Inhibition Mechanisms of Hepatitis C Virus Infection by Caffeic Acid and Tannic Acid

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-sense, single-stranded RNA virus in the genus Hepacivirus within the Flaviviridae family. Approximately 71 million people worldwide are currently infected with HCV. Given that chronic HCV infection is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, it has been recognised as a major global health concern. Although novel HCV therapeutics with direct-acting antivirals have led to remarkably improved treatment outcomes, problems still persist; high costs in particular restrict most patients’ access to these effective therapeutics.

Caffeic acid (CA), a coffee-related organic acid, is a degrad product of chlorogenic acid, abundant in coffee beans. It is involved in the deep aroma, color, and bitterness of coffee. This polyphenol is also contained in a wide variety of other foods, including fruits, vegetables, and grains. For example, red grape juices and wild chokeberries contain 17–30 mg/L and 1.41 mg/g, respectively. It has many beneficial biological effects, including inhibition of cancer cell proliferation and metastasis and antiviral activities against a variety of viruses.

Previously, our group has shown that CA inhibits HCV infection and HCV entry into cells, but the detailed antiviral mechanism has not yet been defined. In the present study, we investigated the inhibitory effect of another coffee-related compound, tannic acid (TA), on HCV infection and further examined how CA and TA inhibit HCV infection.

MATERIALS AND METHODS

Cells and Cell Culture Huh7.5.1–8, which is derived from Huh7.5.1, is a human hepatoma cell line that is highly permissive to HCV infection. Huh7.5.1–8-derived OKH-4 is an occludin (OCLN) knockout cell line that is non-permissive to HCV infection because OCLN is essential for HCV infection. Both cells were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate.

Reagents Chlorogenic acid and CA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methyl 2,5-dihydroxycinnamate and TA were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Purity of CA and TA is ≥98 and 90% (ACS grade), respectively. Kahweol, kahweol palmitate, cafestol, cafestol palmitate, cafestol eicosanate, cafestol stearatate, and cafestol acetate were purchased from LKT Laboratories (St Paul, MN, U.S.A.). p-Coumaric acid was purchased from MP Biomedicals (Santa Ana, CA, U.S.A.). d-(-)-Quinic acid was purchased from Alfa Aesar (Haverhill, MA, U.S.A.). Mouse monoclonal antibody (mAb) against HCV core protein (clone 2H9) was described previously. Goat polyclonal antibodies (pAb) against apolipoprotein E (ApoE) protein were purchased from Merck Millipore (Burlington, MA, U.S.A.). Mouse mAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (clone 5A12) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mouse mAb against HCV NS3 protein (clone 8G2) was purchased from Abcam (Cambridge, U.K.).

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**Viral Stock, Infection, and Quantification** The HCV-JFH1 strain, an HCV genotype 2a isolate, was prepared from the culture supernatant of Huh7.5.1–8 cells that had been transfected with in vitro-transcribed HCV-JFH1 RNA. After serial passages of the HCV-JFH1 in naïve Huh7.5.1–8 cells, infectious culture supernatants were collected and used as virus stock in this study. Virus titers were determined by fluorescent focus assay as described previously. Total RNA was extracted from culture supernatants or cultured cells and purified using the Viral Nucleic Acid Extraction Kit I and Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN Biotech Corp., Pingtung City, Taiwan), respectively. Quantification of HCV-JFH1 RNA was performed by quantitative (q)RT-PCR as described previously.

**Cell Viability Assay** Huh7.5.1–8 cells were seeded at 5 × 10^4 cells per well in 48-well plates and cultured at 37°C for 1 d. Cells were then incubated in medium containing each coffee-related compound at 37°C for 3 d. After washing twice with phosphate buffered saline (PBS), cells were treated with 500 μL phenol red-free complete medium containing cell counting kit-8 reagent (Dojindo, Tokyo, Japan) at 37°C. After 1 or 2 h, culture supernatants were collected and transferred to 96-well plates. For each well, the absorbance at 450 nm was measured using an iMark microplate reader (BIORAD, Hercules, CA, U.S.A.).

**HCV RNA Replication Assay** We used the subgenomic HCV-JFH1 replicon system described previously. In brief, cells were seeded at 5 × 10^4 cells per well in 48-well plates and cultured at 37°C for 1 d. Subgenomic replicon RNA (1 μg each/well) in vitro transcribed from pSGR-JFH1/Luc was transfected into cells using DMRIE-C Transfection Reagent (Life Technologies, Carlsbad, CA, U.S.A.) at 37°C. After incubation for 4 h, the culture medium was replaced with fresh complete medium containing CA or TA. Cells were cultured at 37°C for 3 d and then lysed in 100 μL Cell Culture Lysis Reagent (Promega Corp., Madison, WI, U.S.A.). Luciferase activity was measured using a Luminescencer-PSN luminometer (ATTO Co., Ltd., Tokyo, Japan) with 5 μL aliquots of the cell solution mixed with 10 μL luciferase assay substrate (PicaGene, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan).

**Evaluation of Egress Efficiency of HCV Particles** OKH-4 cells were seeded at 5 × 10^4 cells per well in 48-well plates and cultured at 37°C for 1 d. HCV-JFH1 full genomic RNA (1 μg each/well) from pJFH1 was transfected into cells using DMRIE-C Transfection Reagent (Life Technologies, Carlsbad, CA, U.S.A.) at 37°C. After incubation for 4 h, the culture medium was replaced with fresh complete medium containing CA or TA. Cells were cultured at 37°C for 3 d and then lysed in 100 μL Cell Culture Lysis Reagent (Promega Corp., Madison, WI, U.S.A.). Luciferase activity was measured using a Luminescencer-PSN luminometer (ATTO Co., Ltd., Tokyo, Japan) with 5 μL aliquots of the cell solution mixed with 10 μL luciferase assay substrate (PicaGene, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan).

**Evaluation of Infectivity of Viral Particles** Huh7.5.1–8 cells were seeded at 5 × 10^4 cells in 48-well plate and cultured at 37°C for 1 d. HCV-JFH1-containing medium (corresponding to a multiplicity of infection (MOI) of 1.0; 200 μL) was mixed with various concentrations of CA or TA at 37°C for 4 h. Cells were then infected with a 100-fold dilution of these HCV-JFH1-containing media in order to reduce the direct influence of the compounds on the cells. At 3 d post-infection (p.i.), cellular HCV RNA copies were determined by qRT-PCR as described above.

**HCV Particle Cellular Attachment Assay** Huh7.5.1–8 cells were seeded at 5 × 10^4 cells per well in 48-well plates and cultured for 2 d. HCV-JFH1 (corresponding to an MOI of 10; 900 μL) were pretreated with CA (1 mM) or TA (10 μM) at 37°C for 4 h. Huh7.5.1–8 cells were then incubated with these HCV-JFH1 (200 μL/well) at 4°C for 2 h. After cells were washed three times with PBS, amounts of HCV-JFH1 particles attached to the cells were quantified by qRT-PCR.

**Evaluation of ApoE Associated with HCV Particles by Co-immunoprecipitation Assay** HCV-JFH1-containing medium (corresponding to an MOI of 10; 200 μL) were pretreated with CA (1 mM) or TA (10 μM). Anti-ApoE pAb (5 μL) was then added to the medium. These mixtures (150 μL/each) were treated with 50 μL of 50% (v/v) protein A/G PLUS agarose (Santa Cruz, Dallas, TX, U.S.A.) in PBS at 4°C for 2 h. Immunoprecipitated fractions were washed trice with PBS. Then, 150 μL 10% SDS was added to these tubes and heated at 70°C for 10 min. After centrifugation at 15,000 rpm (20400 × g) for 1 min, supernatants were collected and HCV-JFH1 RNA fractions were purified using FavorPrep Viral nucleic acid extraction kit I (FAVORGEN Biotech Corp.).

**RESULTS**

**Inhibitory Effects of CA and TA on HCV Infection** Previously, we showed that a coffee extract in addition to coffee-related compounds such as CA and p-coumaric acid can inhibit HCV infection. In the present study, we explored the effects of a variety of other coffee-related compounds, including the hydrophilic group (chlorogenic acid, p-coumaric acid, quinic acid, CA, and TA) and the lipophilic group (methyl 2,5-dihydroxy-cinnamate, kahweol palmitate, cafestol, cafestol palmitate, cafestol eicosanate, cafestol stearate, cafestol acetate, and kahweol), on HCV infection. Among the hydrophilic group, chlorogenic acid, p-coumaric acid, CA, and TA had apparent inhibitory effects on HCV infection, but no compounds in the lipophilic group displayed these effects (Fig. 1A). In particular, CA and TA (Fig. 1B) showed stronger inhibitory effects (Fig. 1A), and we examined whether these effects were dose-dependent and whether they resulted in cellular toxicity. We found that both CA (Fig. 1C) and TA (Fig. 1D) suppressed cellular HCV RNA levels in a dose-dependent manner. Based on immunoblot and immunofluorescence analyses, the cellular contents of viral proteins, HCV core and NS3, were also decreased in a dose-dependent manner by treatment with CA and TA (Supplementary Figs. 1A–C). CA and TA exhibited IC₅₀ values of 0.06 ± 0.01 mM and 2.56 ± 1.32 μM and CC₅₀ values of 2.40 ± 1.32 μM and 2.56 ± 1.32 μM, giving selectivity index estimates (SI) of 40 and 11, respectively (Table 1).

**CA and TA Attenuate HCV Infectivity by Acting on HCV Particles** Next, we determined which steps in the HCV lifecycle were affected by CA and TA. First, we tested the effects of CA and TA on HCV replication using Huh7.5.1–8 cells carrying a subgenomic replicon with the luciferase reporter gene, SGR-JFH1/Luc. Even at a concentration of 1 mM (Fig. 2A), at which HCV infection was strongly inhibited by CA (Fig. 1), luciferase activities were unaffected. Meanwhile, luciferase activities in the TA-treated cells were decreased in a dose-dependent manner and reached a plateau of 40–60% at 5 μM TA (Fig. 2B).

Next, we examined the effects of CA and TA on viral egress efficiency (Fig. 2C). OCLN-knockout OKH-4 cells,
which were not permissive to HCV entry and thus did not support secondary infection, were transfected with full-length HCV RNA genomes and then treated with CA and TA. At 1–3 d post-transfection (p.t.), intracellular and extracellular HCV RNA was quantified by qRT-PCR. We calculated HCV regression activity as the ratio of extracellular/intracellular

Fig. 1. Inhibitory Effects of CA and TA on HCV-JFH1 Infection
(A) HuH7.5.1–8 cells were pre-incubated in medium containing the indicated concentrations of each coffee-related compound at 37°C for 30 min and then infected with HCV-JFH1 (MOI of 0.1). At 3 d p.i., cellular HCV-JFH1 RNA contents were measured by qRT-PCR. Values are expressed as percentages of the control (DMSO treatment). Data are presented as means ± standard deviation (S.D.) ($n$ = 4). *$p$ < 0.01. The working concentration of each compound was set as the 10-fold dilution of the concentration at which cytotoxicity was observed. (B) Structural formulas of CA and TA. (C, D) HuH7.5.1–8 cells were pre-incubated in medium containing the indicated concentrations of CA (C) or TA (D) at 37°C for 30 min and then infected with HCV-JFH1 (MOI of 0.1). At 3 d p.i., cellular HCV-JFH1 RNA contents (closed circles) and cell viability (open circles) were determined by qRT-PCR and WST assay, respectively. Values are expressed as percentages of the control (DMSO treatment). Data are presented as means ± S.D. ($n$ = 4). Data are representative of more than two independent experiments.

Table 1. Anti-HCV Activity and Cytotoxicity of CA and TA

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC$_{50}$ (µM)</th>
<th>IC$_{50}$ (µM)</th>
<th>CC$<em>{50}$/IC$</em>{50}$</th>
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<tr>
<td>Caffeic acid (CA)</td>
<td>2.40 ± 0.79 mM</td>
<td>0.06 ± 0.01 mM</td>
<td>40</td>
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<tr>
<td>Tannic acid (TA)</td>
<td>28.21 ± 7.63 µM</td>
<td>2.56 ± 1.32 µM</td>
<td>11</td>
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which were not permissive to HCV entry and thus did not support secondary infection, were transfected with full-length HCV RNA genomes and then treated with CA and TA. At 1–3 d post-transfection (p.t.), intracellular and extracellular HCV RNA was quantified by qRT-PCR. We calculated HCV egression activity as the ratio of extracellular/intracellular
HCV RNA (Fig. 2C). HCV egression efficiency was not affected by treatment with CA, but was significantly reduced by treatment with TA (Fig. 2D).

Furthermore, we investigated the effects of CA and TA on infectious HCV particles themselves (Fig. 3A). HCV-JFH1 particles were treated with various concentrations of CA or TA at 37°C for 4 h and then inoculated into Huh7.5.1–8 cells. We then evaluated the infectivity of the HCV particles by measuring the HCV RNA contents in the inocula and the cellular HCV RNA levels at 3 d p.i. Neither CA nor TA affected the amounts of HCV-JFH1 in the inocula, but both decreased the HCV RNA levels in the infected Huh7.5.1–8 cells (Figs. 3B, C). CA and TA exhibited IC_{50} values of 0.05 ± 0.01 mM and 1.31 ± 0.89 µM, respectively, similar to the values in Table 1. Cellular HCV core proteins were also decreased in a dose-dependent manner by treatment of HCV-JFH1 particles with CA and TA (Supplementary Figs. 2A, B). Furthermore, CA and TA pretreatment of HCV particles decreased the infectivity of other infectious HCV particles, such as HCV-TNS2J1 (genotype 1b/2a chimera) and HCV-Jc1 (genotype 2a/2a chimera), in a dose-dependent manner (Supplementary Figs. 3A–D).

CA-Treated HCV Particles Showed Lower Cellular Attachment Since CA and TA attenuated the infectivity of HCV particles, we investigated whether CA and TA affect the cellular attachment of HCV particles. HCV-JFH1 were pretreated with CA (1 mM) or TA (10 µM) at 37°C for 4 h. Huh7.5.1–8 cells were then incubated with these HCV particles at 4°C for 2 h. After cells were washed thrice with PBS, the amount of HCV-JFH1 particles attached to the cells was quantified by qRT-PCR. The HCV-JFH1 particle egress efficiency was calculated by the equation indicated in (C). Values are expressed as percentages of the control (DMSO treatment). Data are presented as means ± S.D. (n = 6). *p < 0.05. Data are representative of more than two independent experiments.

CA Prevented the Interaction Between HCV Particles and ApoE It is well known that the formation of a complex between HCV particles and ApoE is critical for the infectivity of HCV particles. Therefore, we explored the possibility that CA interrupts the interaction between HCV particles and ApoE. The amounts of HCV particles bound to ApoE in the presence or absence of CA or TA were determined by im-
munoprecipitation assays using anti-ApoE antibody (Fig. 5A). Consistent with the results of a previous study, 33) 70–80% of HCV particles (as viral RNA contents) were immunoprecipitated with anti-ApoE antibody when non-treated or DMSO-treated HCV particles were analyzed (Fig. 5B). A similar result was achieved with TA-treated HCV particles (Fig. 5B), indicating that TA had no effects on the HCV-ApoE interaction. NP40-treated HCV particles, which have no envelopes, were used as a negative control; approximately 24% were immunoprecipitated with anti-ApoE antibody (Fig. 5B); likewise, approximately 29% of CA-treated HCV particles were immunoprecipitated with anti-ApoE antibody.

DISCUSSION

In the present study, we found that TA, in addition to CA,11) strongly prevented HCV-JFH1 infection in the cell culture system (Fig. 1, Supplementary Fig. 1, Table 1). TA affected the whole HCV lifecycle including viral entry, genome replication, and egress (Figs. 2B, D, 3C, Supplementary Figs. 2B, 3C, D). Although further analyses are needed to understand the molecular mechanism underlying these effects, TA mainly acts on viral particles (Fig. 3) and inhibits the late entry step after the cellular attachment of viral particles (Figs. 4, 5). On the other hand, CA showed no effect on the genome replication or egress processes of HCV-JFH1 infection (Figs. 2A, D), but did inhibit viral entry, especially at the early stage before viral attachment to the host cells (Fig. 3B). These effects were also confirmed in other viral strains, including Jcl and TNS2J1 (Supplementary Fig. 3), suggesting that CA and TA have general inhibitory effects on HCV infection.

It is well known that formation of a complex of HCV particles and ApoE is critical for viral infectivity. 27–29) These complexes, named lipoviroparticles (LVPs), enhance the interaction between HCV particles and cellular receptors, including heparan sulfate proteoglycans 34) such as syndecan 4, 35) low density lipoprotein (LDL) receptors, 36) and scavenger receptors class B type I, 37) resulting in efficient viral attachment to and entry into host cells. 28,38) We demonstrated that CA interrupted the association between ApoE and HCV particles (Fig. 5B) and reduced the cellular attachment of viral particles (Fig. 4), and we therefore concluded that this is the major molecular mechanism underlying the inhibition of HCV infection by CA.

Recently, it was reported that CA prevented HCV genome replication,13,14) but we did not observe this effect in the present study (Fig. 2). This might be explained by differences in researchers’ use of host cells and viral/replicon strains. In the present study, we used Huh7.5.1–8 cells and HCV-JFH1 (genotype 2a), while previous studies have used Huh7;13,14) Huh7.5.1,15) Con1 replicon (genotype 1b), J399EM (genotype 2a), a JFH-1-based adaptive strain in which an enhanced green fluorescent protein (EGFP) gene was inserted into NS5A.13)
It has also been reported that CA has inhibitory effects on other viruses such as hepatitis B virus (HBV), canine distemper virus (CDV), herpes simplex virus type 1 (HSV-1), influenza A virus (IAV), polio virus type 1 (PV-1), and severe fever with thrombocytopenia syndrome virus (SFTSV) in cultured cells.12,14–17,19,21,39) CA has also shown inhibitory activity against human immunodeficiency virus (HIV) integrase (IC$_{50}$ = 2.8 µM),20,40) which is a critical enzyme for HIV replication. HBV infection was prevented by CA mainly at the viral genome replication step.21) CDV infection was inhibited by CA treatments at 1 and 2 h p.i., but not at −1 and 0 h p.i.,39) suggesting that CA affects CDV after entry into cells. It is thought that CA primarily affects the early stages of HSV and IAV infection, although its target has not yet been clearly identified.15,16) Unlike in HCV in the present study, CA has not been shown to have virucidal effects on HSV-1 or IAV.15,16) In PV-1 infection, CA treatment of cells during infection resulted in higher viral inhibition than CA treatment at pre- or post-infection.17) These results could be partially explained if one assumes that CA affects PV-1 particles and thus blocks viral entry, as with HCV infection, although further detailed investigations are needed. Very recently, we reported that CA directly acts on SFTSV particles and reduces their infectivity.21) The effect of CA on SFTSV is very similar to that on HCV shown in the present study, suggesting cognate underlying molecular mechanisms.

In the present study, we found that the coffee-related compounds CA and TA act on HCV particles and abrogate their infectivity. Although further investigation, including in vivo experiments, is necessary, intake of coffee or the coffee-related compounds CA and TA may lead to prevention of HCV infection and slower disease progression after HCV infection. Foods and food ingredients such as CA and TA, which are inexpensive and easy to supply, should be considered in global efforts to control HCV-derived diseases.

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### Conflict of Interest
The authors declare no conflict of interest.

### Supplementary Materials
The online version of this article contains supplementary materials.


