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Cinnamic acid derivatives inhibit hepatitis C virus replication via the induction of oxidative stress

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ABSTRACT

Several cinnamic acid derivatives have been reported to exhibit antiviral activity. In this study, we prepared 17 synthetic cinnamic acid derivatives and screened them to identify an effective antiviral compound against hepatitis C virus (HCV). Compound **6**, one of two hit compounds, suppressed the viral replications of genotypes 1b, 2a, 3a, and 4a with EC_{50} values of 1.5–8.1 μM and SI values of 16.2–94.2. The effect of compound **6** on the phosphorylation of Tyr^{705} in signal transducer and activator of transcription 3 (STAT3) was investigated because a cinnamic acid derivative AG490 was reported to suppress HCV replication and the activity of Janus kinase (JAK) 2. Compound **6** potently suppressed HCV replication, but it did not inhibit the JAK1/2-dependent phosphorylation of STAT3 Tyr^{705} at the same concentration. Furthermore, a pan-JAK inhibitor tofacitinib potently impaired phosphorylation of STAT3 Tyr^{705}, but it did not inhibit HCV replication in the replicon cells and HCV-infected cells at the same concentration, supporting the notion that the phosphorylated state of STAT3 Tyr^{705} is not necessarily correlated with HCV replication. The production of reactive oxygen species (ROS) was induced by treatment with compound **6**, whereas N-acetyl-cysteine restored HCV replication and impaired ROS production in the replicon cells treated with compound **6**. These data suggest that compound **6** inhibits HCV replication via the induction of oxidative stress.

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1. Introduction

Hepatitis C virus (HCV), which is the major causative agent of chronic liver diseases such as steatosis, cirrhosis and hepatocellular carcinoma, affects at least 71 million people worldwide (WHO, 2017). Over the past decade, combination therapy using pegylated interferon α and ribavirin has been employed for hepatitis C. Recently, tremendous efforts to develop efficient direct acting antivirals (DAAs) demonstrated the achievement of sustained viral response (SVR) in over 95% of cases with generally minor side effects after 12 to 24 weeks of treatment with DAAs (Asselah et al., 2016). However, several issues regarding DAA therapy remain (Pawlotsky, 2016). 1) Ribavirin, which exhibits moderate side effects, is still required for many groups of hepatitis C patients. 2) Treatment with DAA achieves a low SVR for genotype 3 patients, compared with other genotype patients. 3) DAA medication is extremely expensive in developed countries. 4) Treatment with DAA should be carefully applied to patients with underlying disorders including kidney disorders and patients co-infected with hepatitis B virus (HBV). Thus, further development of effective and safe anti-HCV agents remains necessary for the settlement of these issues.

Cinnamic acid is an organic chemical mainly isolated from

Abbreviations: HCV, hepatitis C virus; IFN, interferon; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; JAK, Janus kinase; IL-6, interleukin-6; OAS3, 2′-5′-oligoadenylate synthetase 3; ISG, interferon stimulated gene; ISRE, interferon stimulated response element; HSV, herpes simplex virus; HBV, hepatitis B virus.

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cinnamon, and its derivatives are known as plant hormones correlated with the regulation of cell growth and differentiation. Natural and synthetic cinnamic acid derivatives were reported to exhibit multiple biological activities including anti-inflammatory, anti-microbial, anti-oncogenic, anti-oxidant and/or kinase-inhibitory effects (De et al., 2011; Mielecki and Lesyng, 2016; Sova, 2012). Several cinnamic acid derivatives have been known to suppress the propagations of several viruses. 3,5-Dicaffeoylquinic acid, 1-methoxyoxalyl-3,5-dicaffeoylquinic acid, and L-chicoric acid inhibited the enzymatic activity of human immunodeficiency virus type 1 integrase (Robinson et al., 1996). Synthetic cinnamyl derivatives of thieo[2,3-*d*]oxazinoes impaired the productions of herpes simplex virus type (HSV) 2, varicella zoster virus, and cytomegalovirus (Jarvest et al., 1999). Carboxylated lignins based on a cinnamic acid scaffold exhibited potent inhibitory effects on HSV-1 entry (Thakkar et al., 2010). The compound synthesized by attaching cinnamic acids to caudatin and its derivatives suppressed HBV promoters and enhancers, resulting in the inhibition of HBV replication (Wang et al., 2012). However, the effects of cinnamic acid on HCV infection have not yet been fully elucidated.

The activation of signal transducer and activator of transcription 3 (STAT3) is dependent on the phosphorylation of Tyr^{705} in response to several cytokines, including interleukin-6 (IL-6), leukemia inhibitory factor, epidermal growth factor and oncostatin M (Bromberg and Darnell, 2000; Wen et al., 1995; Zhong et al., 1994). The binding of IL-6 to its receptor leads to receptor dimerization and the activation of JAK1/2. Activated JAK1 and 2 can phosphorylate STAT3 Tyr^{705}. Phosphorylated STAT3 proteins are homo-dimerized and then translocated into the nucleus for the promotion of targeted gene expression (Heinrich et al., 1998). STAT3 was reported to play a role in HCV replication (McCartney et al., 2013; Waris et al., 2005) and to associate with HCV-induced carcinogenesis (Yoshida et al., 2002). AG490, which is classified in the cinnamic acid group as a JAK2 inhibitor, specifically inhibits the phosphorylation of STAT3 Tyr^{705} (Mielecki and Lesyng, 2016) and suppresses HCV propagation (McCartney et al., 2013; Waris et al., 2005), suggesting that the phosphorylation of STAT3 Tyr^{705} is critical for the HCV life cycle.

In this study, we prepared cinnamic acid derivatives based on an AG490 scaffold and screened them using HCV replicon cells to identify novel antivirals against HCV. We also analyzed the mode of action of hit compounds.

2. Materials and methods

2.1. Cell culture and luciferase reporter assay

The Huh7/Rep-Feo cell line harboring the subgenomic replicon RNA of the N strain (genotype 1b) was reported previously (Yokota et al., 2003). The HCV replicon cell line derived from strain JFH1 (genotype 2a) was described previously (Nishimura-Sakurai et al., 2010). The S52/SG-Feo (AI) cell line (genotype 3a) and ED43/SG-Feo (VYG) cell line (genotype 4a) were kindly provided by C. M. Rice (Saeed et al., 2012, 2015). These replicon cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, nonessential amino acids and 0.5 mg/mL G418. Huh-7 cells were maintained in DMEM containing 10% fetal calf serum, sodium pyruvate and nonessential amino acids (Moriishi et al., 2010).

The replicon cells harboring luciferase transcript on the replicon RNA as described above were treated with various concentrations of compounds and then harvested at 72 h post-treatment for the estimation of luciferase activity (Shen et al., 2013). Transient replicon assay was conducted by the method of Krieger et al. (2001). The subgenomic replicon RNA carrying firefly luciferase and nonstructural genes with GND mutation (Con1 GND RNA) (Krieger et al., 2001) was kindly provided by R. Bartenschlager.

2.2. Oxidative stress-detection assay

The levels of ROS were measured with CELLROX® Green Reagents (Thermo Fisher Scientific). The genotype 1b replicon cells were seeded at a density of 1.6×10^6 cells per well on a 6-well plate. The cells were treated with CELLROX® Green and harvested 27 h post-treatment. Compound **6** or hydroxyl peroxide was added with or without NAC at 3 h before cell harvest. The CELLROX® Green Reagent fluorescence in 20,000 cells was measured with a FACSCalibur flow cytometer (BD Bioscience). Mean fluorescence intensities were calculated as levels of ROS with CellQuest software (BD Bioscience).

2.3. HCV infection and virus titration

An Huh7 subclone that is highly permissive to infection with cell culture-generated HCV strain JFH-1 (HCVcc) was established as described previously (Okamoto et al., 2006). Viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7 cells according to the method of Wakita et al. (2005). Huh7 cells were infected with JFH1 at a multiplicity of infection of 0.1 and then passaged two times for 4 days each time. The resulting cells exhibiting more than 90% NS5A-positive cells were used as HCVcc-infected cells in this study (Kasai et al., 2015). Mouse monoclonal anti-HCV NS5A antibodies (Austral) and Alexa Fluor-488 -conjugated goat anti-mouse IgG antibodies (Thermo Fisher Scientific) were employed for the focus-forming assay (Wakita et al., 2005). HCV-positive foci were observed with fluorescence microscopy (BZ-9000, KEYENCE, Osaka, Japan) and were counted to calculate a focus-forming unit. HCV pseudo-particle (HCVpp) encoding E1 and E2 of H77 strain was prepared by the method reported previously (Bartosch et al., 2003). Firefly luciferase gene was introduced into pMXs (Kitamura et al., 2003). The resulting plasmid was designated as pMXs-luc.

3. Results

3.1. Effect of cinnamic acid derivatives on HCV replication

To identify novel antivirals against HCV infection, we prepared 17 cinnamic acid derivatives based on an AG490 scaffold, and we screened them using genotype 1b replicon cells to identify more potent antiviral than AG490 (Table 1). AG490 could inhibit the replication of genotype 1b replicon with an EC_{50} value of 15.2 ± 0.8 μM (Supplementary Fig. 1). Each compound was added to the culture supernatant at a final concentration of 10 μM, which is the lowest concentration of AG490 among concentrations reported previously (McCartney et al., 2013; Waris et al., 2005). Luciferase activity and cell viability were estimated at 72 h post-treatment. Anti-HCV candidates were defined in the primary screening as compounds exhibiting less than 30% HCV replication of the mock control (Fig. 1A) and more than 80% cell viability (Fig. 1B), since luciferase activity was reduced to about 60% of mock control in cells treated with 10 μM AG490 (Supplementary Fig. 1). Compounds **5** and **6** were finally identified as hit compounds and were then administered into the genotype 1b replicon cells for dose-dependency analysis. Compound **6** suppressed the viral replication with an EC_{50} value of 4.2 μM and an SI value of 48, which were more appropriate for further analysis on anti-HCV compounds compared to the values of compound **5**. (Supplementary Fig. 2 and Table 2). Compound **6** did not affect activities of firefly and *Renilla* luciferase under the control of CAG promoter (Supplementary Fig. 3), suggesting that compound **6**-dependent inhibition of

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