MATERIALS AND METHODS

Chemicals and antibodies

Silymarin (SI) and interferon (IFN) from human leukocytes were from Sigma (Saint Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Silymarin stock solution (100 mM) was prepared in DMSO and filtered through a 0.2 micron filter. Interferon stock solution (~1 x 10^3 units/mL) was prepared in deionized water.

Mouse monoclonal antibody against HCV core protein was purchased from Affinity BioReagents, Inc (Golden, CO, USA). Mouse monoclonal antibody against HCV NS5A was purchased from Virogen Corporation (Watertown, MA, USA). Goat anti-GAPDH polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-STAT1 monoclonal and rabbit anti-PhosphoStat1 polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA). Halt Protease Inhibitor Cocktail was from Pierce (Rockford, IL, USA). 4-15% Tris-HCl READY GEL for Polyacrylamide Electrophoresis was from BIO-RAD Laboratories (Hercules, CA, USA). Enhanced chemiluminescence (ECL)-Plus western blotting detection reagent was from Amersham Biosciences (Piscataway, NJ, USA). TRIzol Plus RNA Purification System was purchased from Invitrogen (Carlsbad, CA, USA).

Routine cell culture

Wild type (WT) human hepatoma Huh-7 cell line was purchased from the Japan Health Research Resources Bank (Osaka, Japan). The CNS3 cell line was a gift from R. Bartenschlager, University of Heidelberg, Heidelberg, Germany. These cells express HCV core to the amino terminal portion of NS3 and were generated by transduction of Huh-7 cells with a retroviral vector (pRV-CNS3-IGZ) which carries an expression cassette for HCV core-E1-E2-p7-NS2-NS3 regions. The 9-13 cell line was also from R.

Bartenschlager. These cells carry a stably replicating HCV subgenomic (NS3-5B) replicon. The three cell lines were maintained in low glucose Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 100 U/mL of penicillin, 100 µg/mL streptomycin, and 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). For CNS3 and 9-13 cells, additional selection antibiotics 10 µg/mL Zeocin (Invitrogen) and 100 µg/mL G418 (Gibco, Grand Island, NY, USA) respectively, were added to the culture media. The cells were maintained in 25 cm^2 (growth area) flasks and passaged routinely 1 to 2 times a week. The three cell lines were washed with 3 mL 1X PBS to remove unattached cells and then 3 mL of 0.25% trypsin were added to the flask for trypsinization. 4 mL of complete medium were added, and 1.5 mL of this trypsinized solution was added to a new 25 cm^2 flask. For RNA experiments, cells were plated on 12-well plates. For western blot experiments, cells were plated on 6-well plates. For MTT assays, cells were plated on 48-well plates.

Treatment and RNA Isolation

Huh-7, CNS3 and 9-13 cells were plated on 12-well plates and grown up to 50% confluence the day before treatment. The treatment was made in triplicate with 0 µL, 1 µL, 2 µL or 3 µL of a 100 mM stock solution of silymarin (SI) dissolved in 1 mL of DMSO, in 5% FBS DMEM for 24 h, 48 h and 72 hours.

After the time of incubation, RNA isolation was made using TRIZOL reagent as follows:

1. Remove media and add 500 µL of TRIZOL per well
2. Pipette up and down with a 1mL pipette and place in a 1.5mL microcentrifuge tube
3. Add 100 µL of chloroform
4. Vortex for 10-15 seconds
5. Let sit on ice for 5 min, then centrifuge at 14000 rpm for 15 min
6. Remove 150 µL of the upper layer and place in a clean 1.5 mL microcentrifuge tube
7. Add 150 µL (equal volume) of cold isopropanol and vortex for 5 sec. Put into the -20°C freezer for ≥ 30min or leave it for 10 min at room temperature
8. Centrifuge (14000 rpm at 4°C) for 10 min

9. Remove supernatant with a 1 mL pipette and discard

10. Add 1 mL of 75% ethanol to wash the pellet, vortex 5 sec and spin 7000 rpm for 5 min

11. Pour off and discard the supernatant, spin at 3000 rpm for 1 min to consolidate the rest of the liquid and remove it with a 100 µL pipette, being careful not to disturb the RNA pellet.

12. Air dry for 5 min in the fume hood

13. Add 50 µL of RNAse-free deionized water and gently mix until the RNA pellet dissolves. Samples can be stored at -80°C or used immediately for cDNA synthesis.

Total RNA concentrations and purity were determined espectrophotometrically:

1. Place 5 µL of the RNA sample into a 96 well UV plate

2. Add 195 µL of deionized water

The absorbance was measured at 260 nm /280 nm with the Synergy HT spectrophotometer (BIO-TEK Instruments INC., Winooski, VT, USA). Samples with a 260/280 nm ratio of 1.8 or higher were considered sufficiently free of contaminating proteins. The concentrations of RNA (µg/µL) were calculated using the following formula:

$$\text{RNA } (\mu g/\mu l) = ((\ 260corr / 0.0225\ ) * 40) \ / \ 1000$$

Reverse Transcription

Reverse transcription was performed by converting 1 µg of total RNA into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in a total volume of 20 µL. According to the manufacturer instructions:

5x iScript Reaction Mix	4 µL
iScript RTase	1 µL
RNA (1 µg)	X µL
Water	Y µL (20-4-1-X)
TOTAL	20 µL

The incubation conditions for the complete reaction mix were:

1. 25°C for 5 min
2. 42°C for 30 min
3. 85°C for 5 min
4. Hold at 4°C

The synthesized cDNA was diluted 1:20 to a final volume of 400 µL and stored at -20°C.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) and iQTM SYBR Green Supermix PCR kit (Bio-Rad). Sequence specific primers for HCV core and NS5A, HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Applied Biosystems Primer Express Software as described [31, 53]. Primers used for HCV NS5A were forward primer, CGG ACG TAG CAG TGC TCA CTT C and HCV NS5A reverse primer, CGG AAG CTG GGA TGG TCA AAC; HCV core forward primer, GGA CGG CGT GAA CTA TGC AAC and HCV core reverse primer, CGG AAG CTG GGA TGG TCA AAC; HO-1 forward primer, CGG GCC AGC AAC AAA GTG and HO-1 reverse primer, AGT GTA AGG ACC CAT CGG AGA A; GAPDH forward primer, TGC ACC ACC AAC TGC TTA GC and GAPDH reverse primer, GGC ATG GAC TGT GGT CAT GAG; Bach1 forward primer, CCG CTT CAG TCT CTA CCA TAT C and Bach1 reverse primer, TCA AAT GCA AAC CCA CAA TAG.

Standard curves to evaluate primer efficiency were run using as a template a random sample of cDNA synthesized from CNS3 and 9-13 cells (Apendix I).

Samples were run in triplicate on the qRT-PCR MyiQ and prepared by adding 5 µL of cDNA and 20 µL of the master mix (2X SYBR Green mix, 12.5 µL; 10 µM Forward primer, 1 µL ; 10 µM Reverse primer, 1µL; deionized water, 5.5 µL) into a 96-well PCR plate.

Treatment and Western Blot

CNS3 and 9-13 cells were plated on 6-well plates and grown up to 70% confluence the day before treatment. The treatment was made in duplicate in 5% FBS DMEM 0 μL, 1 μL, 2 μL or 3 μL of a 100 mM stock solution of silymarin (SI) dissolved in 1 mL of DMSO, 10U IFN, 50 U IFN, 100 μM SI+10U IFN or 100 μM SI+50U IFN, for 24 or 48 h depending on the cell line.

After the time of incubation, samples were prepared as follows:

Sample preparation

Cells grown to near confluence were washed with PBS, lysed in a buffer containing 1% Triton [X-100] with PBS and Halt Protease Inhibitor Cocktail. The lysed cells were sonicated for 10 sec, followed by centrifugation for 10 min at 13,000 rpm at 4 °C. The supernatants were stored at -80 °C until subjected to Western blotting analysis.

Protein concentrations were measured using the bicinchoninic acid method (BCA) (Appendix II) on the Synergy HT spectrophotometer (BIO-TEK Instruments INC., Winooski, VT, USA) using BSA as standard. A protein standard curve was generated in each experiment and used to determine the protein content of each sample.

Total proteins (30-50 μg) were separated on 4-15% gradient SDS-PAGE (Bio-Rad) at 100 volts for approximately 60 min, then electrophoretically transferred onto a Immune-Blot PVDF membrane (Bio-Rad) at 50 volts for 2 hr in ice-cold buffer. The membranes were blocked for 1 h in PBS containing 5% nonfat dry milk, then incubated overnight with the primary antibody at 4 °C. The dilutions of the primary antibodies were as follows: 1:1000 for anti-HCV core antibody; 1:1000 for anti-HCV NS5A antibody; 1:500 for anti-STAT1 and anti-Phospho-STAT1 antibody; 1:1000 for anti-GAPDH antibody.

After four washes with 0.1% Tween 20 in PBS (PBS-T), the membranes were incubated for 1 h with a secondary antibody (anti-rabbit, anti-goat or anti-mouse IgG; dilution 1:10,000). Finally, the membranes were washed four times with PBS-T, and the bound antibodies were visualized with the ECL-Plus chemiluminescence system following the manufacturer's instructions. A computer based imaging system (Kodak, Rochester, NY)

was used to measure the relative optical density of each specific band obtained after Western blotting based on the pixel intensity of the samples.

MTT Assay

This assay is based on the Sigma Kit for cell growth determination (Stock No. CGD-1). The effect of treatments on cellular cytotoxicity in Huh-7, CNS3 and 9-13 cells was assessed by measuring the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to MTT formazan (Sigma) which is catalyzed by mitochondria ldehydrogenase activity in living cells. The MTT assay procedure used:

- MTT stock solution: 5mg/mL MTT in DMEM media

1. After treatment with Silymarin for 24 h (9-13 cells and Huh-7 cells) or 48 h (CNS3 cells), MTT stock solution was added to the cells in an amount equal to 10% of the original media volume.
2. Incubate the cells (37 °C, 5% CO_2) for 4 h.
3. After the incubation period, remove media and add the MTT formazan solvent (0.1N HCl in isopropanol) in an amount equal to half of the original media volume.
4. Gently pipette up and down to completely dissolve the MTT formazan crystals. At this point, the color of the cells will change to clear brown; the color depends on of the quantity of MTT formazan formed.
5. Remove the liquid and place it in a 96 well plate.
6. Spectrophotometrically measure absorbance at a wavelength of 570 nm. Subtract background absorbance measured at 690 nm.
7. To calculate how much MTT was converted to MTT formazan according to the absorbance, a standard curve was performed and measured at the same time as the samples (Appendix III).

Statistical Analysis of Data

The experiments were performed at least three times with similar results. Every experiment included at least triplicate samples for each treatment group. The results shown are the mean and SE of three separate determinations. Independent *t*-tests were employed for testing the differences in mean values. Statistical analyses were performed with JMP 6 software (SAS Institute, Cary, NC). Values of $p < 0.05$ were considered significant.