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 EC50 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 EC50 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 irrespective 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.

Introduction

Hepatitis C virus (HCV) is well known as a major causative agent of chronic liver disease including cirrhosis and hepato-cellular 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 multi-functional 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 morpholine [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
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 then was subjected to Western blotting using antibodies to NS3 and beta-actin.

Figure 1. Effect of CAPE on viral replication in the replicon cell line of genotype 1b.

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

| Compound (Number) | EC50 * (µM) | CC50 b (µM) | SI 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 (CC50/EC50).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).

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corresponding to length of n-alkyl side chain (Fig. 2A). Compounds 10 and 11 exhibit EC50 values of 2.7 and 5.9 μM, respectively, SI values of 29.6 and 9.80, respectively, and Clog 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 Clog 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 Clog P value and CC50, 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 EC50 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 EC50 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, Clog P values of compound 10, 13, 14, 15 and 16 were not correlated with anti-HCV activity (EC50 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 EC50 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...
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 off target 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 EC50 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 showed that treatment with compound 10 reduced HCV viral production (EC50 = 1.8 ± 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 IRF3 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. 4A). The data of the RT-PCR analysis showed that the transcriptional expressions of ISGs including Mx1, MxA, IFTT4, 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 ratios 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 EC50 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 antagonistic (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 2. Effect of caffeic acid esters 7, 9–14, including 1, on HCV replication.

| Compound No. | R     | EC50 * (μM) | CC50 b (μM) | SI * | Clog P d |
|--------------|-------|-------------|-------------|------|---------|
| 7            | CH3   | 28.6 ± 1.2  | 122.1 ± 5.0 | 4.2  | 1.20    |
| 8            | C6H5  | 13.5 ± 2.1  | 39.0 ± 1.1  | 2.9  | 2.79    |
| 9            | C6H13 | 7.3 ± 0.2   | 37.6 ± 1.2  | 5.1  | 3.85    |
| 10           | CH3   | 2.7 ± 0.1   | 71.7 ± 8.5  | 26.6 | 4.90    |
| 11           | CH3   | 5.9 ± 0.9   | 57.9 ± 2.9  | 9.8  | 5.96    |
| 1            | (C6H5)Ph | 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 (CC50/EC50).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
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### Table 3. Effect of caffeic acid esters 7 and 8 on HCV replication.

| Compound No. | EC50 * (μM) | CC50 b (μM) | SI * | 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 (CC50/EC50).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
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### Table 4. Effect of octyl esters 10 and 13–16 on HCV replication.

| Compound No. | R1, R2, R3 | EC50 * (μM) | CC50 b (μM) | SI * | Clog P d |
|--------------|-------------|-------------|-------------|------|---------|
| 10           | R1 = R2 = R3 = H | 2.7 ± 0.1   | 71.7 ± 8.5  | 26.6 | 4.90    |
| 13           | R1 = CH3, R2 = R3 = H | 10.2 ± 1.1  | 60.3 ± 1.6  | 5.9  | 5.35    |
| 14           | R1 = H, R2 = CH3 | 19.6 ± 0.8  | 59.2 ± 1.4  | 3    | 5.35    |
| 15           | R1 = CH3, R2 = H | 48.5 ± 1.7  | 212.4 ± 6.9 | 4.4  | 5.82    |
| 16           | R1 = H, R2 = 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 (CC50/EC50).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
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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'), 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’.
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 EC50 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.

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.

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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 EC50 value of the derivative decreased depending 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 EC50 value and Clop P value. Compound 10, Caffeic acid n-octyl ester, exhibited the highest anti-HCV activity among the tested compounds with an EC50 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'-(2-Morpholine-4-carboxyl)-2-naphthalene-1-yl acetimidamidine 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 NSSA, 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 NSSA. 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 EC50 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-methyl caffeic acid n-octyl ester (n-octyl-3-methyl caffeate) (13), 4-O-methyl caffeic acid n-octyl ester (n-octyl-3-methyl caffeate) (14), and 3, 4-O-dimethyl caffeic acid n-octyl ester (n-octyl-3, 4-methyl caffeate) (15) were from LKT Laboratories (St. Paul, MN, USA).

Caffeic acid esters 7, 8, 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)
was heated at 70°C for 1 h. The reaction mixture was concentrated under a vacuum to give a residue, which was dissolved in CHCl₃-IPA (3.1, v/v) and then washed with 10% HCl and water. The organic layer was dried over Na₂SO₄ 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 (35 mg, 13.8%) as a pale powder.

FT-IR vmax [KB]: 3309, 3239, 2923, 1675, 1627, 1606 cm⁻¹.

1H NMR (400MHz, CD₃OD) 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), 6.35 (2H, s), 7.40 (2H, d, \( J = 15.6 \) Hz).

13C NMR (100 Hz, CD₃OD) 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.

The resulting luminescence was detected by a Luminescencer-JNR cell lysate was estimated using a luciferase assay system (Promega).

The replicon cells were seeded at a density of 1×10⁴ cells per well in a 96-well plate and then incubated at 37°C for 24 h. Luciferase assays were carried out as described in Materials and Methods.

The replicon cells were seeded at a density of 1×10⁴ cells per well in a 48-well plate 24 h before treatment. Compounds were added to the culture medium to give various concentrations and were then harvested at 72 h post-treatment. The cellular viability was measured using a dimethylthiazol carboxymethoxy-phenylsulfonylphenyl tetrazolium (MTS) assay on a CellTiter 96 aqueous one solution cell proliferation assay kit (Promega).

The replicon cells were seeded at a density of 1×10⁴ 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 at 72 h post-treatment. Cell viability was measured using a dimethylthiazol carboxymethoxy-phenylsulfonylphenyl tetrazolium (MTS) assay on 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, mousenoclonal, 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 follows: 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′-AGCCACTGGACT-GACGAC-3′ and 5′-GAGGGCTGAAAATCCCTTC-3′; Mxa: 5′-GTCAAGGATGTCCTCCGCA-3′ and 5′-ATT-GCCATTCCCTCCCCG-3′; IFIT4: 5′-CCCCGCCAGGGCATAGGGCA-3′ and 5′-GAGGTGGTTGGAAATGTTG-3′; OAS1: 5′-CAAGGCAGCCCTCATTCCAT-3′ and 5′-TGAGGGTTGTGGAATG-3′; OAS2: 5′-ACAGGTCAGAATACTTTGGGA-3′ and 5′-GAC-TAACAGGGCAGAAC-3′; OAS3: 5′-CACTGACATCCCAGACGAT-3′ and 5′-GAGGTGCAGCT-3′; GAPDH: 5′-GAAGGTGAAGGTTCAGAGTC-3′ and 5′-GAATGATGTAGGATT-3′.

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

Huh7 OK1 cells were transfected with pEF.Rluc.HCV.IRES.-Feo or pEF.Rluc.EMCV.IRES.Feo [39]. These transfected cells were seeded at 2×10⁵ 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-α2b, 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 CloqP for compounds**

The CloqP value deduced from chemical structure roughly corresponds to a value of hydrophobicity. The CloqP 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, EC95 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 EC50, EC75, and EC90 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.

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