JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2012, **19**, 364–370 doi:10.1111/j.1365-2893.2011.01558.x

Anti-hepatitis C virus activity of 3-hydroxy caruilignan C from *Swietenia macrophylla* stems

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Received July 2011; accepted for publication October 2011

SUMMARY. Chronic hepatitis C virus (HCV) infection ultimately leads to chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (HCC). As the standard treatment is not completely efficacious, a safer and more effective agent against HCV infection needs to be developed. In this report, we demonstrated that 3-hydroxy caruilignan C (3-HCL-C) isolated from *Swietenia macrophylla* stems exhibited high anti-HCV activity at both protein and RNA levels at nontoxic concentrations, with an EC_{50} value of 10.5 ± 1.2 μM. Combinations of 3-HCL-C and interferon-α (IFN-α), an HCV NS5B polymerase inhibitor (2′-C-methylcytidine; NM-107) or an HCV NS3/4A protease inhibitor (Telaprevir; VX-950) increased the suppression of HCV RNA replication. The results suggested that 3-HCL-C may be a potential anti-viral agent. We then demonstrated that 3-HCL-C interfered with HCV replication by inducing IFN-stimulated response element transcription and IFN-dependent anti-viral gene expression.

Keyword: hepatitis C virus, interferon, lignan, *Swietenia macrophylla*.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped virus belonging to the Hepacivirus genus within the *Flaviviridae* family [1]. It has a 9.6-kb genome, which comprises an open reading frame flanked by highly structured 5′ and 3′ untranslated regions [2]. The virus polyprotein is cleaved by both host and viral proteases into 10 mature individual proteins: three structural proteins (C, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [3].

Over 170 million people worldwide are infected with HCV, which can lead to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) [4]. The current standard of therapy [5], a combination of pegylated interferon-α (PEG-IFN-α) with ribavirin, is usually accompanied with extensive side effects that have often led to discontinuation of therapy [6]. Moreover, a sustained virological response is achieved in approximately 50% of patients infected with HCV genotype 1. Consequently, it is important to develop a less toxic and more effective agent for the treatment of HCV.

Swietenia macrophylla belongs to the Meliaceae family, and is related to the red-brown wood of the species *S. mahogany*, known as mahogany. The fruit of *S. macrophylla* (sky fruit) has been comprehensively used as a folk medicine to treat diabetes and hypertension in Malaysia [7]. The crude extract from the seeds of *S. macrophylla* has been reported to possess biological activities such as anti-inflammatory, anti-mutagenec [8], anti-malarial [9], anti-diarrhoeal [10], anti-diabetic [11], anti-microbial [12] and anti-tumor properties [13]. Until now, the anti-viral activity of the phytochemicals of *S. macrophylla* has not been investigated. In this study, we performed an anti-viral activity-guided fractionation and isolation procedure to screen for anti-HCV components of *S. macrophylla* using a cell-based HCV replicon system [14]. A bioactive compound was isolated from the most active ethyl acetate extract of *S. macrophylla* stems (SMS), and was identified as 3-hydroxy caruilignan C (3-HCL-C). 3-HCL-C effectively inhibited HCV replication with no apparent cytotoxicity, and

Abbreviations: 3-HCL-C, 3-hydroxy caruilignan C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN-α, interferon-α; ISG, IFN-stimulated gene; ISRE, interferon-stimulated response element; PKR protein kinase R.

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possible mechanisms underlying anti-HCV activity were further elucidated. Combinations of 3-HCL-C and IFN-α or promising HCV enzyme inhibitors were tested to determine their enhancement of anti-HCV activity.

MATERIALS AND METHODS

Extraction and isolation

The stems of *S. macrophylla* were collected from Kaohsiung Metropolitan Park in September 2007, and were powdered and extracted with methanol (MeOH). The MeOH extract was partitioned into *n*-butanol (BuOH), ethyl acetate (EA) and water-soluble fractions. The *n*-BuOH-soluble fraction (100 g) was subjected to column chromatography on a porous polymer polystyrene (Diaion HP-20) and eluted with water (8 L), 25% MeOH (12 L), 50% MeOH (16 L), 75% MeOH (6 L), 100% MeOH (6 L) and 100% acetone (4 L), to give six fractions designated as SMS-D1–SMS-D6. The SMS-D4 (10 g) fraction was then successively partitioned with EA. The EA-soluble fraction (5.54 g) was further separated by silica column chromatography (4.5 × 25 cm) using the gradient solvent system [dichloromethane (CH_2Cl_2):MeOH (50:1–0:100)] to yield 10 subfractions (SMS-D4-N1–SMS-D4-N10). Subsequently, SMS-D4-N3 was purified by high-performance liquid chromatography using a C18 column (Spherical, 250 × 10 mm, 5 μm) to obtain 3-hydroxy caruilignan (3-HCL-C) (11.09 mg). The chemical structure was identified using 1D and 2D nuclear magnetic resonance and confirmed using mass spectrometry.

Cell culture and reagents

Ava5 cells are human hepatoma cells (Huh-7) that contain HCV subgenomic replicon RNA [14]; these were cultured as described previously [15]. Interferon alfa-2a (Roferon©-A) was purchased from Hoffmann-La Roche Inc., Nutley, NJ, USA. Telaprevir (VX-950) and 2′-C-methylcytidine (NM-107) were purchased from Kouting Chemical Co., Ltd., Shanghai, China and Toronto Research Chemicals Inc., Ontario, Canada, respectively, and were stored at 10 mM in 100% DMSO.

Western blotting

The standard procedure was used for Western blotting [16]. The membranes were probed with either anti-NS3 antibody (1:2000; Virostat, Portland, ME, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (1:10000; GeneTex, Irvine, CA, USA).

Determination of cytotoxicity

The CellTiter 96® Aqueous One Solution Cell Proliferation assay system (Promega, Madison, WI, USA) was used to assess cell viability as described previously [15].

Quantification of HCV and cellular gene RNAs

The quantification of the viral and cellular gene RNA was performed by a previously described methodology [17]. The polymerase chain reaction (PCR) primers used are given in Table 1. Each sample was normalized using endogenous cellular GAPDH.

Analysis of the combination treatment

Ava5 cells were treated with diluted 3-HCL-C (12.5 and 15 μM) in combination with diluted IFN-α (30 and 60 U/mL), NM107 (0.75 and 1.5 μM) or VX-950 (0.3 and 0.6 μM). After 3 days of incubation, the total cellular RNA was collected and analyzed using quantitative real-time RT-PCR (RT-qPCR), in which endogenous cellular GAPDH served as a loading control.

Transfection and luciferase activity assay

Ava5 cells were transfected with 0.5 μg of the plasmid pIS-RE-Luc (BD Biosciences Clontech, Palo Alto, CA, USA) using T-Pro™ reagent (Ji-Feng Biotechnology Co., Ltd., Taipei, Taiwan) in accordance with the manufacturers' instructions. Each transfection complex contained 0.1 μg of secreted alkaline phosphatase (SEAP) reporter vector (pCMV-SEAP) to serve as an internal control for normalization of the transfection efficiency. The transfected cells were then incubated with various concentrations of 3-HCL-C or vehicle control (0.1% DMSO) for 3 days. The luciferase activity assay was performed using the Bright-Glo™ Luciferase assay system (Promega) according to the manufacturer's instructions.

Table 1 Oligonucleotide sequences for real-time RT-PCR

Oligonucleotide name	Sequence 5′–3′
5′GAPDH	5′-GTC TTC ACC ACC ATG GAG AA
3′GAPDH	5′-ATG GCA TGG ACT GTG GTC AT
5′NS5B	5′-GGA AAC CAA GCT GCC CAT CA
3′NS5B	5′-CCT CCA CGG ATA GAA GTT TA
5′OAS1	5′-CAA GCT TAA GAG CCT CAT CC
3′OAS1	5′-TGG GCT GTG TTG AAA TGT GT
5′OAS2	5′-ACA GCT GAA AGC CTT TTG GA
3′OAS2	5′-GCA TTA AAG GCA GGA AGC AC
5′OAS3	5′-CAC TGA CAT CCC AGA CGA TG
3′OAS3	5′-GAT CAG GCT CTT CAG CTT GG
5′PKR	5′-ATG ATG GAA AGC GAA CAA GG
3′PKR	5′-GAG ATG ATG CCA TCC CGT AG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OAS, oligoadenylate synthetase; PKR, protein kinase R.

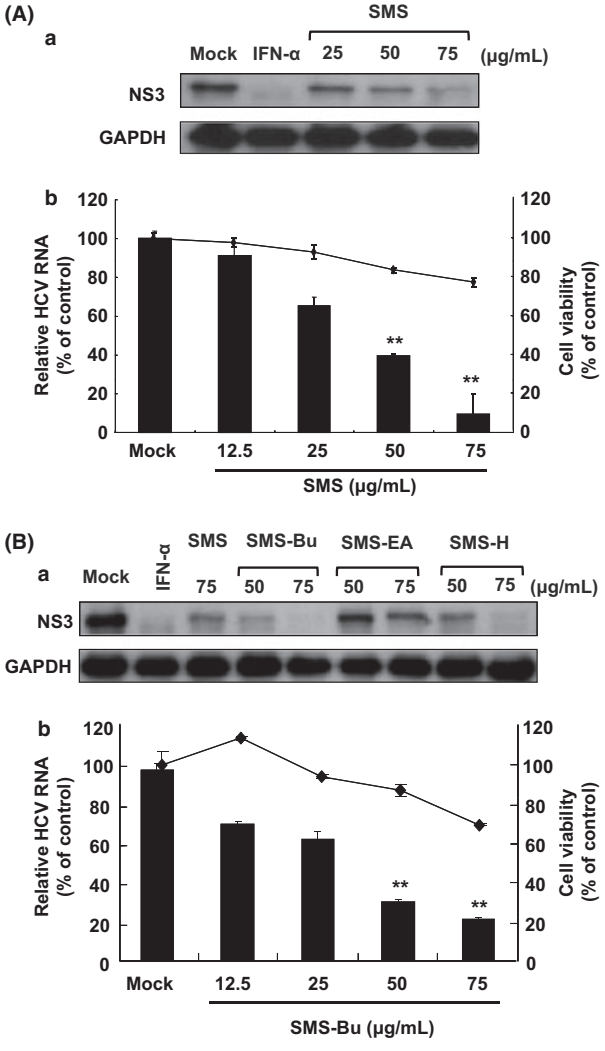
Statistical analysis

Data are presented as the mean ± standard deviation (SD) from triplicate experiments. Statistical comparison of the data from the various samples was carried out using the Student's *t*-test. The differences were considered to be significant at $^{*}P < 0.05$ or $^{**}P < 0.01$.

RESULTS AND DISCUSSION

Extract of SMS suppressed HCV RNA replication

At first, SMS were dried and extracted with MeOH. Then, HCV replicon cells (Ava5 cells) [14] were treated with SMS at concentrations of 25, 50 and 75 μg/mL for 4 days. Western blotting was used to examine the inhibitory effect of the crude extract on the synthesis of HCV protein. As shown in Fig. 1A-a, SMS reduced the synthesis of HCV NS3 in a dose-dependent manner compared with the mock control (0.1% DMSO), in which the treatment of IFN-α served as positive control of anti-HCV activity. Quantification of HCV replicon RNA levels using RT-qPCR confirmed the anti-viral activity of SMS (Fig. 1A-b, left axis) with no apparent cytotoxicity, as revealed using the MTS assay (right axis). Therefore, the crude SMS extract was further partitioned into BuOH-soluble (SMS-Bu), EA-soluble (SMS-EA) and water-soluble (SMS-H) fractions for anti-HCV activity assay. As shown in Fig. 1B-a, the SMS-Bu fraction demonstrated significant inhibition of HCV NS3 protein synthesis and a concentration-dependent reduction in HCV RNA levels (Fig. 1B-b, left axis). Slight cytotoxicity was observed among cells incubated with the SMS-Bu fraction at effective concentrations (right axis).



Reduction of HCV protein synthesis and RNA levels by a lignan compound

Each fraction from the SMS-Bu crude extract was examined for anti-HCV activity and cytotoxicity. Finally, a bioactive compound, 3-hydroxy caruilignan (3-HCL-C), was obtained (Fig. 2A). To determine the anti-viral effect of 3-HCL-C, Ava5 cells were treated with various concentrations of 3-HCL-C for 4 days. As shown in Fig. 2B-a, 3-HCL-C displayed an inhibitory effect on viral protein synthesis at a concentration of 15 μM. Similarly, RT-qPCR analysis clearly demonstrated that 3-HCL-C decreased HCV RNA levels in a concentration-dependent manner, with an EC_{50} value of 10.5 ± 1.2 μM (Fig. 2B-b, left axis). The level of HCV RNA was suppressed by approximately 80% at a concentration of

Fig. 1 Reduction of hepatitis C virus (HCV) replication by the crude extracts and three partitioned fractions derived from *S. macrophylla* king stems (SMS). (A) (a) Concentration-dependent reduction of HCV protein synthesis. Ava5 cells were incubated with crude extracts of SMS at indicated concentrations for 4 days, and total protein was analysed using Western blotting with specific antibody against HCV NS3 or GAPDH. Treatment with 100 U/mL IFN-α and 0.1% DMSO served as the positive and mock controls respectively. (b) Concentration-dependent reduction of HCV RNA levels. HCV RNA levels in the SMS-treated Ava5 cells were quantified using RT-qPCR following incubation for 3 days. Relative HCV RNA levels were normalized by cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Cellular toxicity was evaluated using the MTS assay. (B) (a) Concentration-dependent reduction of HCV protein synthesis by the partitioned fractions of various solvents; *n*-butanol (SMS-Bu), ethyl acetate (SMS-EA), and water (SMS-H). HCV protein synthesis was measured using Western blotting analysis. (b) Concentration-dependent reduction of HCV RNA levels with no apparent cytotoxicity. HCV RNA levels were quantified using RT-qPCR analysis. The errors reflect the standard deviation (SD) following triplicate experiments. An asterisk (*) represents a significant difference from controls at $^{*}P < 0.05$ or $^{**}P < 0.01$.

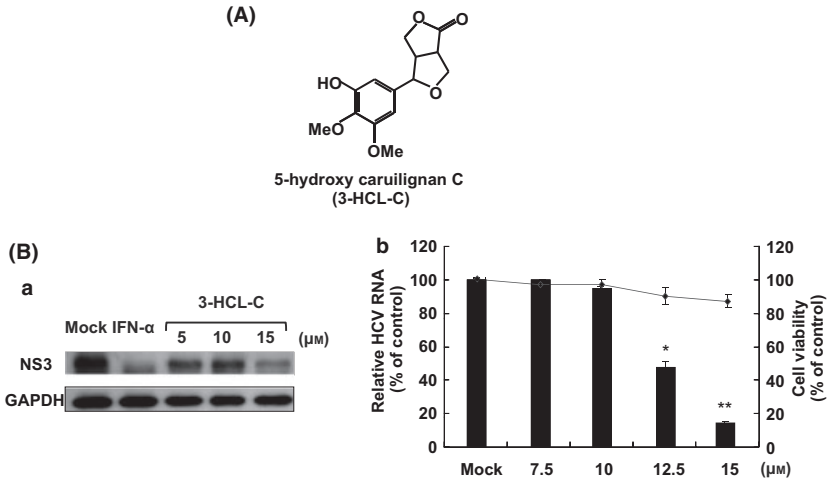


Fig. 2 The inhibitory effect of 3-hydroxy caruilignan C compound (3-HCL-C) on hepatitis C virus (HCV) RNA levels and protein synthesis was dose-dependent. (A) Chemical structure of 3-HCL-C. (B) (a) Concentration-dependent reduction of HCV protein synthesis by 3-HCL-C. Ava5 cells were treated with 3-HCL-C at indicated concentrations for 4 days. Western blotting was performed with specific antibody against HCV NS3 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Treatment with 100 U/mL IFN-α and 0.1% DMSO served as the positive and mock controls respectively. (b) Concentration-dependent suppression of HCV RNA levels by 3-HCL-C. HCV RNA levels in the 3-HCL-C-treated Ava5 cells were quantified using RT-qPCR analysis following incubation for 3 days. The efficacy of inhibition is shown as a percentage relative to the level of HCV RNA in cells incubated with 0.1% DMSO (mock control). Cell viability was analysed using MTS assay. The errors reflect SD following triplicate experiments. An asterisk (*) represents a significant difference from control at $^{*}P < 0.05$ or $^{**}P < 0.01$.

15 μM, at which no apparent cytotoxicity was observed (right axis).

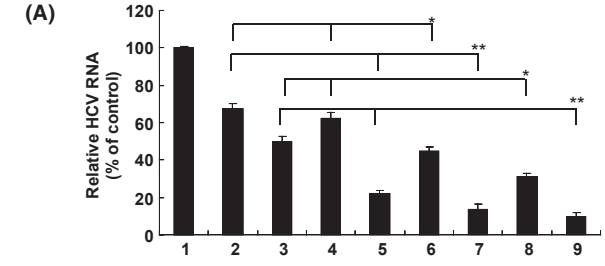
The anti-viral effect of a combination of 3-HCL-C with either IFN-α or viral enzyme inhibitors on HCV replication

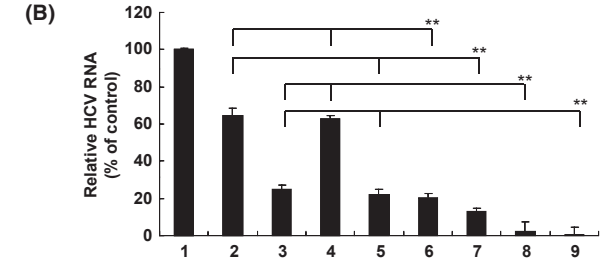
To examine the anti-viral effect of 3-HCL-C in combination with IFN-α, Ava5 cells were incubated with 3-HCL-C and various anti-HCV inhibitors at fixed concentrations for 3 days. As shown in Fig. 3A, the combination of 3-HCL-C and IFN-α induced a more significant decrease in HCV RNA levels (lanes 6–9), when compared with the treatment with each agent alone (lanes 2–5) and the DMSO vehicle control (lane 1). Moreover, the anti-HCV effects of 3-HCL-C combined with other potential HCV enzyme inhibitors, the NS5B polymerase inhibitor 2′-C-methylcytidine (NM-107) [18] and the NS3/4A protease inhibitor Telaprevir (VX-950), a phase III clinical compound [19,20], were also examined. Similar to the results described above, an additive effect on the inhibition of HCV RNA replication was also observed for treatments with 3-HCL-L in combination with either NM-107 (Fig. 3B) or VX-950 (Fig. 3C). Therefore, 3-HCL-C has the potential to be developed into a potent adjuvant for anti-HCV therapy.

The inhibitory effect of 3-HCL-C on HCV replication by induction of anti-viral IFN response

To gain insight into the molecular mechanism by which 3-HCL-C reduced HCV replication, we examined the influence of 3-HCL-C on the IFN signaling pathway, which is suppressed during HCV infection [21]. At first, a transient interferon-stimulated response element (ISRE) activity assay using the ISRE-mediated firefly luciferase expression vector pISRE-Luc was performed to verify the induction of IFN-associated genes by 3-HCL-C. The pISRE-Luc-transfected Ava5 cells were treated with various concentrations of 3-HCL-C for 3 days. The results revealed that 3-HCL-C significantly increased the ISRE-mediated luciferase activity by 2.54- and 3.07-fold at concentrations of 12.5 and 15 μM, respectively, compared with the mock control (0.1% DMSO) (Fig. 4A). In addition, several IFN-mediated anti-viral genes, including protein kinase R (PKR), 2′-5′-oligoadenylate synthetase (OAS) 1, OAS2 and OAS3, were examined at levels of mRNA transcription using RT-qPCR analysis in 3-HCL-C-treated Ava5 cells. Compared with DMSO-treated Ava5 cells, 3-HCL-C significantly induced the expression of PKR, OAS1, OAS2 and OAS3 (Fig. 4B). These observations suggested that the partial restoration of IFN-dependent anti-viral genes may contribute to the IFN-based antiviral activity of 3-HCL-C.

Plants are important sources of biologically active substances, such as coumarins, alkaloids, flavonoids, terpenes, phenols and lignans. Currently, a mixture of flavonoligands contained in silymarin (milk thistle extract) have been used in eradicating chronic hepatitis C through a multitude of hepatoprotective functions in initial trials [22]. Lignan derivatives are common substances present in the roots, stems, bark, fruit and seeds of many plant species, which





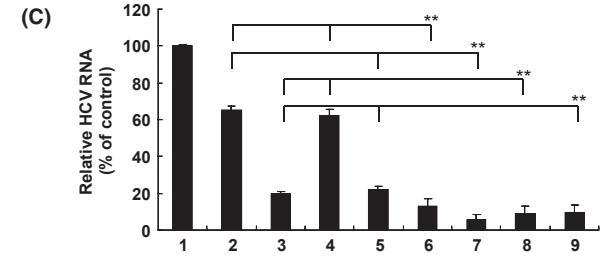


Fig. 3 Enhanced inhibition of the combined treatment with 3-HCL-C 3-hydroxy caruilignan C (3-HCL-C) and interferon (IFN)-α, NM-107, or VX-950 in hepatitis C virus (HCV) replicon cells. Ava5 cells were treated with 3-HCL-C combined with (A) IFN-α, (B) NM-107 or (C) VX-950 at indicated concentrations for 3 days. HCV RNA levels were quantified using RT-qPCR analysis. The efficacy of inhibition is shown as a percentage relative to level of HCV RNA in cells incubated with 0.1% DMSO (mock control). The errors reflect the SD from triplicate experiments. An asterisk (*) represents a significant difference from control at $^{*}P < 0.05$ or $^{**}P < 0.01$.

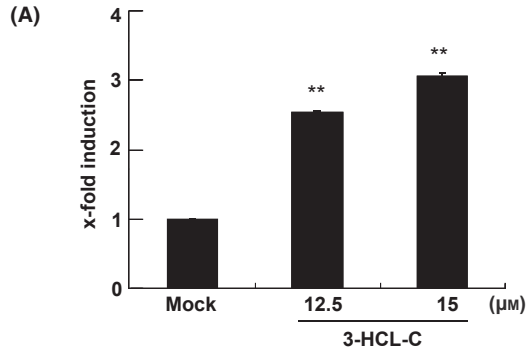


Fig. 4 Reduction of hepatitis C virus replication by 3-HCL-C 3-hydroxy caruilignan C (3-HCL-C) correlated with the induction of anti-viral interferon (IFN) response. (A) Concentration-dependent induction of IFN-stimulated response element (ISRE) activity by 3-HCL-C. The pISRE-Luc-transfected Ava5 cells were incubated with 3-HCL-C at 12.5 and 15 μM for 3 days, and then the total cell lysates were analysed for luciferase activity. The enhanced luciferase activity compared with the signal of mock-treated cells (0.1% DMSO) is represented as the mean fold of induction ± SD from triplicate experiments. The RNA level in the pISRE-Luc-transfected Ava5 cells without the drugs treatment served as the basal control of ISRE promoter activity, which was defined as 1. (B) Concentration-dependent induction of IFN-mediated anti-viral gene expression. Ava5 cells were exposed to 3-HCL-C at indicated concentrations for 3 days. ''Mock'' indicates the treatment with 0.1% DMSO. Expression of protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS) 1–3 was determined using RT-qPCR analysis. The induced gene expression compared with the mRNA level of mock-treated cells, defined as 1, is represented as the mean fold of induction ± SD from triplicate experiments. An asterisk (*) represents a significant difference from controls at $^{*}P < 0.05$ or $^{**}P < 0.01$.

have been shown to exhibit a variety of biological activities, including anti-tumor [23], anti-oxidative [24], anti-hepatitis [25] and anti-virus [26]. Recent studies have demonstrated that lignan derivatives exerted inhibitory activity against several viruses including human immunodeficiency virus-1 [27,28], hepatitis B virus [29] and Japanese encephalitis virus [30], although the detailed mechanisms of this

anti-viral activity are still debatable. In the present study, we clearly demonstrated that a phytochemical constituent of SMS, 3-HCL-C, which is a lignan derivate, is promisingly effective in its anti-HCV activity at both protein and RNA levels (Fig. 2).

IFNs comprise a family of multifunctional cytokines that prevent viral infections through the activation of IFN-stimulated gene (ISG) expression [31]. Conversely, several of the HCV proteins, such as core, E2 and NS3/4A and NS5A, have been reported to block IFN-α signaling after HCV infection through interaction with the signal transducer and activator of transcription 1 (STAT1), PKR or 2′-5′-OAS, leading to evasion of innate cellular defense pathways [32–34]. In this report, 3-HCL-C resulted in considerable induction of ISRE-controlled promoter activity and the expression of several known ISGs with anti-viral activity (Fig. 4), which indicated that lignans can have an alternative effect on HCV replication by activating important mediators of intracellular the host anti-viral response. The induction of ISGs is regulated by transcription factors such as phosphorylated STAT1/STAT2 and IFN regulatory factor 3 complexes through binding of the consensus sequence of ISRE within the promoter region of many anti-viral ISGs [35]. More recently, the inhibition of the Janus kinase [36]-STAT pathway has been reported to be involved in anti-HCV activity in IFN responses [37,38]. Therefore, it is essential to further explore whether the JAK-STAT pathway, the upstream effector of the ISRE, could be influenced by 3-HCL-C.

ACKNOWLEDGEMENTS

We are grateful to Dr. Charles Rice at Rockefeller University and Aapth, LCC, USA, for kindly supplying the Con1b replicon plasmid, the Human hepatoma cell line Huh-7 and the HCV subgenomic replicon containing cell line Ava5.

DECLARATION OF FUNDING INTERESTS

This work was supported by the National Science Council of Taiwan (grant number: NSC 100-2311-B-037-001) and the Council of Agriculture, Executive Yuan, Taiwan (grant number: 100AS-1.1.5-HI-H3).

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