound **1**. The replication level of the HCV RNA was measured as an enzymatic activity of luciferase, which is bicistronically, encoded on the replicon RNA. Compound **1** suppressed HCV RNA replication at concentrations from 1.3 to 40 μM in a dose-dependent manner, but did not affect cell viability (Fig. 1B). HCV NS3, which is a viral protease, was decreased at the protein level by treatment with CAPE in a dose-dependent manner, corresponding to the viral replication, whereas beta-actin was not changed in the replicon cell line (Fig. 1C). Based on the calculation using a dose dependency of CAPE, compound **1** exhibited an EC_{50} value of 9.0 μM and a CC_{50} value of 136.1 μM, giving a selectivity index estimate (SI) of 17.9 (Table 1). These results suggest that treatment with CAPE inhibits HCV replication in HCV subgenomic replicon cells.

Structure-activity relationship of CAPE analogues

To clarify the structure-activity relationship of CAPE analogues, we examined the effect of hydroxyl groups on the aromatic ring (catechol moiety), the alkenyl moieties on alpha, beta-unsaturated esters, and the ester parts as follows (Figure S1).

We tested whether commercially available CAPE-related compounds **2** to **6** (Fig. S1) affected HCV replication (Table 1). All these compounds showed weaker inhibitory activity than CAPE (**1**), but are not toxic. Compound **2**, which is the acid component of CAPE, showed a slightly lower value of EC_{50} than compound **3**, which is the compound **2** derivative replaced a hydroxyl group with a methoxyl group of catechol moiety, while compound **4**, which is the derivative lacking two hydroxyl groups within catechol moiety, exhibits a higher value of EC_{50} than compounds **1** and **2**. These data suggest that the catechol moiety of CAPE is required for anti-HCV activity. Interestingly, compounds **5** and **6**, which are natural products including polyhydroxylated acid moieties in the ester parts, showed much weaker inhibitions than compound **1** and exhibits low *C*log *P* values. The position of hydroxyl group or/and the structure of the ester part may affect the inhibitory activity and/or hydrophobicity.

We next examined the effects of caffeic acid ester compounds **7** to **11**, which include various lengths of alkyl side chains, on HCV replication (Table 2 and Figure S2). The EC_{50} values decreased in the order methyl ester (compound **7**), n-butyl ester (compound **8**), n-hexyl ester (compound **9**), and n-octyl ester (compound **10**), suggesting that elongation of the n-alkyl side chain increased the inhibitory activity. However, the EC_{50} value of n-dodecyl ester (compound **11**) was higher than that of compound **10**. Thus, n-octyl ester (compound **10**) showed the lowest EC_{50} value and the highest SI among the tested compounds shown in Tables 1 and 2. Compounds **7** to **11** gradually increased own *C*log *P* values,

Table 1. Effect of CAPE (**1**) and related compounds **2–6** on HCV replication.

Compound (Number)	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	*C*log *P* [d]
CAPE (**1**)	9.0±0.7	136.1±1.9	17.9	3.30
caffeic acid (**2**)	36.6±6.7	>320	>8.7	0.98
ferulic acid (**3**)	71.9±5.8	>320	>4.5	1.42
cinnamic acid henethyl ester (**4**)	86.1±6.3	>320	>3.7	4.56
chlorogenic acid (**5**)	103.0±3.4	>320	>3.1	−0.96
rosmarinic acid (**6**)	109.6±1.1	>320	>2.9	1.10

a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t001

corresponding to length of n-alkyl side chain (Fig. 2A). Compounds **10** and **11** exhibit EC_{50} values of 2.7 and 5.9 μM, respectively, SI values of 29.6 and 9.80, respectively, and *C*log *P* values of 4.90 and 5.96, respectively, suggesting that high hydrophobic property of n-alkyl side chain decreases anti-HCV activity. The appropriate *C*log *P* value of caffeic acid ester containing unsaturated side chain may be around 5.

Dihydrocaffeic acid methyl ester (compound **12**) showed less activity than caffeic acid methyl ester (compound **7**) regardless of values of *C*log *P* value and CC_{50}, suggesting that the alpha, beta-unsaturated part attached to ester affects the anti-HCV activity level (Table 3 and Figure S3).

We further examined the effect of the hydroxyl groups on the aromatic ring on HCV replication (Table 4 and Figure S4). The EC_{50} values of *O*-methylated caffeic acid n-octyl esters (compounds **13** and **14**) were higher than that of compound **10**. Compounds **15** including 3, 4-di-*O*-methylated caffeic acid n-octyl ester exhibited higher EC_{50} than values of compounds **10**, **13** and **14**. However, addition of a third hydroxyl group to 3, 4, 5-trihydroxy derivative (compound **16**) of compound **10** resulted in a reduction of anti-HCV activity. Furthermore, *C*log *P* values of compound **10**, **13**, **14**, **15** and **16** were not correlated with anti-HCV activity (EC_{50} value) (Fig. 2B). These results suggest that the catechol moiety plays an important role in anti-HCV activity, and that the 4-hydroxy moiety is more important for the activity than the 3-hydroxy moiety.

Thus, compound **10**, which exhibits the lowest EC_{50} value and the highest SI value, is the most effective compound among CAPE analogues used in this study.

Effect of CAPE derivatives on virus production

The structure of compound **10** is shown in Fig. 3A. Treatment with compound **10** reduced HCV replication and NS3 protein in a dose-dependent manner at a higher anti-HCV level than

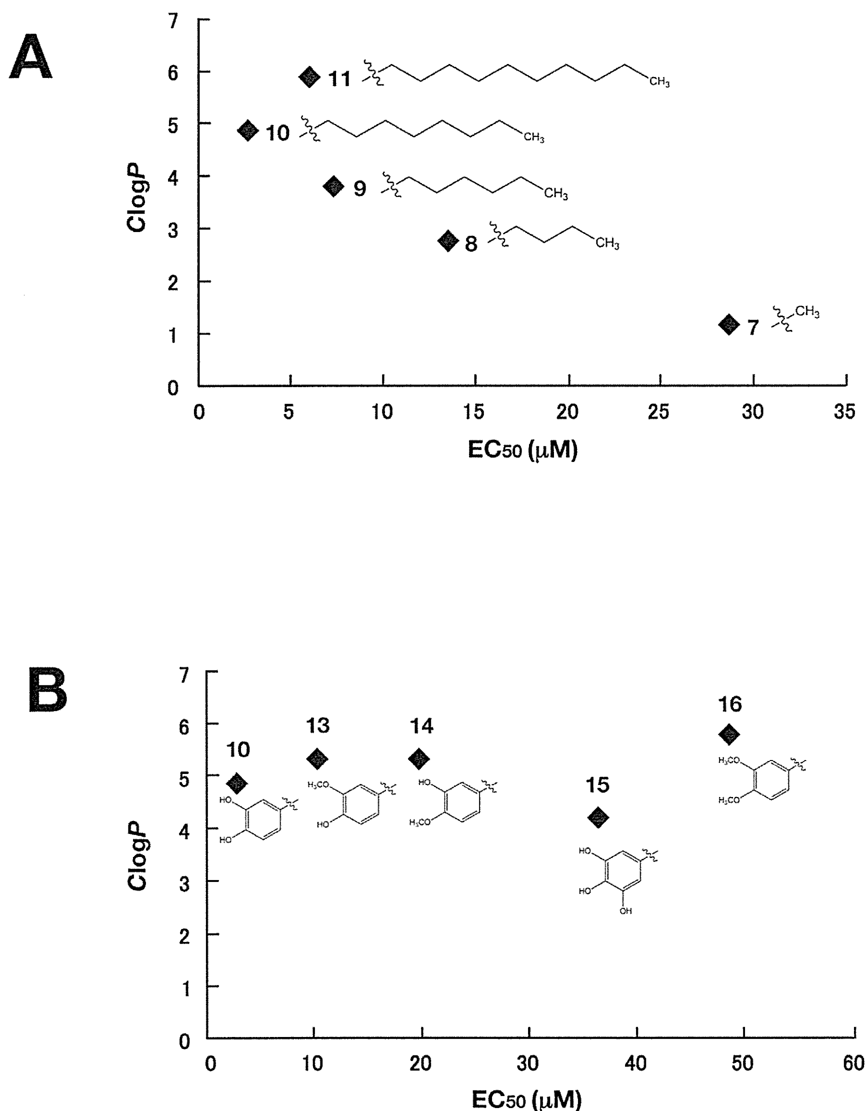


Figure 2. Correlation between the inhibitory effect on HCV replication *and C*log *P* of CAPE analogues. Values of *x*-axis indicate EC_{50} values of CAPE analogues, while values of *y*-axis show *C*log *P* values. (A) Correlation between the inhibitory effect on HCV replication and *C*log *P* of CAPE analogues (Compound 7–11). (B) Correlation between the inhibitory effect on HCV replication and *C*log *P* of CAPE analogues (Compound 10 and 13–16).
doi:10.1371/journal.pone.0082299.g002

Table 2. Effect of caffeic acid esters **7, 9–14**, including **1**, on HCV replication.

Compound No.	R	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
7	CH_3	28.6±1.2	122.1±5.0	4.2	1.20
8	C_4H_9	13.5±2.1	39.0±1.1	2.9	2.79
9	C_6H_{13}	7.3±0.2	37.6±1.2	5.1	3.85
10	C_8H_{17}	2.7±0.1	71.7±8.5	26.6	4.90
11	$C_{10}H_{21}$	5.9±0.9	57.9±2.9	9.8	5.96
1	$(CH_2)_2Ph$	9.0±0.7	136.1±1.9	17.9	3.30

The basic structure and side moieties are shown in Figure S2.
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t002

compound **1** (Figs. 3B and C), but not effect enzymatic activities of firefly and *Renilla* luciferases (Fig. 3D) and IRES-dependent translation (Fig. 3E), suggesting that inhibition of HCV replication by compound **10** is not due to offtarget effect. We evaluated the inhibitory effect of compound **10** on three different subgenomic replicon cell lines (1b: N strain, Con1 strain, 2a: JFH-1 strain) and one full genome replicon cell line (1b: O strain). Compound **10** inhibited the viral replication of all replicon cell lines at similar level, and exhibited the lowest EC_{50} value of 1.0 μM and an SI value of 63.1 by using Con1 replicon cells (Table 5). We next examined the effect of compound **10** on virus production by using HCVcc, since subgenomic replicon mimics HCV replication, but not the whole viral cycle. The Huh7 OK1 cell line, which is highly permissive to the HCV JFH1 strain [22], was infected with HCVcc and then treated with compound **10** at 24 h post-infection. The supernatant was harvested 72 h post-infection from the culture supernatant and then the RNA that prepared from the supernatant was estimated by real time qRT-PCR. Figure 3F shows that treatment with compound **10** reduced HCV viral production ($EC_{50} = 1.8 \pm 0.4$ μM) in a similar way to the data obtained by using a replicon cell line. To clarify whether or not compound **10** inhibited HCV replication via interferon-signaling pathway, we analyzed ISRE activity and the expression of interferon stimulated gene (ISG) by using reporter assay and RT-PCR, respectively. The replicon cells were harvested at 48 h post-treatment. There were no significant effects of compound **1**, **6** and **10** on ISRE-promoter activities, while interferon alpha 2b significantly enhanced it as a positive control (Fig.4 A). The data of the RT-PCR analysis showed that the transcriptional expressions of ISGs including Mx1, MxA, IFIT4, ISG15, OAS1, OAS2, and OAS3 were induced with interferon alpha 2b, but not with compound **1**, **6** and **10** (Fig. 4B). These data suggest that the CAPE derivatives have an inhibitory effect on virus production and replication, irrespective of interferon signaling induction.

Synergistic effect of caffeic acid n-octyl ester on interferon and direct-acting antiviral agents

To estimate the effects of drug combinations on anti-HCV activity, we examined the antiviral activity of compound **10** in combination with IFN-α 2b, telaprevir (NS3 protease inhibitor), danoprevir (NS3 protease inhibitor), daclatasvir (NS5A inhibitor) or VX-222 (NS5B polymerase inhibitor). Con1 LUN Sb #26 replicon cells were treated with compound **10** in combination with each anti-HCV agent at various concentration rations for 72 h. The effect of each drug combination on HCV replication was analyzed by using CalcuSyn. An explanatory diagram of isobologram was shown at a right end of lower panels of Fig. 5A as described in Materials and Methods. As shown in the resulting isobologram, all plots of the calculated EC_{90} values of compound **10** with IFN-alpha 2b, daclatasvir, or VX-222 are located under the additive line, while the plots of compound **10** with telaprevir, or danoprevir are located above the additive line and closed to the additive line (Fig. 5A). Additionally, we determined the degree of inhibition for each drug combination was analyzed as the combination index (CI) calculation at 50, 75 and 90% of effective concentrations by using CalcuSyn. An explanatory diagram was shown at a right end of lower panels of Fig. 5B as described in Materials and Methods. On the basis of the CalcuSyn analysis, the combination of compound **10** with daclatasvir exhibited strong synergistic effect on inhibition of HCV replication in the replicon cells (Fig. 5B, upper middle). The combination of compound **10** with VX-222 exhibited an additive to synergistic effect, suggesting that it trends toward synergistic (Fig. 5B, upper right), and with IFN-alpha 2b exhibited an antagonistic to synergistic effect, suggesting that it trends toward synergistic (Fig. 5B, upper left). In contrast, the combination of compound **10** with telaprevir resulted in antagonistic effect (Fig. 5B, lower left), and with danoprevir resulted in an antagonistic to additive effect, suggesting it trends toward antagonistic (Fig. 5B, lower middle). These calculated data

Table 3. Effect of caffeic acid esters **7**and **8** on HCV replication.

Compound No.	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
7	28.6±1.2	122.1±5.0	4.2	1.20
12	77.0±1.6	140.7±3.4	1.8	1.02

Chemical structures of both compounds are shown in Figure S3
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t003

Table 4. Effect of octyl esters **10** and **13–16** on HCV replication.

Compound No.	R^1, R^2, R^3	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
10	$R^1=R^2=R^3=H$	2.7±0.1	71.7±8.5	26.6	4.90
13	$R^1=CH_3$, $R^2=R^3=H$	10.2±1.1	60.3±1.6	5.9	5.35
14	$R^1=R^3=H$, $R^2=CH_3$	19.6±0.8	59.2±1.4	3	5.35
15	$R^1=R^2=CH_3$, $R^3=H$	48.5±1.7	212.4±6.9	4.4	5.82
16	$R^1=R^2=H$, $R^3=OH$	36.3±2.9	59.8±6.9	1.6	4.24

The basic structure and side moieties are shown in Figure S4.
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t004

Figure 3. Effect of compound 10 on the viral replication in the replicon cell line and HCVcc. (A) Molecular structure of compound **10**. (B) Huh7/Rep-Feo cells were incubated for 72 h in a medium containing various concentrations of compound **10**. Luciferase and cytotoxicity assays were carried out by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (C) Protein extract was prepared from Huh7/Rep-Feo cells treated for 72 h with the indicated concentration of compound **10** and it was then subjected to Western blotting using antibodies to NS3 and beta-actin. (D) Huh7 cell line was transfected with pEF Fluc IN encoding firefly luciferase or pEF Rluc IN encoding *Renilla* luciferase. Both transfected cell lines were incubated with DMSO (Control) or 40 µg/ml compound **10**. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data were represented from three independent experiments. (E) Schematic structure of RNA transcribed from the plasmids was shown (Top). The bicistronic gene is transcribed under the control of elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV or HCV IRES. Huh7 cell line was transfected with each plasmid and incubated for 72 h post-treatment with DMSO (control) or 40 µg/ml of compound **10**. Firefly and *Renilla* luciferase activities were measured. Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity was represented as percentage of the control condition. Error bars indicate standard deviation. The data were represented from three independent experiments. (F) Huh7 OK1 cell line was infected with HCVcc derived from JFH-1 strain and then treated with several concentrations of compound **10** at 24 h post-infection. The resulting cells were harvested 72 h post-infection. The viral RNA of supernatant was purified and estimated by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. Treatment with DMSO corresponds to '0'.
doi:10.1371/journal.pone.0082299.g003

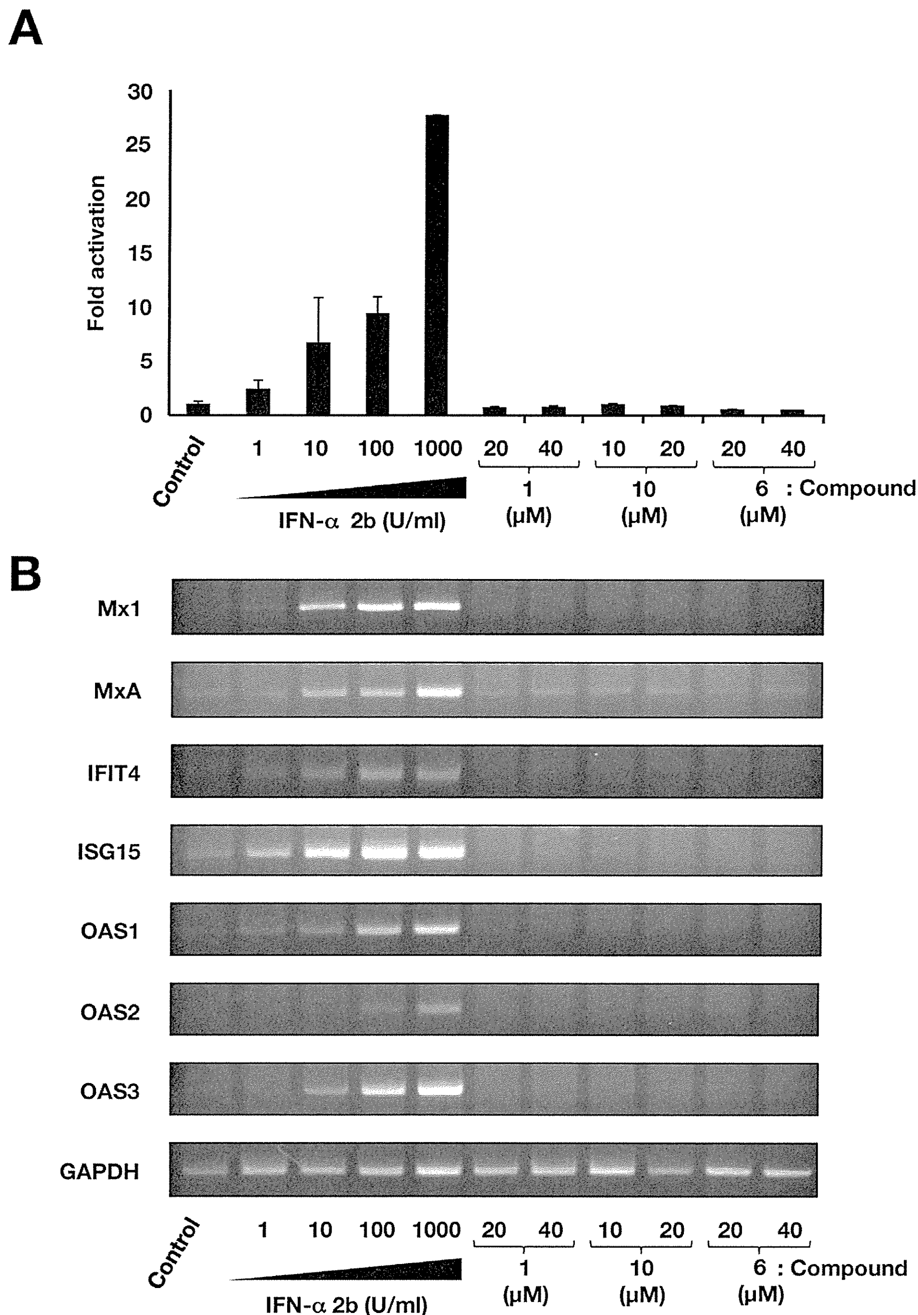


Figure 4. Effect of CAPE derivatives on the interferon-signaling pathway. (A) Plasmids pISRE-TA-Luc and phRG-TK were co-transfect into Huh7 OK1 cells. The transfected cells were cultured with 1, 10, 100, or 1000 U/mL of interferon-alpha 2b, and compounds **1**, **6** and **10**. Treatment with DMSO corresponds to '0'. After 48 h of treatment, luciferase activities were measured, and the value were normalized against *Renilla* luciferase activities. Error bars indicate standard deviation. The data represent three independent experiments. (B) Huh7 replicon cell line of genotype 1b was treated with 1, 10, 100, or 1000 U/mL of interferon-alpha 2b, and compounds **1**, **6** and **10** for 48 h. Treatment with DMSO corresponds to the control. The mRNAs of Mx1, MxA, IFIT4, ISG15, OAS1, OAS2, OAS3, and GAPDH as an internal control were detected by RT-PCR.
doi:10.1371/journal.pone.0082299.g004

of combination tests suggest that daclatasvir, IFN-alpha 2b, and VX-222 synergistically, but telaprevir and danoprevir antagonistically, inhibit HCV replication in combination with compound **10**.

Discussion

CAPE is an active component of propolis, which possesses broad-spectrum biological activities [14–19]. In this study, CAPE suppressed HCV RNA replication in a dose-dependent manner (Fig. 1A and B). Treatment with CAPE inhibited HCV replication with an EC_{50} of 9.0 μM and an SI of 17.9 in Huh7/Rep-Feo cells (Table 1). The treatment of the replicon cell line with CAPE did not induce expression of the IFN-inducible gene (Fig. 4), suggesting that the inhibition of HCV replication by CAPE is independent of the IFN signaling pathway.

Table 5. Anti-HCV activity of compound **10** in replicon cell lines of genotypes 1b and 2a.

Cell line	Replicon type	Strain (Genotype)	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]
Huh7 Rep/Feo-1b	Subgenome	N (1b)	2.7±0.1	71.7±8.5	26.6
Con1 LUN Sb #26	Subgenome	Con1 (1b)	1.0±0.1	63.1±3.1	63.1
Huh7 Rep/Reo-2a	Subgenome	JFH1 (2a)	1.0±0.3	60.0±2.3	60.0
OR6	Full genome	O (1b)	1.5±0.4	61.7±0.6	41.1

a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the inhibition of HCV replication.
c: Selectivity Index (CC_{50}/EC_{50}).
doi:10.1371/journal.pone.0082299.t005

We also examined the effect of CAPE derivatives on HCV replication. Our data suggest that the n-alkyl side chain and catechol moiety of the CAPE derivative are critical in its anti-HCV activity (Tables 2 and 3). The EC_{50} value of the derivative decreased dependently on the length of the n-alkyl side chain until reaching octyl ester length (Table 2), while longer chains than octyl ester of a derivative led to an increase in the EC_{50} value and *C*log *P* value. Compound **10**, Caffeic acid n-octyl ester, exhibited the highest anti-HCV activity among the tested compounds with an EC_{50} value of 2.7 μM and an SI value of 26.6. Cyclosporine A and its analogues could suppress the viral replication of genotype 1b at a higher level than that of genotype 2a [23]. Interestingly, compound **10** could inhibit HCV replication of genotype 1b and 2a at a similar level, irrespective of expression of the interferon-inducible gene (Fig. 4). CAPE and its derivatives may therefore possess a mechanism different from cyclosporine A and its analogues with respect to anti-HCV activity.

CAPE has been reported to be an inhibitor of NF-kappaB [14,20]. Lee et al. reported that the catechol moiety in CAPE was important for inhibition of NF-kappaB activation [24]. The data shown in Table 3 suggest that the catechol moiety in CAPE is critical to the anti-HCV activity of compound **10**. Previous studies have implicated the inhibition of NF-kappaB in anti-HCV activity. Treatment with an extract prepared from *Acacia confusa* [25] or San-Huang-Xie-Xin-Tang [26] could suppress HCV replication and inhibit NF-kappaB activation. However, Chen et al. reported that curcumin-mediated inhibition of NF-kappaB did not contribute to anti-HCV activity [11]. Furthermore, treatment with *N*′-(Morpholine-4carbonyloxy)-2(naphthalene-1-yl) acetimidamide could activate NF-kappaB and downstream gene expression in the same Huh7/Rep-Feo replicon cell line as the cell line used in this study and exhibited potent inhibition of HCV replication without interferon signaling [27]. These reports support the notion that CAPE derivatives do not mainly target NF-kappaB activity as part of their anti-HCV activity.

Several host proteins have been reported to regulate function of NS5A, leading to supporting HCV replication (review in [2,28]). Daclatasvir exhibited potent synergistic effect on anti-HCV activity in combination of compound **10** (Fig. 5). Anti-HCV activity of compound **10** might associate with intrinsic functions of host factors that interact with NS5A. NS3 protease inhibitors exhibited antagonistic effect in combination of compound **10** (Fig. 5). The inhibitory effect of compound **10** might be mediated by the activation of an unknown endogenous protease that is nonspecifically suppressed by NS3 protease inhibitors. Further study to clarify the mechanism by which compound **10** suppresses HCV replication might contribute to identification of a novel host factor as a drug target for development of the effective compound supporting an effect of other anti-HCV drugs.

In conclusion, we showed that CAPE and its analogue possess a significant inhibitory effect against HCV replication. The length of n-alkyl side chains and the catechol moiety of CAPE are critical to its inhibitory activity against HCV replication. The most effective derivative among the tested compounds was caffeic acid n-octyl ester, which exhibited an EC_{50} value of 1 μM and an SI value of 63.1 in the replicon cell line of genotype 1b strain Con1. Treatment with caffeic acid n-octyl ester reduced the viral replication of genotype 1b and 2a at a similar level and inhibited viral production of HCVcc. Treatment with caffeic acid n-octyl ester could synergistically enhance the anti-HCV activities of IFN-α 2b, daclatasvir, and VX-222, but neither telaprevir nor danoprevir. Further investigation to clarify the mechanism of anti-HCV activity and further modification of the compound to improve anti-HCV activity will lead to novel therapeutic strategies to treat chronic hepatitis C virus infection.

Materials and Methods

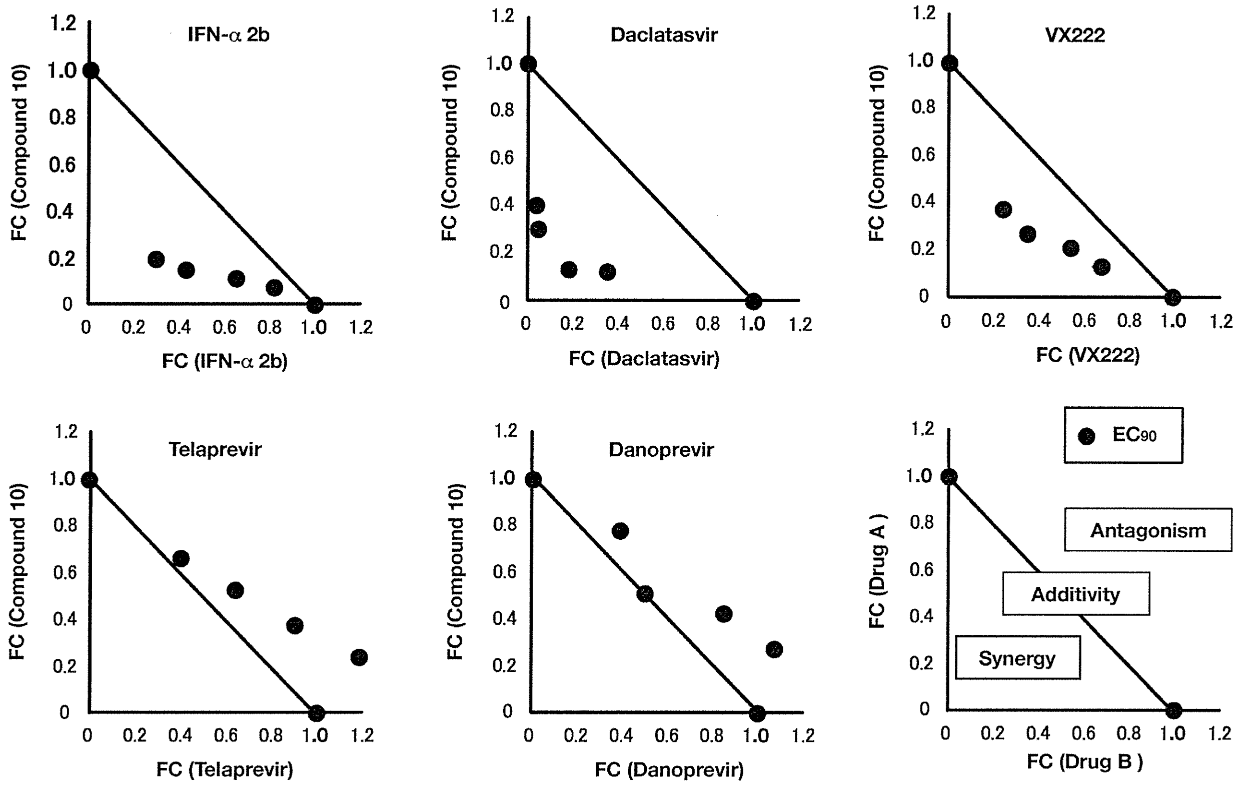
Compounds

Boldface numbers in this text indicate the compound numbers shown in Tables. All chemical structures of compounds used in this study are shown in figure S1. CAPE (**1**), caffeic acid (**2**), ferulic acid (**3**), and chlorogenic acid (**5**) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cinnamic acid phenethyl ester (**4**) was from Tokyo Chemical Industry (Tokyo, Japan). Rosmarinic acid (**6**) was from Wako Pure Chemical (Tokyo, Japan). Caffeic acid n-octyl ester (n-octyl caffeate) (**10**), 3-O-methylcaffeic acid n-octyl ester (n-octyl-3-methylcaffeate) (**13**), 4-O-methylcaffeic acid n-octyl ester (n-octyl-3-methylcaffeate) (**14**), and 3, 4-O-dimethylcaffeic acid n-octyl ester (n-octyl-3, 4-methylcaffeate) (**15**) were from LKT Laboratories (St. Paul, MN, USA).

Caffeic acid esters **7**, **8**, **9**, and **11** were synthesized by preparing caffeic acid chloride followed by treatment with corresponding alcohols [29]. Dihydrocaffeic acid ester **12** was prepared by hydrogenation of **7**. Compound **16** is a newly synthesized ester. Spectroscopic data of known esters **7–9**, and **11** prepared here were identical to those reported [30-32] Interferon alfa-2b (IFN-α 2b) was obtained from MSD (Tokyo, Japan). Telaprevir and daclatasvir were purchased from Selleckchem (Houston, TX, USA). Danoprevir and VX-222 were from AdooQ BioScience (Irvine, CA, USA).

Chemistry of 3,4,5-Trihydroxycinnamic acid n-octyl ester

3,4,5-Trihydroxycinnamic acid n-octyl ester (**16**) was prepared by condensation of corresponding benzaldehydes with malonic acid n-octyl monoester [33]. A solution of malonic acid n-octyl monoester (432 mg, 2 mmol), 3,4,5-trihydroxybenzaldehyde (462 mg, 3 mmol) and piperidine (0.2 mL) in pyridine (2 mL)


A
IFN-α 2b
FC (Compound 10)
FC (IFN-α 2b)
Daclatasvir
FC (Daclatasvir)
VX222
FC (VX222)
Telaprevir
FC (Telaprevir)
Danoprevir
FC (Danoprevir)
EC90
Antagonism
Additivity
Synergy
FC (Drug A)
FC (Drug B)

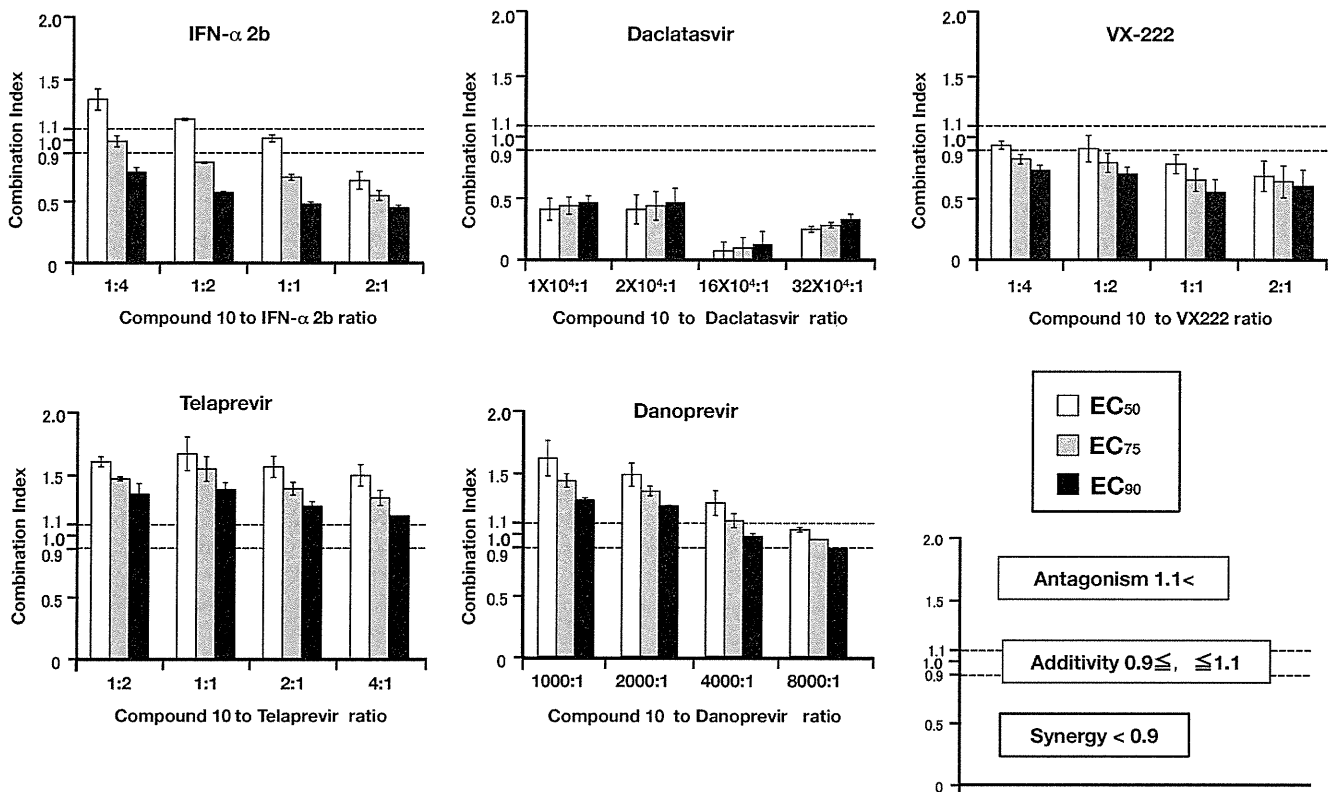

B
IFN-α 2b
Combination Index
1:4
1:2
1:1
2:1
Compound 10 to IFN-α 2b ratio
Daclatasvir
1X10⁴:1
2X10⁴:1
16X10⁴:1
32X10⁴:1
Compound 10 to Daclatasvir ratio
VX-222
Compound 10 to VX222 ratio
Telaprevir
4:1
Compound 10 to Telaprevir ratio
Danoprevir
1000:1
2000:1
4000:1
8000:1
Compound 10 to Danoprevir ratio
EC50
EC75
EC90
Antagonism 1.1<
Additivity 0.9≦, ≦1.1
Synergy < 0.9

Figure 5. Synergistic effect analyses for the combination of compound 10 with IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir. The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was treated for 72h with combinations of compound 10 and IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir. Luciferase assay were carried out as described in Materials and Methods. (A) The calculated EC_{90} values for combination were plotted as the fractional concentration (FC) of compound 10 and one of IFN-α 2b, daclatasvir, VX-222, telaprevir, and danoprevir on the *x* and *y* axes, respectively. Synergy, antagonism and additivity are indicated in a representative graph as a right end of lower graphs and are described in Materials and Methods. (B) Combination indexes of compound 10 with IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir at the EC50, EC75, and EC90 values were measured at various drug rations. Synergy, antagonism and additivity are indicated in a representative graph as a right end of lower graphs and are described in Materials and Methods.
doi:10.1371/journal.pone.0082299.g005

was heated at 70°C for 1 h. The reaction mixture was concentrated under a vacuum to give a residue, which was dissolved in $CHCl_3$-IPA (3:1, v/v) and then washed with 10% HCl and water. The organic layer was dried over Na_2SO_4 and evaporated to give a residue, which was purified by silica gel column chromatography using AcOEt-hexane (1:1, v/v) as eluent to give the corresponding n-octyl ester (85 mg, 13.8%) as a pale powder. FT-IR νmax (KBr): 3389, 3239, 2923, 1675, 1627, 1606 cm^{-1}. ^1H NMR (400MHz, CD_3OD) δ: 0.86 (3H, t, *J* = 7.2 Hz), 1.20–1.40 (10H, m), 1.65 (2H, quintet, *J* = 6.4 Hz), 4.11, (2H, t, *J* = 6.4 Hz), 6.16 (2H, d, *J* = 15.6 Hz), 6.55 (2H, s), 7.40 (2H, d, *J* = 15.6 Hz). 13C NMR (100 Hz, CD_3OD) δ: 14.4, 23.7, 27.1, 29.8, 30.3, 30.4, 32.9, 65.6, 108.5, 115.3, 126.6, 137.5, 147.1, 169.4. CI MS *m/z*: 309 (M^++H). High-resolution CI MS calcd. for $C_{17}H_{25}O_5$ (M^++H) for 309.1702. Found: 309.1686.

Replicon cell lines and virus infection

The Huh7/Rep-Feo cell line, which harbors the subgenomic replicon RNA composed of HCV IRES, the gene of the fusion protein consisting of neomycin phosphotransferase and firefly luciferase, EMCV IRES and a nonstructural gene of genotype 1b strain N in order in Huh7 cell line, was previously established [34]. Thus, the luciferase activity corresponds to the level of HCV RNA replication. The cell line was maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 0.5 mg/mL G418 and cultured in absence of G418 when they were treated with compounds. The Lunet/Con1LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was described previously [35]. The OR6 cell line, which harbors the full genomic replicon RNA of the O strain (genotype 1b), was described previously [36]. The HCV replicon cell line derived from the genotype 2a strain JFH1 was described previously [37]. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [38]. The virus was amplified by the several times passages. The cells were infected with the virus at a multiplicity of infection (moi) of 1 and then treated with each compound at 24 h post-infection. The culture supernatants were harvested 72 h post-treatment to estimate the viral RNA as described below.

Determination of luciferase activity in HCV replicon cells

The replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. Compounds were added to the culture medium to give various concentrations. The resulting cells were harvested 72 h post-treatment and lysed with cell culture lysis reagent (Promega, Madison, WI). The luciferase activity of each cell lysate was estimated using a luciferase assay system (Promega). The resulting luminescence was detected by a Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan).

Determination of Cytotoxicity in HCV replicon cells

The replicon cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and then incubated at 37°C for 24 h. Compounds were added to the culture medium to give various concentrations and were then harvested 72 h post-treatment. Cell viability was measured using a dimethylthiazol carboxymethoxyphenylsulfophenyl tetrazolium (MTS) assay with a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Western Blotting

Western blotting was carried out by the method described previously [39]. The antibodies to NS3 (clone 8G-2, mousemonoclonal, Abcam, Cambridge, UK), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study.

RNA analysis

Total RNAs were prepared from cells by using the RNAqueous-4PCR kit (Life Technologies, Carlsbad, CA). Viral RNA were prepared from culture supernatants by using the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany). The viral RNA genome was estimated by the qRT-PCR method described previously [40]. RT-PCR was carried out by the method described previously [41] which was slightly modified at the PCR step. The PCR samples were incubated once for 10 min at 95°C for an initial activation step of the AmpliTaq Gold DNA Polymerase (Life Technologies), and then subjected to an amplification step of 30 repeats of the cycle consisting of three segments as follow: 0.5 min at 95°C, 1 min at 55°C and 1 min at 72°C. The primers used in this study were as follows: Mx1: 5′-AGCCACTGGACTGACGACTT-3′ and 5′-GAGGGCTGAAAATCCCTTTC-3′;

MxA: 5′-GTCAGGAGTTGCCCTTCCCA-3′ and 5′-ATTCCCATTCCTTCCCCGG-3′;

IFIT4: 5′-CCCTTCAGGCATAGGCAGTA-3′ and 5′-CTCCTACCCGTCACAACCAC -3′; ISG15: 5′-CGCAGATCACCCAGAAGAT-3′ and 5′-GCCCTTGTTATTCCTCACCA-3′;

OAS1: 5′-CAAGCTCAAGAGCCTCATCC-3′ and 5′-TGGGCTGTGTTGAAATGTGT-3′;

OAS2: 5′-ACAGCTGAAAGCCTTTTGGA-3′ and 5′-GCATTAAAGGCAGGAAGCAC-3′;

OAS3: 5′-CACTGACATCCCAGACGATG-3′ and 5′-GATCAGGCTCTTCAGCTTGGC-3′;

GAPDH: 5′-GAAGGTGAAGGTCGGAGTC and 5′- GAAGATGGTGATGGGATTTC-3′

Effects on activities of internal ribosome entry site (IRES) and luciferases

Huh7 OK1 cells were transfected with pEF.Rluc.HCV.IRES.-Feo or pEF.Rluc.EMCV.IRES.Feo [39]. These transfected cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with DMSO or compound **10**, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize

luciferase activity. To evaluate the interferon response, Huh7OK1 cells were seeded on a 48 well plate at a density of 2×10^4 cells per well, and transfected with pISRE-TA-Luc (Takara bio, Shiga, Japan) and phRG-TK (Promega). These transfected cells were incubated in the presence of compounds, IFN-α 2b, or DMSO, and then harvested at 48 h post-treatment. The firefly luciferase and *Renilla* luciferase activities were quantified by using Dual luciferase reporter assay system (Promega).

Prediction of Clog*P* for compounds

The *C*log*P* value deduced from chemical structure roughly corresponds to a value of hydrophobicity. The *C*log*P* values of compounds used in this study were calculated using the computer software Chem Bio Office Ultra 2008 (PerkinElmer, Cambridge, MA, USA).

Synergistic effect of caffeic acid n-octyl ester on anti-HCV activities of other drugs

The effects of drug-drug combinations were evaluated by using the Con1 LUN Sb #26 replicon cells, and were analyzed by using the computer software CalcuSyn (Biosoft, Cambridge, United Kingdom). Dose inhibition curves of two different drugs were plotted with each other. In each drug combination, EC_{90} values of several combinations of two different drugs were plotted as the fractional concentration (FC) of both drugs on the *x* and *y*-axes. Additivity indicates the line linked between 1.0 FC value points of both drugs in the absence of each other. Synergy and antagonism are indicated by values plotted under and above, respectively, an additivity line. The explanatory diagram of isobologram is shown in a right end of lower panels of Figure 5A. Combination indexes (CIs) were calculated at the EC_{50}, EC_{75}, and EC_{90} by using CalcuSyn. A CI value of less than 0.9 indicates synergy. A CI value ranging from 0.9 to 1.1 indicates additivity. A CI value of more than 1.1 indicates antagonism. The explanatory diagram was shown in a right end of lower panels of Figure 5B.

Supporting Information

Figure S1 Molecular structure of CAPE and commercial CAPE-related compounds. CAPE structure is divided into three parts: (I) the catechol moiety, (II) the alkenyl moiety on alpha, beta -unsaturated ester, and (III) the ester part. Molecular structures of CAPE and its commercial derivatives are shown.
(TIF)

Figure S2 The basic structure and side moieties of compounds shown in Table 2. Each compound structure is represented on the basis of the basic structure (top).
(TIF)

Figure S3 The molecular structures of compounds 7 and 12, which are shown in Table 3. Both compounds are different in alpha, beta-unsaturated or saturated part attached to ester.
(TIF)

Figure S4 The basic structure and side moieties of compounds shown in Table 4. Each compound structure is represented on the basis of the basic structure (top).
(TIF)

Acknowledgments

We thank T. Wakita for kindly providing a plasmid.

Author Contributions

Conceived and designed the experiments: MT KM. Performed the experiments: H. Shen AY MN HY MS H. Shindo SM. Analyzed the data: HK TT NE. Contributed reagents/materials/analysis tools: YF MI NK NS. Wrote the paper: H. Shen AY MT KM.

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JOURNAL OF VIRAL HEPATITIS JVH

Journal of Viral Hepatitis, 2012 doi:10.1111/j.1365-2893.2012.01649.x

IL-28B (IFN-λ3) and IFN-α synergistically inhibit HCV replication

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Received March 2012; accepted for publication June 2012

SUMMARY. Genetic variation in the IL-28B (interleukin-28B; interferon lambda 3) region has been associated with sustained virological response (SVR) rates in patients with chronic hepatitis C treated with peginterferon-α and ribavirin. However, the mechanisms by which polymorphisms in the IL-28B gene region affect host antiviral responses are not well understood. Using the HCV 1b and 2a replicon system, we compared the effects of IFN-λs and IFN-α on HCV RNA replication. The anti-HCV effect of IFN-λ3 and IFN-α in combination was also assessed. Changes in gene expression induced by IFN-λ3 and IFN-α were compared using cDNA microarray analysis. IFN-λs at concentrations of 1 ng/mL or more exhibited concentration- and time-dependent HCV inhibition. In combination, IFN-λ3 and IFN-α had a synergistic anti-HCV effect; however, no synergistic enhancement was observed for interferon-stimulated response element (ISRE) activity or upregulation of interferon-stimulated genes (ISGs). With respect to the time course of ISG upregulation, the peak of IFN-λ3-induced gene expression occurred later and lasted longer than that induced by IFN-α. In addition, although the genes upregulated by IFN-α and IFN-λ3 were similar to microarray analysis, interferon-stimulated gene expression appeared early and was prolonged by combined administration of these two IFNs. In conclusion, IFN-α and IFN-λ3 in combination showed synergistic anti-HCV activity *in vitro*. Differences in time-dependent upregulation of these genes might contribute to the synergistic antiviral activity.

Keywords: HCV, IFN-λ, IL-28B, ISG, synergistic inhibition, microarray.

INTRODUCTION

In 2009, reports from three genome-wide association studies revealed that several single-nucleotide polymorphisms (SNPs) (rs12979860, rs12980275 and rs8099917) around the IL-28B (interleukin-28B; interferon lambda 3) gene are strongly associated with sustained viral response (SVR) to PEG-IFN and RBV treatment for chronic hepatitis C [1–3]. Specifically, patients with the TG or GG genotype at rs8099917 infected with genotype 1b are more resistant to PEG-IFN and RBV treatment than patients with the TT genotype. IL-28B haplotypes were also reported to be strongly associated with spontaneous HCV clearance [1, 4, 5].

IL-28B is a member of the type III IFN family [6], consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). IFN-λs bind to their cognate receptor, composed of IL28R1 and IL10R2, and then activate the receptor-associated Janus-activated kinases (Jak) 1 and tyrosine kinase (Tyk) 2, leading to the activation of downstream signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2. Similar to type I IFN signalling, the Jak-STAT signalling pathway activates the IFN-stimulated response element (ISRE) within the promoter region of interferon-stimulated genes (ISGs) [7].

Concerning the functional role of IL-28B in HCV infection, two of *in vivo* studies assessed the correlation of IL-28A/B mRNA levels in whole blood and peripheral blood mononuclear cells (PBMC) with IL-28B haplotypes at position rs8099917. IL-28A mRNA and IL-28B mRNA levels in subjects with the TT genotype were higher than in subjects with other genotypes (TG or GG), suggesting an association between higher amounts of endogenous IFN-λs and HCV clearance [2, 3]. On the other hand, subjects

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; ISG, interferon-stimulated genes; MTS, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium; PBMC, peripheral blood mononuclear cells; SNP, single-nucleotide polymorphisms; STAT, signal transducer and activator of transcription; SVR, sustained viral response.

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with the TT genotype at SNP rs8099917 were reported to have lower expression levels of ISGs in the liver during the pretreatment period as compared with subjects with the TG or GG genotypes [8]. Several *in vitro* studies support a direct role of IFN-λs in the control of HCV replication through the innate immune pathway. In a cell culture system, Marcello *et al.* [9] showed that IFN-λ1 inhibited HCV replication with similar kinetics to that of IFN-α, although IFN-λ1 induced stronger upregulation of ISGs and this effect lasted longer. Moreover, combinations of IFN-λ1 and IFN-α had a greater inhibitory effect on HCV replication compared with the individual agents [10].

As suggested by the studies performed to date, a change in IFN-λ3 expression might be a key mechanism by which IL-28B SNPs determine the response to PEG-IFN and RBV. Considering that IFN-λ1 plays a direct role in the control of HCV replication and that IFN-λ1 enhances the antiviral activity of IFN-α, it seems reasonable to speculate that IFN-λ3 plays a similar antiviral role. Therefore, in this study, we investigated the direct antiviral role of IFN-λ3 alone and in combination with IFN-α using an HCV replicon system. In addition, we used microarray analysis to investigate the influence of IFN-λ3, alone or in combination with IFN-α, on the regulation of ISG-mediated antiviral pathways.

MATERIALS AND METHODS

Cell culture and HCV replicon

The human hepatoma cell lines OR6 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) at 37°C in 5% CO_2. JFH-1-infected Huh7.5.1 cells were grown in DMEM supplemented with 10% FBS. The OR6 cell line, harbouring full-length genotype 1b HCV RNA and co-expressing *Renilla* luciferase (ORN/C-5B/KE) [11], was established in the presence of 500 μg/mL G418 (Promega, Madison, WI, USA).

Reagents

IL-28A (IFN-λ2), IL-28B (IFN-λ3) and IL-29 (IFN-λ1) were obtained from R&D Systems (Minneapolis, MN, USA). IL-28A and IL-29 are recombinant proteins generated from an NSO-derived murine myeloma cell line, and IL-28B is a recombinant protein generated from the CHO cell line. Interferon alpha-2b (INTRON®A 300 IU) was obtained from Schering-Plough Corporation (Kenilworth, NJ, USA).

Reporter plasmids and luciferase assay

HCV replication in OR6 replicon cells was determined by monitoring *Renilla* luciferase activity (Promega). To monitor IFN signalling directed by the interferon-stimulated response element (ISRE), the plasmids pISRE-luc expressing firefly luciferase were cotransfected using FuGENE®6 Transfection Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Luciferase activity was quantified using the dual-luciferase assay system (Promega) and a GloMax 96 Microplate Luminometer (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD percentage of the control values.

Quantification of HCV core protein and RNA

We quantified HCV core protein in culture supernatant using Lumipulse Ortho HCV Ag (Ortho Clinical Diagnostics, Tokyo, Japan) as specified by the manufacturer. The principle of the measurement method is based on the chemiluminescent enzyme immunoassay (CLEIA) [12].

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Intracellular genomic JFH-1 HCV RNA as well as cellular mRNA of IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 was quantified by TaqMan RT-PCR. The cells were lysed and subjected to reverse transcription without purification of RNA using a Cells-to-Ct kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed in triplicate using a 7500 Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The sequences of the sense and antisense primers and the TaqMan probe for 5'UTR region of HCV were 5'-TGCGG AACCGGTGAGTACA-3', 5'-CTTAAGGTTTAGGATTCGTGCT CAT-3' and 5'-(FAM)CACCCTATCAGGCAGTACCACAAGG CC(TAMRA)-3', respectively. TaqMan probes for IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 were purchased from Applied Biosystems. Primers for 18s rRNA (Applied Biosystems) were used as internal control.

Microarray analysis

OR6 replicon cells were harvested by centrifugation after exposure to 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both for 6, 12, 24 and 48 h. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality control of extracted RNA was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

The RNA was then amplified and labelled using the Ambion® WT Expression Kit and GeneChip® WT Terminal Labelling and Control Kit (Affymetrix, Santa Clara, CA, USA). cDNA was synthesized, labelled and hybridized to the GeneChip® array according to the manufacturer's protocol, starting with 200-ng total RNA. The GeneChips were finally washed

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and stained using the GeneChip® Fluidics Station 450 (Affymetrix) and then scanned with the GeneChip® Scanner 3000 7G (Affymetrix).

Affymetrix CEL files were imported into GeneSpring GX v.11.5 (Agilent Technologies, Santa Clara, CA, USA) analysis software. Data were normalized using robust multichip average analysis (RMA).

Dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays

To evaluate the cell viability, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. P values of < 0.05 were considered to be statistically significant.

RESULTS

IFN-λ1, IFN-λ2 and IFN-λ3 demonstrate antiviral activity against HCV

To determine the antiviral effect of IL-29 (IFN-λ1), IL-28A (IFN-λ2) and IL-28B (IFN-λ3) against HCV, OR6/ORN/C-5B/KE cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various concentrations for another 24, 48 and 72 h. In this system, the *Renilla* luciferase activity reflects the amount of HCV RNA synthesized. As shown in Fig. 1, at concentrations of 1 ng/mL or more, all IFN-λs led to a concentration- and time-dependent decrease in luciferase activity of the OR6/C-5B replicon. IFN-λ3 at 10 ng/mL inhibited HCV replication (32% reduction, $P < 0.05$) to a similar extent as 0.01 ng/mL IFN-α (49% reduction, $P < 0.05$) by 48 h.

We also assessed the effects of IFN-λ1, IFN-λ2 and IFN-λ3 on Huh7.5.1/JFH-1 cells. JFH-1 cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various doses for another 48 h. To determine their antiviral effect, HCV core protein in the medium and intracellular HCV RNA were measured by CLEIA and quantitative real-time RT-PCR, respectively. HCV RNA quantitative PCR assays were multiplexed for 18s ribosomal RNA to control for the amount of input RNA. As shown in Fig. 2, all IFN-λs inhibited HCV replication in JFH-1 cells in a concentration-dependent manner. Similarly, all of the IFN-λs caused suppression of HCV core protein secretion into the cell culture medium (Figure S1).

In C-5B system, there was no evident cytotoxicity below 100 ng/mL in any interferons except for IFN-λ1 (Figure S2). On the other hand, cytotoxicity was observed in lesser concentrations by those IFNs in JFH-1 system. However, as demonstrated in Fig. 2 and Figure S3, antiviral effect exceeded the cytotoxicity in the JFH-1 system.

Synergistic inhibition of HCV replication by IFN-λ3 and IFN-α in combination

We examined whether the combination of IFN-λ3 and IFN-α induces greater antiviral activity as compared with the individual cytokines alone. OR6/ORN/C-5B/KE cells were treated with the combinations of IFN-λ3 and IFN-α at various concentrations for 48 h. As shown in Fig. 3a, the relative concentration–inhibition curves of IFN-α were plotted for each fixed concentration of IFN-λ3, and the curves shifted to the left with increasing concentrations of IFN-λ3. The results indicate a synergistic effect of IFN-λ3 and IFN-α against HCV replication. We confirmed the synergistic effect of IFN-λ3 and IFN-α by isobologram (Fig. 3b). The inhibitory effects of the combination were quantified according to the method of Chou *et al.* using the CalcuSyn software program (Biosoft, Cambridge, UK). At the ED_{50} of each drug, the combination index was 0.40-0.61, indicating significant synergism. We also assessed the effect of the combination on Huh7.5/JFH-1 cells by HCV RNA quantitative PCR assays and HCV core protein secretion. At the ED_{50} of each drug, the combination index was 0.55 and 0.48, respectively (Table S1). The cytotoxicity was not observed at the range of concentration tested (Fig. S2E, S3E).

IFN-λ3 induces ISRE promoter activity

We used the ISRE luciferase reporter assay to assess activity downstream of the JAK-STAT signalling pathway. The ISRE-firefly luciferase plasmid was transfected into OR6/ORN/C-5B/KE cells for 24 h, and these cells were cultured with various concentrations of IFN-λ3 and IFN-α for another 12, 24 or 48 h. Firefly and *Renilla* luciferase activity was then measured.

IFN-λ3 induced ISRE luciferase activity in a time-dependent manner; activity was elevated threefold after treatment with 100 ng/mL IFN-λ3 for 48 h (Fig. 4). In contrast, IFN-α induced ISRE luciferase more rapidly, producing maximal activation of the response to IFN-α at 12 h. The combination of IFN-λ3 and IFN-α induced ISRE luciferase activity similarly to IFN-λ3 alone.

IFN-α and IFN-λ3 induce expression of similar genes in HCV 1b replicon cells

OR6/ORN/C-5B/KE cells were stimulated for 6, 12, 24 and 48 h with 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both, while controls were left unstimulated for the same time interval. Induction of gene expression by IFNs was analysed in microarray experiments.

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