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Anti-hepatitis C virus activity of *Acacia confusa* extract via suppressing cyclooxygenase-2

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ABSTRACT

Chronic hepatitis C virus (HCV) infection continues to be an important cause of morbidity and mortality by chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) throughout the world. It is of tremendous importance to discover more effective and safer agents to improve the clinical treatment on HCV carriers. Here we report that the *n*-butanol–methanol extract obtained from *Acacia confusa* plant, referred as ACSB-M4, exhibited the inhibition of HCV RNA replication in the HCV replicon assay system, with an EC_{50} value and CC_{50}/EC_{50} selective index (SI) of 5 ± 0.3 μg/ml and >100, respectively. Besides, ACSB-M4 showed antiviral synergy in combination with IFN-α and as HCV protease inhibitor (Telaprevir; VX-950) and polymerase inhibitor (2′-*C*-methylcytidine; NM-107) by a multiple linear logistic model and isobologram analysis. A complementary approach involving the overexpression of COX-2 protein in ACSB-M4-treated HCV replicon cells was used to evaluate the antiviral action at the molecular level. ACSB-M4 significantly suppressed COX-2 expression in HCV replicon cells. Viral replication was gradually restored if COX-2 was added simultaneously with ACSB-M4, suggesting that the anti-HCV activity of ACSB-M4 was associated with down-regulation of COX-2, which was correlated with the suppression of nuclear factor-kappaB (NF-κB) activation. ACSB-M4 may serve as a potential protective agent for use in the management of patients with chronic HCV infection.

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1. Introduction

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus belonging to the family *Flaviviridae* (Lindenbach and Rice, 2005). It has a 9.6-kb genome encoding a single polyprotein that is subsequently cleaved by both host and virus protease into at least 10 mature individual proteins: four structural proteins (C, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Penin et al., 2004). Approximately 170 million people worldwide are chronically infected with HCV, which is leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Alter, 2007; Levrero, 2006). To date, there is no prophylactic vaccine available to prevent HCV infection. The current standard of care for chronic hepatitis C involves the administration of pegylated interferon-α (IFN-α) in combination with the nucleoside analog ribavirin (Ferenci, 2006). However, this regimen has an unfavorable side-effect profile (including flu-like symptoms, hemolytic anemia, and depression), which often leads to discontinuance of therapy (Schaefer and Mauss, 2008). Thus, there is a strong medical need to discover novel agents with a high therapeutic index and few side-effects to treat chronic HCV infection.

Constitutive NF-κB activation, caused by infection with viruses, is recognized as a risk factor for virally induced hepatic failure due to chronic inflammation or proliferation of hepatoma cells (Sun and Karin, 2008). Cyclooxygenase-2 (COX-2) is a critical NF-κB-mediated factor that participates in inflammatory disorders and is associated with human cancer (Pikarsky et al., 2004; Tang et al., 2005). Recent studies have shown that HCV proteins, including core, E2, NS3 and NS5A, promote the improper up-regulation of hepatic NF-κB and COX-2 signaling pathway leading to HCC (Lu et al., 2008; Nunez et al., 2004; Waris and Siddiqui, 2005). Thus, the NF-κB–COX-2 signaling pathway represents a pharmacological

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target in the therapy of HCV-related inflammation and carcinogenesis (El-Bassiouny et al., 2007).

Acacia confusa species are indigenous to Taiwan and have been used as a traditional medicine, such as wound healing and anti-blood-stasis (Kan, 1978; Wu et al., 2008). The main bioactivities shown by a number of phenolic compounds isolated from crude extracts of *A. confusa* bark, flowers or heartwood were antioxidation, anti-inflammation, and anti-xanthine oxidase effects, suggesting that the *A. confusa* might be a valuable source for the pharmacotherapy of cancer and inflammatory disease (Tung and Chang, 2010a,b; Tung et al., 2009a,b; Wu et al., 2005, 2008). Nevertheless, there has been no investigation on the prevention of infectious disease by *A. confusa*. In this study, we investigated the efficacy against HCV replication of constituents of extracts from *A. confusa* stem using a bioassay-guided fractionation and isolation procedure. Column chromatography was used to separate and purify active fraction(s), which were evaluated for anti-HCV activity in a cell-based HCV replicon system (Blight et al., 2000). A partially purified fraction, referred to here as ACSB-M4, obtained by extraction with *n*-butanol–methanol displayed high anti-HCV activity in cultured cells, and its inhibitory effects may be due to down-regulation of cyclooxygenase-2 (COX-2) by suppression of nuclear transcriptional factor-κB (NF-κB) activation.

2. Materials and methods

2.1. Preparation of crude extract and various fractions

Dried stems of *A. confusa* (5 kg) were collected in September 2007, and then extracted with MeOH (20 L × 5) at room temperature and concentrated under reduced pressure at 35 °C to yield a viscous extract (340 g). The viscous extract was partitioned into *n*-hexane and 95% MeOH soluble-fractions. The latter fraction was further partitioned into *n*-butanol and H_2O soluble-fractions after being concentrated. Using bioactivity-guided fractionation and isolation method, the *n*-butanol soluble-fraction (132.6 g) was subjected to Diaion HP-20 column chromatography (14.5 cm × 30 cm) and eluted with H_2O, 100% acetone, 25% MeOH, 50% MeOH, 75% MeOH, and 100% MeOH, six fractions were obtained and designed as ACSB-H (0.5 g), ACSB-A (1.4 g), ACSB-M1 (20.2 g), ACSB-M2 (70.2 g), ACSB-M3 (25.2 g), and ACSB-M4 (10.1 g), respectively. The characteristics of ACSB-M4 by nuclear magnetic resonance (NMR) are shown in Fig. 5.

2.2. Cell culture and reagents

Ava5 cells are the human hepatoma cells (Huh-7) harboring HCV subgenomic replicon RNA (Blight et al., 2000) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 1% Antibiotic–Antimycotic, 1% Non-essential amino acids, 1 mg/ml G418. The interferon alfa-2a (Roferon©-A) was purchased from Roche Ltd. 2′-C-methylcytidine (NM-107) and Telaprevir (VX-950) was purchased from Toronto Research Chemicals Inc. and Kouting Chemical Co. Ltd, respectively, which were stored as 10 mM in 100% dimethylsulfoxide (DMSO). The final concentration of DMSO in all reactions was maintained constantly at 0.1% in the experiments.

2.3. Plasmid construction

The cDNA of human COX-2 (GenBank Accession no.: BC013734) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA) and cloned into pcDNA™4/myc-His A by EcoRI and ApaI, designed as pCMV-COX-2-Myc. COX-2 promoter fragment was amplified from human genomic DNA as described (Tazawa et al., 1994). The PCR product (−891/+9) flanked with KpnI was inserted into the promoterless luciferase vector pGL3-Basic (Promega Co, Madison, WI), designed as pCOX-2-Luc. pNF-κB-Luc and pAP-1-Luc are the reporter vectors to measure NF-κB and AP-1-dependent transcription activity (Stratagene, La Jolla, CA). The cloned DNA fragments were verified by DNA sequencing.

2.4. Western blotting assay

A standard procedure was used for Western blotting (Lee et al., 2010). Membranes were probed with either anti-NS5B antibody (1:5000; Abcam, Cambridge, MA) or anti-GAPDH antibody (1:10,000; GeneTex, Irvine, CA) or anti-C-Myc antibody (1:1000; GeneTex, Irvine, CA) or anti-COX-2 antibody (1:1000; Cayman, Ann Arbor, MI). The signal was detected using an ECL detection kit (PerkinElmer, CT).

2.5. Quantification of HCV RNAs

Total cellular RNA was extracted by using RNA Trizol reagent (Invitrogen, Carlsbad, CA) according to the Manufacturer's instructions. The expression of HCV subgenomic RNA was detected by quantitative real-time RT-PCR (RT-qPCR) with primers corresponding to NS5B gene; Forward primer: 5′-GGA AAC CAA GCT GCC CAT CA-3′ and Reverse primer: 5′-CCT CCA CGG ATA GAA GTT TA-3′. Each sample was normalized by an endogenous reference gene glyceraldehydes-3-phosphate dehydrogenase (*gapdh*); Forward primer: 5′-GTC TTC ACC ACC ATG GAG AA-3′ and Reverse primer: 5′-ATG GCA TGG ACT GTG GTC AT-3′. The cDNA quantification was measured by the ABI Step One Real-Time PCR-System (ABI Warrington, UK).

2.6. Cytotoxicity assay

The cell viability was evaluated by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS) method (Promega, Madison, WI) according to the Manufacturer's instructions. The absorbance was detected at 490 nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK).

2.7. Analysis of drug synergism

Ava5-EG(Δ4AB)SEAP cells (Lee et al., 2004) were treated with serially diluted ACSB-M4 (2.5, 5, 10, and 25 μg/ml) in combination with serially diluted IFN-α (5, 10, 30, and 60 U/ml), NM-107 (1.25, 2.5, 5, and 10 μM), or VX-950 (0.125, 0.25, 0.5, and 1 μM). Two days later, culture medium was replaced with fresh medium containing the same concentration of inhibitors and cells were incubated for another 1 day. Culture medium was collected and subjected to measurement of secreted alkaline phosphatase (SEAP) activities by using Phospha-Light assay kit (Tropix, Foster City, CA), according to Manufacturer's instruction. Combination index (CI) values were analyzed using CalcuSyn™ software (Biosoft, Cambridge, UK) (Chou and Talalay, 1984). Briefly, according to the percentage inhibition of SEAP activity, CI value is calculated with the formula: $CI = (Da + Db)/(Dxa + Dxb) + DaDb/DxaDxb$. Da and Db are the doses of drugs A (for example, the ACSB-M4) and B (for example, IFN-α) to inhibit X% of SEAP activity as single drugs, whereas Dxa and Dxb are the doses of A and B to inhibit X% of SEAP activity in a combination treatment, in which treatment of 0.1% DMSO is served as a negative control for the inhibitory effect. The effect of multiple drug combination is presented as antagonism ($CI > 1$), additivity ($CI = 1$), or synergism ($CI < 1$). In addition, traditional isobologram analysis was used to confirm the drug-drug interaction (Tallarida, 2001).

2.8. Transfection and luciferase activity assay

For evaluation of COX-2, NF-κB, and AP-1 regulated by ACSB-M4, Ava5 cells were transfected with 1 μg of plasmid pCOX-2-Luc, pAP-1-Luc or pNF-κB-Luc (BD Biosciences Clontech, Palo Alto, CA) by using T-Pro™ reagent (Ji-Feng Biotechnology Co. Ltd., Taiwan) in accordance with the Manufacturer's instructions. Each transfection complex contained 0.1 μg of SEAP expression vector (pCMV-SEAP) to serve as an internal control. Subsequently, the transfected cells were incubated with different concentrations of ACSB-M4. For evaluation of COX-2 regulated by ACSB-M4, Ava5 cells were transfected with increased concentrations of COX-2 expression vector (pCMV-COX-2-Myc) from 0.25 to 1.5 μg in the presence of ACSB-M4 at 25 μg/ml. After 3 days of incubation, cell lysates were prepared for luciferase activity with the Bright-Glo™ Luciferase Assay System (Promega, Madison, WI) in accordance with the Manufacturer's instructions and Western blotting with specific antibodies.

2.9. Intracellular prostaglandin E_2 (PGE_2) measurements

Cells were seeded in 96-well plates at a density of 5×10^3, and treated with ACSB-M4 at various concentrations. After 3 days incubation, cell membranes were broken to release intracellular PGE_2. PGE_2 expression levels were detected with the PGE_2 enzyme-linked immunosorbent assay system (Biotrak, Amersham Bioscience) according to the Manufacturer's protocol.

2.10. Statistical analysis

Data were presented as means ± SD for at least three independent experiments. The statistical significance was analyzed by using Student's *t*-test. A significant difference was considered as $^*P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Suppression of HCV subgenomic RNA replication by extracts of Acacia confusa stem

Based on bioactivity-guided screening, Huh7 cells harboring an HCV subgenomic replicon (Blight et al., 2000), designed Ava5 cells, were used to assess activity against HCV replication of various fractions extracted from *A. confusa*. Initially, Ava5 cells were treated with the partitioned fractions from an *n*-butanol-soluble extract of *A. confusa* stem at a fixed concentration of 50 μg/ml for 4 days. The treatment with 100 U/ml IFN-α served as a positive control for anti-HCV activity. The inhibitory effect of plant extracts on the synthesis of HCV proteins was analyzed by Western blotting. As shown in Fig. 1, the *n*-butanol-soluble crude extract, designated ACSB, showed significant inhibition of the synthesis of HCV NS5B proteins when compared with the mock control (0.1% DMSO) and the IFN-α treatment (lanes 1–3). Therefore, subsequent fractionation of ACSB crude extract was performed on a Diaion HP-20 chromatograph and eluted with 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH, H_2O and 100% acetone. Ava5 cells were then treated with each fraction at 50 μg/ml for 4 days and cell lysates were analyzed by Western blotting. The fractions eluted with 75% MeOH (ACBS-M3, lane 6) and 100% MeOH (ACSB-M4, lane 7) showed greater anti-HCV activity than those eluted from 25% MeOH (ACSB-M1, lane 4), 50% MeOH (ACSB-M2, lane 5), H_2O (ACSB-H, lane 8), and 100% acetone (ACSB-A, lane 9). To verify the antiviral activity of this fraction, Ava5 cells were incubated with ACSB-M4 either at different concentrations (1, 5, 10, 25, and 50 μg/ml) for 4 days or at the single concentration of 25 μg/ml for various times of incubation (1–4 days). Then, cell lysates were analyzed by Western blotting. As shown in Fig. 2A and B, the synthesis of HCV NS5B proteins was suppressed by ACSB-M4 in a concentration- and time-dependent manner. Furthermore, quantitative RT-PCR (RT-qPCR) showed a concentration-dependent reduction of the HCV RNA level (Fig. 2C), which exhibited an EC_{50} value of 5 ± 0.3 μg/ml, as normalized by cellular *gapdh* mRNA. A cell viability assay showed no significant cytotoxicity at high concentrations up to 500 μg/ml (Fig. 2C, right axis). Thus, the ACSB-M4 fraction displayed the best selective index (SI) for anti-HCV activity, with a CC_{50}/EC_{50} ratio of more than 100.

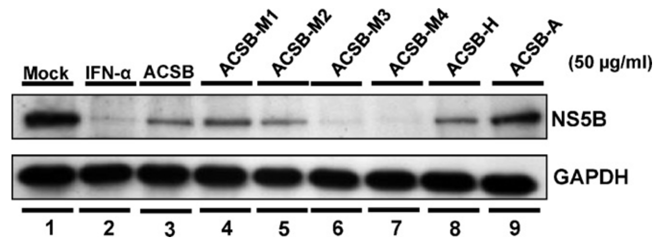

Fig. 1. Inhibition of HCV protein expression in HCV replicon cells by fractions derived from *Acacia confusa* stem. Extract of the *n*-butanol fraction (ACSB) was subfractionated by different solvents: 25% MeOH (ACSB-M1), 50% MeOH (ACSB-M2), 75% MeOH (ACSB-M3), 100% MeOH (ACSB-M4), H_2O (ACSB-H), and acetone (ACSB-A). Huh7 cells harboring HCV subgenomic replicon RNA (Ava5 cells), were seeded at a density of 5×10^4 cells per well in 24-well plates and treated with different extracts at 25 μg/ml for 4 days. Cells were treated with 100 U/ml IFN-α and 0.1% DMSO for a positive control and a mock control on anti-HCV activity, respectively. Western blotting was performed using anti-HCV NS5B antibody and anti-GAPDH antibody. GAPDH protein levels showed equal loading of cell lysates.

3.2. The antiviral effect of ACSB-M4 extract combined with IFN-α or viral enzyme inhibitors in HCV subgenomic replicon cells

Combination therapy with drugs with different modes of action is regarded as a promising way to eliminate the development of viral escape mutants and to reduce side effects. Therefore, the antiviral activities of ACSB-M4 combined with either IFN-α, the NS3/4A protease inhibitor Telaprevir (VX-950) (Lin et al., 2006), and the NS5B polymerase inhibitor 2′-*C*-methylcytidine (NM-107) (Bassit et al., 2008) were examined in an HCV replicon–reporter system (Lee et al., 2004). Both VX-950 and NM-107 have shown promising efficiency of anti-HCV activity in the most advanced phase of clinical trials when combined with current standard of care treatment (IFN-α plus ribavirin) (Gardelli et al., 2009; Peese, 2009). Ava5-EG(Δ4AB)SEAP cells were treated with ACSB-M4 combined with anti-HCV agents at various concentration ratios, as described in Section 2. Dose–response inhibition of HCV RNA replication was determined via the quantification of SEAP activity in culture medium (Supplementary Table S1) (Lee et al., 2004). The isobologram method and the CalcuSyn™ software (Chou and Talalay, 1984; Tallarida, 2001) were used to determine the combination effect. As shown in Table 1, a double combination of ACSB-M4 with the various inhibitor exerted a synergic inhibitory effect on HCV replication, as revealed by the combination index (CI) values of <1 for ED_{50}, ED_{75}, and ED_{90} (range, 0.40–0.73). The traditional isobologram analysis also confirmed a synergistic effect on the inhibition of HCV replication (Supplementary Fig. S1). The results greatly increased the possibility that ACSB-M4 might be considered as a good adjuvant to treat HCV infection in a combination regimen.

3.3. ACSB-M4 down-regulates cox-2 gene expression in HCV replicon cells

To test whether ACSB-M4 inhibits HCV replication by influencing viral targets, for example through internal ribosome entry site (IRES) translation, NS3/4A protease activity, or NS5B poly-

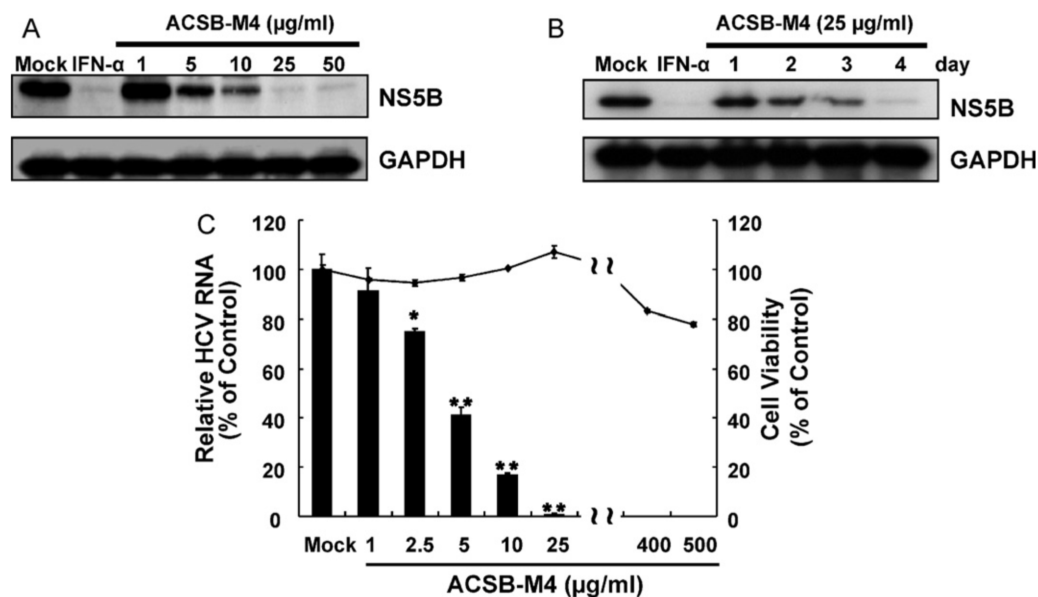

Fig. 2. Effect of ACSB-M4 on HCV protein expression and RNA replication in HCV replicon cells. Ava5 cells were exposed to ACSB-M4 at (A) different concentrations (1, 5, 10, 25, and 50 μg/ml) for 4 days or (B) different lengths of time (1, 2, 3, and 4 days) at the concentration of 25 μg/ml. Treatment with 100 U/ml IFN-α and 0.1% DMSO served as a positive and a mock control for anti-HCV activity, respectively. Western blotting was performed using anti-HCV NS5B antibody and anti-GAPDH antibody. GAPDH protein levels showed equal loading of cell lysates. (C) Suppression of HCV RNA replication by ACSB-M4. Total RNA of ACSB-M4-treated Ava5 cells was extracted to quantify HCV RNA levels by RT-qPCR. Relative HCV RNA levels were normalized by cellular *gapdh* mRNA. Cellular toxicity was simultaneously evaluated by the MTS assay. The EC_{50} of ACSB-M4 was about 5 ± 0.3 μg/ml, and no significant cytotoxicity was observed at more than 500 μg/ml. Results are expressed as means ± SD (error bar) for triplicate experiments. *$P < 0.05$; **$P < 0.01$.

merization activity, those activities were analyzed based on our earlier reporter-based assay systems (Lee et al., 2005a,b, 2010). There was no significant change in reporter activity even when ACSB-M4 was used at the highest concentration, 50 μg/ml (data not shown), suggesting that the ACSB-M4 did not directly target viral translation, proteolysis of polyproteins, or polymerization. Currently, the preventive effects of *A. confuse* extracts have been shown to effectively down-regulate lipopolysaccharides-induced inflammation by blocking nitric oxide synthase (iNOS), COX-2, and prostaglandin E2 (PEG_2) production (Wu et al., 2008). Moreover, recent reports revealed that production of cellular COX-2 induced by HCV proteins was an important factor in the efficient replication of HCV (Trujillo-Murillo et al., 2007, 2008). To test whether expression of COX-2 is mediated by ACSB-M4, Ava5 cells were incubated with concentrations of ACSB-M4 from 2.5 to 50 μg/ml for 4 days. Then, cell lysates were subjected to Western blotting. As shown in Fig. 3A(a), a concentration-dependent reduction of the COX-2 protein level was observed in ACSB-M4-treated cells (lanes 2–7) when compared with 0.1% DMSO-treated cells (mock control) (lane 1). Induction of COX-2 protein level was almost eliminated at an ACSB-M4 concentration of 50 μg/ml (lane 7). Furthermore, we used a transient COX-2 promoter activity assay using the COX-2 promoter-driven firefly luciferase expression vector pCOX-2-Luc to confirm the inhibitory effect of ACSB-M4 on COX-2 gene transcriptional level, as revealed by a concentration-dependent reduction of luminescent signal (Fig. 3A(b)). Moreover, similar result were observed in the reduction of prostaglandin E_2 (PEG_2) levels caused by ACSB-M4 (Fig. 3A(c)). To further investigate whether the inhibitory effect of ACSB-M4 on HCV replication depended on specific interference with COX-2 gene expression, we used a combination of transiently overexpressed COX-2 and ACSB-M4 to evaluate the antiviral action at the molecular level. Ava5 cells were transiently transfected with the plasmid pCMV-COX-2-Myc at different concentrations (0.25, 0.5, 1, and 1.5 μg), and then were incubated with ACSB-M4 at 25 μg/ml. Endogenous COX-2 induction was strongly eliminated by ACSB-M4 in Ava5 cells at this concentration (Fig. 3A(a) lane 6). After 3 days of incubation, cells lysates were subjected to Western blotting analysis using anti-Myc and anti-NS5B antibody to indicate the levels of input COX-2-Myc and HCV protein synthesis, respectively. As shown in Fig. 3B(a), the level of HCV NS5B (upper panel) increased as the concentration of COX-2-Myc (middle panel) increased, revealing that the overexpression of COX-2 reversed the inhibitory effect of ACSB-M4 on viral protein expression in a concentration-dependent manner (lanes 3–6), compared with untransfected cells in the presence of ACSB-M4 (lane 2). It is noteworthy that the recovery of the NS5B protein level at high concentrations of COX-2-Myc was comparable to the recovery of protein levels in Ava5 cells without ACSB-M4 treatment (lanes 1 and 6). In parallel, quantitative RT-PCR was performed to determine the levels of intracellular HCV subgenomic RNA. Consistent with our results on protein expression, the percentage recovery of HCV RNA levels was correlated to the increase in the amount of COX-2-Myc protein as compared with ACSB-M4-treated Ava5 cells without extraneous COX-2-Myc expression (Fig. 4B(b)). Based on complementary method describe above, we used specific COX-2 inhibitor NS398, HCV NS5B polymerase inhibitor NM-107, and HCV NS3 protease inhibitor BILN-2061 to test the hypothesis that the extraneous COX-2-Myc activity can only recover HCV protein synthesis when

Table 1
Effects of ACSB-M4 combined with various inhibitors on HCV replication. Ava5-EG(Δ4AB)SEAP cells were treated with combinations of various concentrations of ACSB-M4 and IFN-α, VX-950, and NM-107 for 3 days. Anti-HCV activity was determined using a SEAP activity assay to reflect the reduction in HCV replication level, and the combination index (CI) value for an effective dose of 50% (ED_{50}), 75% (ED_{75}), or 90% inhibition (ED_{90}) was calculated using the CalcuSyn™ program (Chou and Talalay, 1984). CI values indicate the degree of interaction of potential drugs; values of <1, =1 and >1 are indicative of synergistic, additive, and antagonistic effects, respectively.

Combination compound	CI values at			Influence
	ED_{50}	ED_{75}	ED_{90}	
IFN-α	0.68	0.51	0.40	Synergistic
VX-950	0.73	0.69	0.68	Synergistic
NM-107	0.45	0.43	0.42	Synergistic

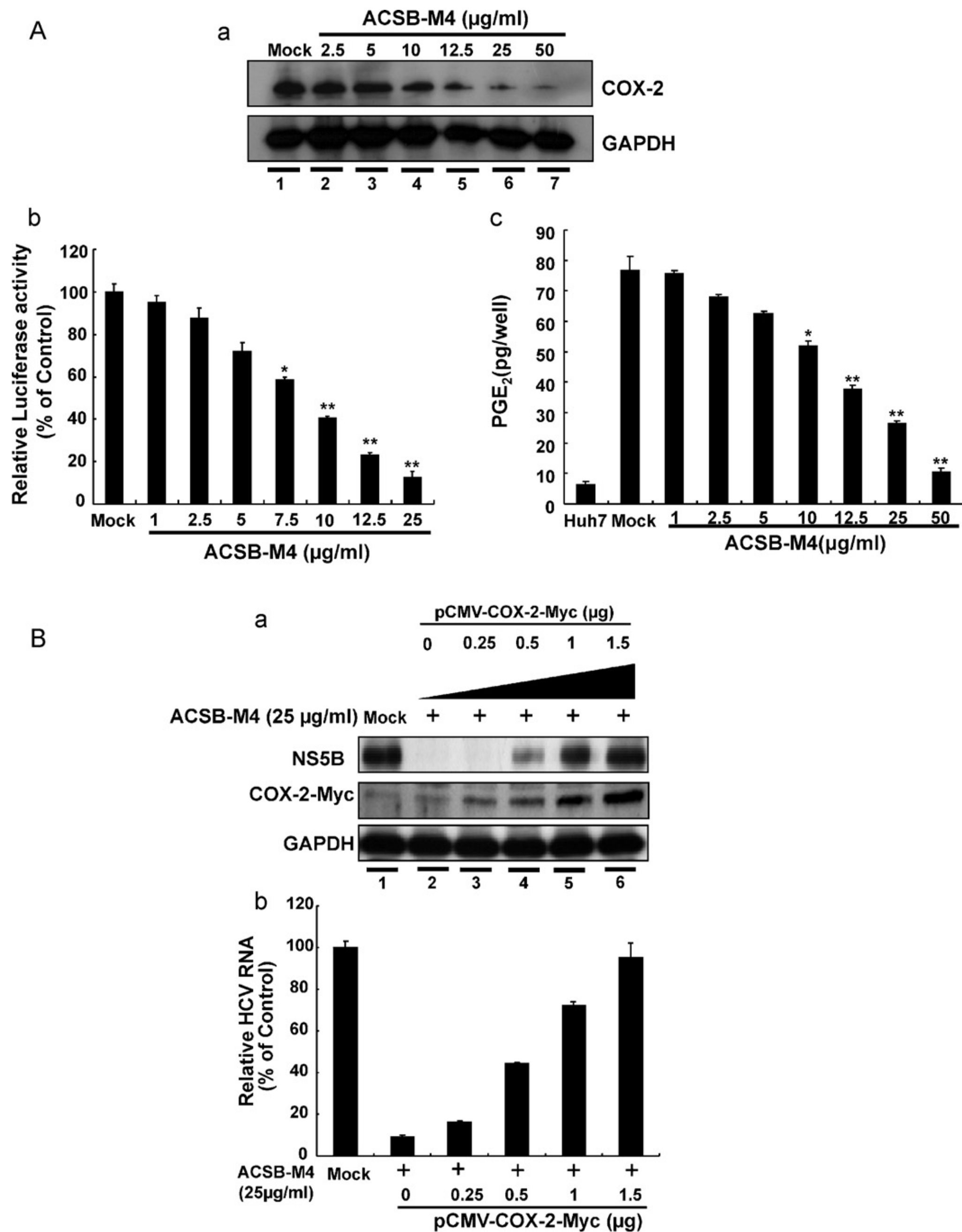

Fig. 3. Involvement of reduction of COX-2 expression in the inhibitory effect of ACSB-M4 on HCV replication. (A) (a) Concentration-dependent reduction of COX-2 expression by ACSB-M4. Ava5 cells were incubated with the indicated concentrations (2.5–50 μg/ml) of ASCB-M4 for 4 days. 'Mock' indicates treatment with 0.1% DMSO. Total cell lysate was extracted and analyzed by Western blotting with anti-COX2 and anti-GAPDH (loading control) antibodies, respectively. (b) Concentration-dependent reduction of COX-2 promoter activity by ACSB-M4. Ava5 cells were transiently transfected with 1.0 μg of the COX-2 promoter reporter vector pCOX-2-Luc. Subsequently, the transfected cells were treated with the indicated concentrations (1–25 μg/ml) of ASCB-M4 for 3 days, and total cell lysates were analyzed for luciferase activity. (c) Dose-dependent reduction of prostaglandin E2 (PGE_2) production by ACSB-M4. After 3 days of treatment, the intercellular PGE_2 levels were assayed with the Biotrak PGE_2 enzyme immunoassay system (Amersham). (B) Restoration of (a) HCV protein synthesis and (b) RNA replication by overexpression of COX-2 in ACSB-M4-treated Ava5 cells. Ava5 cells were transfected with different amounts (0.25–1.5 μg) of the COX-2 expression vector pCMV-COX-2-Myc. After incubation for 12 h, cells were refreshed with complete medium with or without 25 μg/ml ACSB-M4 for an additional 3 days. 'Mock' indicates treatment with 0.1% DMSO. Total cell lysates were subjected to immunoblot analysis to detect NS5B, COX-2-Myc, and GAPDH proteins levels. Total cellular RNA was extracted and analyzed by RT-qPCR. The ratio of HCV RNA expression was normalized by cellular *gapdh* mRNA. Each value represents the mean ± SD of triplicate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

COX-2 is a target for the inhibitor. The results demonstrated that HCV NS5B protein level in NS398-treated Ava5 cells was gradually recovered when extraneous COX-2-Myc increased (Supplementary Fig. S2A, lanes 3–5), which is similar to the results observed in ACSB-M4-treated Ava5 cells. In contrast, HCV NS5B protein level cannot be restored in either NM-107- or BILN-2061-treated Ava5 cells under the same experimental conditions (Supplementary Fig. S2B and C, lanes 3–5). Taken together, these results support our conclusion that down-regulation of COX-2 expression contributes to the antiviral activity of ACSB-M4.

3.4. ACSB-M4 suppresses COX-2 expression through down-regulation of NF-κB activity

Many consensus *cis*-regulatory elements on the COX-2 gene promoter, including two NF-κB binding sites and one AP-1 binding

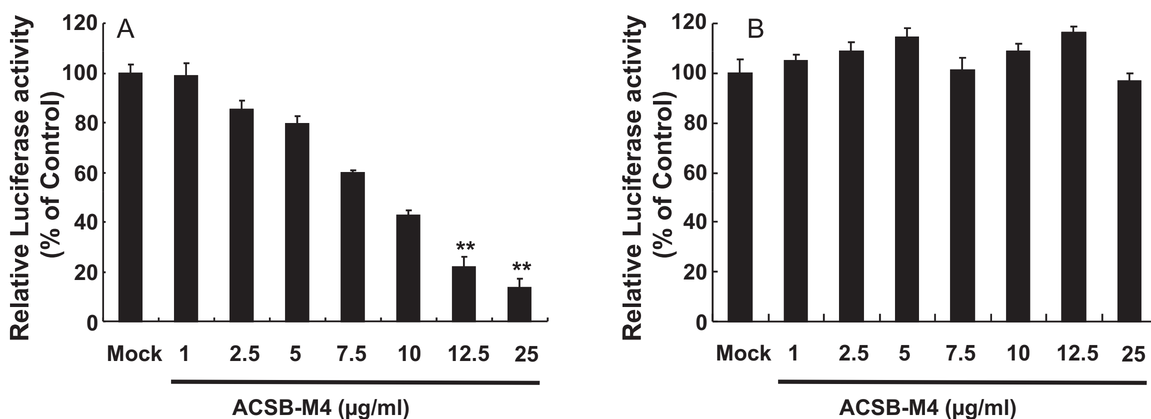

Fig. 4. Effect of ACSB-M4 on the transcriptional activity of (A) NFκB and (B) AP-1 in HCV replicon cells. Reporter plasmid DNA pNFκB-Luc or pAP-1-Luc (0.5 μg/well in 24-well plates) was co-transfected with pCMV-SEAP (0.1 μg/well) into Ava5 cells. Transfected cells were treated with the indicated concentrations (1–25 μg/ml) of ASCB-M4 for 3 days, and total cell lysates were analyzed for luciferase activity. 'Mock' indicates treatment with 0.1% DMSO. Each value represents the mean ± SD of triplicate experiments after normalization of SEAP activities. $^{*}P < 0.05$; $^{**}P < 0.01$.

site, contribute to the activation of COX-2 (Tazawa et al., 1994). Therefore, ACSB-M4-mediated down-regulation of COX-2 may be related to the modulation of transcription factor NF-κB or AP-1. We measured the transcriptional levels of NF-κB and AP-1 using reporter assays in the absence and presence of ACSB-M4 in HCV-expressing cells. Ava5 cells were transiently transfected with the *cis*-reporting plasmid pNF-κB-Luc or pAP-1-Luc, carrying the firefly luciferase gene driven by a synthetic promoter containing consensus binding motifs for NF-κB or AP-1. Then, the plasmid-transfected cells were treated with ACBS-M4 at different concentrations for 3 days. As shown in Fig. 4A, the luciferase activity assay revealed that ACSB-M4 significantly inhibited NF-κB-mediated transcriptional activity in a concentration-dependent manner, which supports that the idea that a decrease in HCV replication effected by ACSB-M4 may result from inhibition of the NF-κB–COX-2 signaling pathway. In contrast, AP-1-mediated transcriptional activity was not significantly influenced by ACSB-M4 (Fig. 4B), suggesting that AP-1 is not involved in the reduction of COX-2 regulated by ACSB-M4.

3.5. Characterization of ACSB-M4 extract by nuclear magnetic resonance (NMR) analysis of ACSB-M4

In order to realize the major ingredients in ACSB-M4, we analyzed the fraction by nuclear magnetic resonance (NMR). The ^{1}H NMR of the active fraction (ACSB-M4) was shown in Fig. 5. In comparison with the spectra database of our laboratory, signals at δ_H 1.2–1.3 indicated characteristic signals of aliphatic methylene and signals at δ_H 0.83 represented terminal methyl protons (region I). These signals indicated the existence of aliphatic long chains obviously. Furthermore, signals at δ_H 4.0–4.8 and δ_H 3.6–3.8 indicated

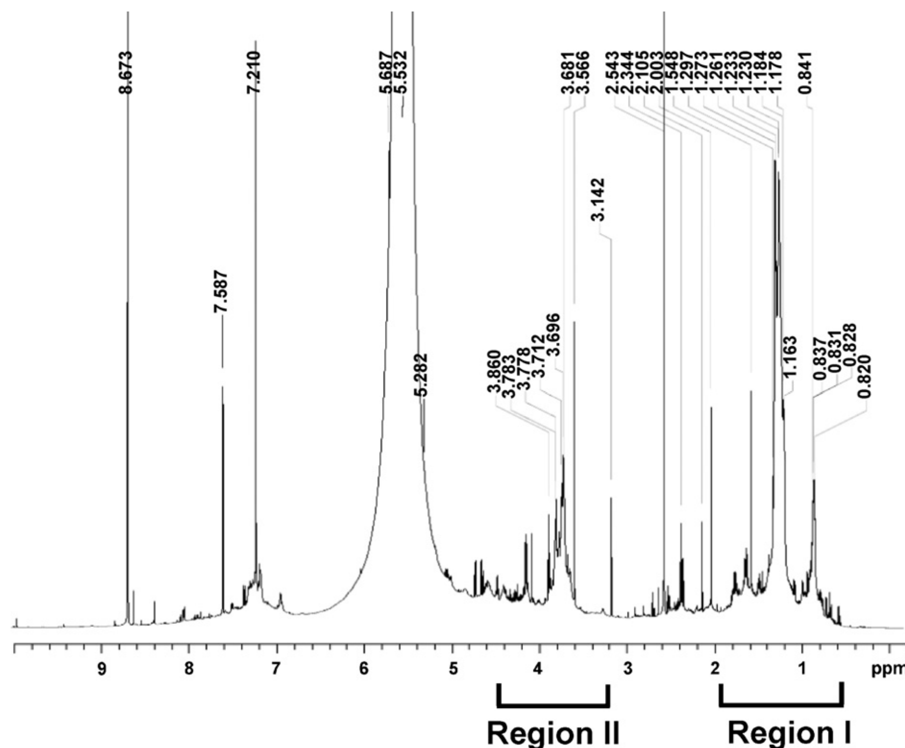

Fig. 5. Characterization of ACSB-M4 extract. ^{1}H NMR (C5D5N, 600 MHz) spectrum of ACSB-M4 fraction. The NMR spectrum was identified by Varian VNMRS 600 MHz FT-NMR. The signals in region I (δ_H 0.8–1.3) represented the aliphatic long chains. The signals in region II (δ_H 4.0–4.8 and δ_H 3.6–38) represented the signal of oxymethine.

the characteristic signals of oxymethine (region II). On the basis of the characteristic signals, the major components of this active fraction will be glycosyl lipidoids, ceramides, which are different from the phenolic compounds described previously.

4. Discussion and conclusion

The etiologic proteins of HCV, including the structural protein, core, and nonstructural proteins, particularly NS3 and NS5A, markedly modulate the activation of NF-κB and COX-2, which may in turn increase HCV replication (Lu et al., 2008; Trujillo-Murillo et al., 2007, 2008). Modulation of the activation of NF-κB and COX-2 also increases the replication of other viruses, such as cytomegalovirus (Zhu et al., 2002), herpesvirus (Reynolds and Enquist, 2006), respiratory syncytial virus (RSV) (Liu et al., 2005), and enterovirus 71 (Tung et al., 2010), although the detailed mechanism is not clearly defined. Consequently, blocking the NF-κB-mediated COX-2 pathway may prove effective in the chemoprevention of infectious diseases. We demonstrated in this study that ACSB-M4 can cause a dramatic reduction of HCV replication through elimination of the virus-induced COX-2 expression (Fig. 3), which is consistent with recent results of Trujillo-Murillo et al. (2008), Gretton et al. (2010), and Okamoto et al. (2009) who used selective COX-2 inhibitors, including acetylsalicylic acid, NS398, COX-2 inhibitor III, and SC-560. By the use of a complementary approach involving the overexpression of COX-2 protein in ACSB-M4- or NS398-treated HCV replicon cells, we confirmed that the molecular mechanism by which ACSB-M4 suppressed HCV replication was associated with the down-regulation of COX-2 expression (Fig. 3 and Supplementary Fig. S2). Moreover, our findings revealed that the inhibitory effect of ACSM-4 on COX-2 consisted of the transcriptional suppression of NF-κB in HCV-replicating or HCV NS5A-expressing cells (Fig. 4 and Supplementary Fig. S3), demonstrating that the target of ACSB-M4 is the NF-κB-mediated COX-2 signaling pathway. In addition to NF-κB and AP-1, induction of COX-2 is mediated by a variety of transcription factors, including nuclear factor of activated T cells (NFAT), cyclic AMP-response element-binding protein (CREB), activating enhancer-binding protein-2 (AP-2), specific protein-1 (SP-1), and CCAAT enhancer-binding protein (C/EBP) (Chun and Surh, 2004). Therefore, it will be important to further investigate additional genes or transcription factor targets involved in suppression of COX-2 synthesis by ACSB-M4 for clearly determining the role COX-2 in the anti-HCV effect of ACSB-M4. In addition to be a COX-2 mediator, NF-κB activation is required for liver homeostasis, pathophysiology, inflammation, and activation of immune responses upon diverse physiological and pathological stimuli, such as bacterial and viral infections (Ahn and Aggarwal, 2005). In the preliminary analysis of inhibitory effect of ACSB-M4 on the transcriptional suppression of NF-κB in Ava5 cells, it was found that transcriptional activity of NF-κB gradually returned to nearly baseline values, as compared with parental Huh-7 cells, when the concentration of ACSB-M4 was increased to the highest concentration, 500 μg/ml (data not shown). This could rule out the possibility that cell viability could be interfered by the suppression of virus-induced NF-κB by ACSB-M4. In fact, the use of drugs to target the host cofactors required for viral replication is suggested as a promising strategy to overcome the problem of drug resistance because of less genetic mutation in the host genome compared with the viral genome (Wohlfarth and Efferth, 2009).

Using the HCV replicon system and an infectious model system (Blight et al., 2000; Wakita et al., 2005), numerous cellular signaling pathways or proteins have now been identified as effective targets for anti-HCV therapy (Georgel et al., 2010; Khattab, 2009). From the present study we cannot exclude the possibility that multiple targets in these pathways were blocked by ACSB-M4 because this is a crude extract that may contain several active constituents. Indeed, multiple inhibitors within the plant extract may be possible to contribute synergistic actions against viral replication through broad modulating effects. In the case of silymarin (milk thistle extract), numerous studies recently showed that a mixture of flavonoligands contained in silymarin exhibited an inhibitory effect on HCV enzyme and replication in different models. These flavonoligands have demonstrated their utility in eradicating chronic hepatitis C through a multitude of the hepatoprotective functions in initial trials (Wagoner et al., 2010). In addition to silymarin used in the treatment of chronic liver disease for many years, glycyrrhizin (licorice root extract) is another commercially available herbal preparation used in treating patients with chronic hepatitis C, specially the interferon-resistant patients (Ikeda et al., 2006). Moreover, polyphenolic catechins extracted from green tea, particularly epigallocatechin-3-gallate (EGCG), and procyanidin B1 purified from *Cinnamomi cortex* inhibit in vitro HCV replication (Li et al., 2010; Zuo et al., 2007). Consequently, plants have been considered as potential sources of new bioactive compounds against HCV infection.

In conclusion, we first demonstrated that the *n*-butanol–methanolic extracts of *A. confusa* stem possess a strong inhibitory effect on HCV replication. In particular, it is desirable to exploit the synergistic effects of combinations of drugs with different modes of anti-HCV activity for improving therapy of HCV-related liver cancer and eliminating side effects during therapeutic regimens. However, our current data cannot discriminate the individual constituents of the extract we used in this study (Fig. 5), and future studies should focus on the development of methods of isolating pure components in order to facilitate studies on the mechanism of action and the chemical modification of these components.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.11.003.

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