Catechol Group of Cinnamic Acid Derivative Is Essential for Its Anti-Hepatitis C Virus Activity

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Abstract: We previously reported that several cinnamic acid derivatives exhibited antiviral effects on hepatitis C virus (HCV). However, further analysis on the chemical structural features of these compounds are required for development of an effective anti-HCV agent. In this study, we examined the relationship between anti-HCV activity and chemical structure using 14 cinnamic acid derivatives to identify an effective anti-HCV compound. Cinnamic acid derivatives with catechol rings showed higher antiviral activities than other compounds. In addition, pyrocatechol, 1,2-dihydroxybenzene, showed antiviral activity. The most effective derivative among the compounds used in this study and our previous report synergistically inhibited HCV replication in combination with daclatasvir or interferon-α2b, but not with telaprevir. These findings suggest that the catechol ring of cinnamic acid derivatives is critical for anti-HCV activity and that the cinnamic acid derivative synergistically inhibits HCV replication in combination with a clinically-used direct-acting antiviral agent for HCV.

Key Words: Hepatitis C virus, cinnamic acid, antiviral

INTRODUCTION

Hepatitis C virus (HCV) infects at least 71 million people worldwide, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma1). Current therapy for hepatitis C using effective direct-acting antivirals (DAA) can achieve sustained viral response (SVR) in over 95% of cases without any serious side effects2). However, several issues still remain to be solved3).

1) DAA therapy of patients infected with the viral genotype 3, which is a major genotype in South Asia and Europe, is limited. 2) Therapy using DAA with ribavirin improves the achievement rate of SVR, although ribavirin causes moderate side effects and is a contraindication for elderly patients and patients with renal failure. 3) Various drugs including antiepileptic drugs are contraindicated when use in combination with some DAA. Thus, development of more efficient and safe anti-HCV agents with fewer side effects is still needed.

Cinnamic acid derivatives are reported to have multiple biological activities such as antimicrobial, anti-cancer, anti-oxidant, anti-fungal,
and anti-diabetic activities\textsuperscript{4–8}. We recently reported that the derivatives inhibit HCV replication via the induction of oxidative stress\textsuperscript{9}.

In this study, we tested the effects of other cinnamic acid derivatives on anti-HCV activity and synergic effects of an effective derivative with currently available anti-HCV agents to identify an effective antiviral agent and to develop an effective therapy. The cinnamic acid derivatives used in this study are different from the derivatives used in our previous report\textsuperscript{9} in combination of residues (OH, CH\textsubscript{3}, CN and others) at R1, R2, R3, and R4 (Table I) in order to clarify the effect of catechol-ring structure on antiviral effect.

### MATERIALS AND METHODS

**Cell culture and luciferase reporter assay**

The Huh7/Rep-Feo cell line, which harbors the subgenomic replicon RNA of the N strain (genotype 1b), was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum, 1% of penicillin-Streptomycin solution (Sigma-Aldrich, St. Louis, USA), and 0.5 mg/mL of G418 (Nakarai, Tokyo)\textsuperscript{9}. These cells were seeded at a density of 2.0 \times 10\textsuperscript{4} cells per well on 48-well plates, were treated with various concentrations of compounds, and then harvested at 72 h post-treatment. Luciferase assay was carried out as described previously\textsuperscript{9}. The cytotoxicity was evaluated by the

### Table I. List of compounds used for screening

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (µM)</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>R\textsubscript{3}</th>
<th>R\textsubscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>37.0 ± 0.4</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>OC\textsubscript{2}H\textsubscript{4}Th</td>
</tr>
<tr>
<td>B</td>
<td>33.7 ± 1.2</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>NH\textsubscript{2}</td>
</tr>
<tr>
<td>C</td>
<td>14.5 ± 0.7</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>NHPh</td>
</tr>
<tr>
<td>D</td>
<td>7.5 ± 0.5</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}PhCF\textsubscript{3}</td>
</tr>
<tr>
<td>E</td>
<td>9.5 ± 0.2</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}Ph</td>
</tr>
<tr>
<td>F</td>
<td>6.5 ± 0.2</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}H\textsubscript{2}Ph</td>
</tr>
<tr>
<td>G</td>
<td>14.3 ± 0.6</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>C\textsubscript{2}N\textsubscript{2}H\textsubscript{4}Ph(OH)\textsubscript{2}</td>
</tr>
<tr>
<td>H</td>
<td>21.1 ± 0.3</td>
<td>OH</td>
<td>OH</td>
<td>CN</td>
<td>Ph(OH)\textsubscript{2}</td>
</tr>
<tr>
<td>I</td>
<td>8.7 ± 1.0</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>NHCH\textsubscript{3}H\textsubscript{2}Ph</td>
</tr>
<tr>
<td>J</td>
<td>73.8 ± 2.4</td>
<td>H</td>
<td>OH</td>
<td>CN</td>
<td>NHCH(CH\textsubscript{3})\textsubscript{2}</td>
</tr>
<tr>
<td>K</td>
<td>78.6 ± 3.8</td>
<td>H</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}H\textsubscript{2}</td>
</tr>
<tr>
<td>L</td>
<td>67.5 ± 2.6</td>
<td>H</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}H \textsubscript{2}</td>
</tr>
<tr>
<td>M</td>
<td>59.7 ± 1.0</td>
<td>H</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}H\textsubscript{2}Ph</td>
</tr>
<tr>
<td>N</td>
<td>51.3 ± 1.0</td>
<td>H</td>
<td>OH</td>
<td>CN</td>
<td>NHCH\textsubscript{3}H\textsubscript{2}Ph</td>
</tr>
</tbody>
</table>
method described previously\(^9\).

**HCV infection and Quantification of viral RNA**

The Huh7OK1 cell line is highly permissive for cell-cultured HCV (HCVcc) as described previously\(^10\). The viral RNA *in vitro* transcribed from the plasmid pJFH1 was transfected into Huh7OK1 cells for HCVcc preparation according to the method of Wakita *et al*\(^11\). Huh7OK1 cells were infected with HCVcc at a multiplicity of infection of 0.1 and then passaged once every 4 days. These infected cells cultured 8 days post-transfection were employed as the HCVcc-infected cells, which exhibited more than 90% NS5A-positive cells\(^10\). HCVcc-infected cells were seeded at a density of 4.0 \(\times\) 10\(^5\) cells per well on 6-well plates and treated with various concentrations of compound 6 as reported previously\(^9\).

Viral RNA was isolated from supernatants after 24 h treatment using the QIAamp mini viral RNA extraction Kit (Qiagen, Hilden, Germany) and reverse-transfected into cDNA using the Thermoscript reverse transcriptase kit (Thermo Fisher Scientific, Waltham, USA). The copy numbers of viral genomes were quantified using the KOD SYBR qPCR Mix Kit (TOYOBO, Osaka) and Step One Plus Real-Time PCR System (Thermo Fisher Scientific). HCV RNA and GAPDH mRNA were estimated by quantitative real-time polymerase chain reaction (qRT-PCR) utilizing the primer pairs 5\'-GAGTGTCGTACAGCCTC-CA -3' and 5\'-CAGTCGAAAGGCCCTATCA-3' and 5\'-GAAGGTGAAGGTCGGAGTC-3' and 5\'-GAAGATGATTGATGGGATTTC -3', respectively.

**Combination studies**

The combined effect of compound 6 with telaprevir, daclatasvir, or IFN-\(\alpha\)2b was determined using the ED43/SG-Feo (VYG) cell line, which was kindly provided by C. M. Rice\(^12,13\). The cells were seeded at a density of 2.0 \(\times\) 10\(^3\) cells per well on 48-well plates and treated with each drug alone, or combinations of compound 6 with telaprevir, daclatasvir, or IFN-\(\alpha\)2b. The treated cells were harvested 72 h after treatment. The luciferase was expressed dependently of HCV replication because the luciferase gene was polycistronically encoded on the replicon RNA together with the HCV subgenomic genome and was translated via HCV IRES. HCV nonstructural proteins were expressed by EMCV IRES located between the luciferase gene and the HCV nonstructural ORF. The luciferase activity was estimated in triplicate. The data were represented from three independent experiments.

**Reagents**

Compounds A, B, C, and F were purchased from TOCRIS Bioscience (Bristol, UK). Compounds G and H were from Sigma-Aldrich (St. Louis, MO, USA). Compound D was from Merck Millipore (Billerica, MA, USA). Cinnamic acid amide compound I was prepared by amidation between caffeic acid\(^14\) and phenylpropylamine. 2- Cyanocinnamic acid amides amides compounds J, K\(^15\), L\(^16\), M\(^17\), and N\(^18\) were synthesized by Knoevenagel condensation of corresponding cyanoacetamides with \(p\)-hydroxybenzaldehyde. Cyanoacetic acid, \(p\)-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, phenylethylamine, phenylpropylamine, isopropylamine, propylamine, and butylamine were purchased from Wako Pure Chemical (Tokyo). Compound J was also commercially available from Aurora Building Blocks (San Diego, CA, USA). Spectroscopic data of the above known amides prepared were identical to those reported. Interferon \(\alpha\)-2b (IFN-\(\alpha\)2b) was obtained from MSD (Tokyo).

**Data analysis**

Each estimated value represented the mean
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standard deviation (SD). The statistical analysis was performed using Student’s t-test. A value of P < 0.05 was defined as significant. Data of combination studies were analyzed for synergistic effects utilizing the computer software CalcuSyn software (HULINKS, Tokyo). A combination index (CI) of 0.9–1.1, CI < 0.9, and CI > 1.1 indicate an additive effect, a synergistic effect, and an antagonistic effect, respectively.

Prediction of ClogP for compounds

The ClogP value roughly corresponds to actual hydrophobicity. The ClogP values of compounds used in this study were calculated from the chemical structures using the computer software Chem Bio Office Ultra 2008 (PerkinElmer, Cambridge, USA).

RESULTS AND DISCUSSION

Inhibitory effects of cinnamic acid derivatives on HCV replication

To examine effective inhibitory effects of cinnamic acid derivatives, we screened 14 derivatives using HCV replicon cells. Each compound was added at various concentrations to the culture medium. HCV replicon RNA basically codes HCV 5’UTR (IRES), luciferase gene, EMCV IRES, HCV nonstructural gene, and HCV 3’UTR and autonomously replicated in the permitted cells. Thus, luciferase activity corresponded to HCV replication. Luciferase assays were carried out at 72 h post-treatment to determine the half maximal effective concentration (EC50). Treatment with 1,2-dihydroxybenzene, which is called pyrocatechol, inhibited the viral replication with an EC50 value of 13.1 ± 0.4 μM (Fig. 1). EC50 values of compounds that lack the hydroxyl group at R1 (Compounds J to N) are markedly lower than those of compounds with the catechol ring (Compounds A-I) (Table I and Fig. 2), suggesting that the catechol ring is essential for antiviral activity of cinnamic acid derivatives. Compound F was identified as the most effective antiviral compound among the compounds used in this study (Table I and Fig. 3). However, the EC50 value of compound F was slightly higher than that of compound 6, which...
Anti-HCV Activity of Cinnamic Acid Derivative was previously reported as an anti-HCV agent by our group 9). Compound 6 (Table II, Fig. 3) may be more effective as an antiviral agent against HCV and less toxic than compound F, because the CLogP value of compound 6 is higher than that of compound F (Table II). However, the possibility that other factors affect the antiviral activity of cinnamic acid-derived compounds could not be denied.

**Compound 6 impairs the production of HCVcc in culture supernatant**

We previously reported that compound 6 exhibits a significant inhibitory effect on the intracellular viral production and replication 9). Production of infectious virus particles was impaired by treatment with compound 6 9). However, compound 6 included in the supernatant might affect viral titration. Next, we examined the effect of compound 6 on the amount of supernatant viral RNA. HCVcc-infected cells were seeded on the culture plate at 24 h before treatment. Fresh medium containing an indicated concentration of compound 6 was exchanged with the medium. These culture supernatants were harvested at 24 h post-treatment. Viral RNAs in culture supernatant were purified and quantified using qRT-PCR. The amount of viral
RNA in the medium was significantly decreased by treatment with compound 6 (Fig. 4), suggesting that compound 6 impairs the production of viral particles due to viral replication.

**Compound 6 possesses a synergistic antiviral effect with HCV inhibitors**

The combination of compound 6 with telaprevir, daclatasvir, or IFN-α2b was analyzed to determine the effect of compound 6 on the antiviral activity of a known anti-HCV agent. Synergistic antiviral effects (CI < 0.9) were observed in replicon cells treated with compound 6 and daclatasvir or IFN-α2b (Fig. 5). When cells were treated with both compound 6 and daclatasvir, CI values ranged from 0.55 (EC_{90}, 13,000: 1 = compound 6: daclatasvir) to 0.87 (EC_{75}, 50000: 1 = compound 6: daclatasvir). On the other hand, the combination resulted in CI values ranging from 0.52 (EC_{90}, 1: 1 = compound 6: IFN-α2b) to 0.79 (EC_{75}, 1: 1 = compound 6: IFN-α2b). A synergistic effect was not observed in the cells treated with compound 6 and telaprevir. These data suggest that compound 6 may enhance DAA therapy for hepatitis C.

In this study, we tested the anti-HCV activity of cinnamic acid derivatives to determine a chemical motif critical for antiviral activity (Fig. 1 and Table I). Our data suggest that catechol group is critical for the anti-HCV activity of cinnamic acid derivatives, although we could not find a more effective compound than compound 6 with regard to anti-HCV activity. In a previous report, compound 6 was identified as a potent HCV inhibitor, which is a synthetic cinnamic acid derivative derived from AG490. Compound 6 suppresses HCV replication via induction of oxidative stress rather than inhibition of tyrosine kinase activity. Compound 6 exhibited the similar antiviral effect on the replications of genotype 1b, 2a, 3a, and 4a replicons. Compound 6 may suppress the HCV replication regardless of viral genotype and/or sensitivity against current DAA. The further study will be needed to clarify the spectrum of antiviral activity for compound 6. In this study, we tested the antiviral activity of compound 6 on HCVcc in the culture medium and combinations of compound 6 with...
clinically used anti-HCV agents. The antiviral effect of compound 6 on production of HCVcc in the culture medium was confirmed (Fig. 4). Compound 6 also showed synergistic antiviral effects with daclatasvir or IFN-α2b (Fig. 5). Oxidative stress is associated with impairment of HCV replication as well as replication of other viruses20). An increase in nitric oxide downregulated viral RNA synthesis on dengue virus type 221). Induction of oxidative stress attenuated infectivity of influenza virus22). Therefore, induction of oxidative stress by treatment with compound 6 may affect not only replication of HCV but also other viral infections. Elevation of ROS can increase host genetic damage, but not influence much on in vivo healthy cells due to high production of catalase. Ascorbate radical and hydrogen peroxide are produced by intravenous administration of high-dose vitamin C and exhibits cytotoxicity against tumor cells or IFN-treated cells but not healthy cells23–25). Thus, compound 6 may be non-toxic for healthy cells but toxic for virus-infected or tumor cells.

Development of an antiviral agent on the basis of compound 6 will advance establishment of a more effective HCV therapy in the future.

CONCLUSION

In this study, our data suggest that the catechol group of a cinnamic acid derivative is essential for its anti-HCV activity and that compound 6
possesses synergistic anti-HCV effects with daclatasvir and IFN-α2b. Induction of oxidative stress by a newly developed antiviral may lead to a novel therapeutic strategy for HCV therapy.

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REFERENCES


