BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of T-Cell Inflammatory Cytokines, Hepatocyte NF-κB Signaling, and HCV Infection by Standardized Silymarin

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Background & Aims: **Chronic hepatitis C is a serious global medical problem necessitating effective treatment. Because standard of care with pegylated interferon plus ribavirin therapy is costly, has significant side effects, and fails to cure about half of all infections, many patients seek complementary and alternative medicine to improve their health, such as Silymarin, derived from milk thistle (*Silybum marianum*). Milk thistle's clinical benefits for chronic hepatitis C are unsettled due to variability in standardization of the herbal product.** ***Methods:*** **In the current study, we focused on the anti-inflammatory and antiviral properties of a standardized Silymarin extract (MK-001).** ***Results:*** **MK-001 inhibited expression of tumor necrosis factor-alpha in anti-CD3 stimulated human peripheral blood mononuclear cells and nuclear factor kappa B-dependent transcription in human hepatoma Huh7 cells. Moreover, MK-001 dose dependently inhibited infection of Huh7 and Huh7.5.1 cells by JFH-1 virus. MK-001 displayed both prophylactic and therapeutic effects against HCV infection, and when combined with interferon-α, inhibited HCV replication more than interferon-α alone. Commercial preparations of Silymarin also displayed antiviral activity, although the effects were not as potent as MK-001. Antiviral effects of the extract were attributable in part to induction of Stat1 phosphorylation, while interferon-independent mechanisms were suggested when the extract was biochemically fractionated by high-performance liquid chromatography. Silybin A, silybin B, and isosilybin A, isosilybin B elicited the strongest anti-NF-κB and anti-HCV actions. These effects were independent of MK-001-induced cytotoxicity.** ***Conclusions:*** **The data indicate that Silymarin exerts anti-inflammatory and antiviral effects, and suggest that complementary and alternative medicine-based approaches may assist in the management of patients with chronic hepatitis C.**

Globally, HCV infects an estimated 170 million people, and causes an estimated 500,000 deaths per year due to complications of late-stage liver disease. In the United States, about 1.8% of the general population (~4 million persons) is infected. Of those acutely infected with HCV, around 85% develop chronic infection. Approximately 70% of patients with chronic viremia develop histologic evidence of chronic liver disease. Hepatitis C is the most frequent indication for liver transplantation in the United States.[1,2] Moreover, in the next 20 years, HCV-related complications, including hepatic decompensation, hepatocellular carcinoma, and liver-related deaths, are expected to increase by 106%, 81%, and 180%, respectively.[3] Thus, chronic hepatitis C is a serious global medical problem necessitating effective treatment.

Interferon-alpha (IFN-α)-based therapy is the only U.S. Food and Drug Administration-approved option for the patient with chronic hepatitis C. Pegylated IFN plus ribavirin therapy is now the standard of care.[4–6] However, 50% of treated patients still do not clear viremia when treated with peg-IFN plus ribavirin. This is particularly evident in patients infected with genotype 1 virus, the most prevalent genotype in the US (~75%). Moreover, IFN therapy is costly and has significant side effects, making many patients seek complementary and alternative medicine-based strategies to improve their health.

Silymarin, the seed extract of milk thistle (*Silybum marianum*), is an ancient herbal remedy used to treat a range of liver and gallbladder disorders, including hepa-

Abbreviations used in this paper: CXCL-8, interleukin 8; IFN, interferon; Jak–Stat, Janus activated kinase-signal transducer and activator of transcription; MOI, multiplicity of infection; NF-κB, nuclear factor kappa B; NS, nonstructural; PBMC, peripheral blood mononuclear cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-alpha.

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titis, cirrhosis, and as a hepatoprotectant against poisoning from wild mushroom, alcohol, chemical, and environmental toxins.[7] Milk thistle is 1 of the best-studied medicinal plants for the treatment of liver disease.[7–10] Its hepatoprotective property is substantiated by experimental data.[11–14] However, its clinical benefits are controversial, because several randomized trials have produced inconsistent results, most likely due to the unavailability of a standardized product. Moreover, the mechanism(s) of action of this botanical are not well characterized. Based on Western medical criteria, the available data are inconclusive for a clinical effect in chronic hepatitis C.[10,15] Thus, mechanism of action studies and well-designed clinical trials are needed to bridge the gap between Eastern and Western medicine. It is also clear that to derive meaningful conclusions, it is essential that botanical preparations be highly standardized in terms of plant source, extraction procedure, and characterization of bioactive molecules.

The correct identification and authentication of a plant species is an essential component of medicinal plant research.[16,17] Proper identification and/or authentication reduces the risk of misusing botanicals and ensures consumer safety. Recent examples of compromised safety and adverse reactions as a result of adulteration, contamination, and substitution of raw drug material can be found in the literature.[16,18–21] Although most crude drugs are traditionally identified on the basis of organoleptic characterization (sensory: taste, feel, odor, color) and microscopic examination of the plant and/or powders, modern chromatographic techniques are now routinely applied. We have previously demonstrated that a standardized extract of Silymarin, MK-001, contains various disasteroisomers of flavononlignans.[22,23]

HCV encodes a single polyprotein precursor that is cleaved within infected cells into 11 proteins. Structural proteins include the core protein, an alternative reading frame protein, 2 envelope proteins (E1, E2), and a protein with ion channel function (P7). Nonstructural (NS) proteins are derived from the remaining two thirds of the polyprotein and include NS2, NS3 (a serine protease/helicase), NS4A, NS4B, NS5A, and NS5B (the RNA-dependent RNA polymerase).[24] The NS proteins have functions in HCV replication.

The HCV replicon system replicates a modified HCV genome to high levels in human hepatoma (Huh-7) cells.[25,26] Original genomic length replicons do not produce infectious virus. However, recent studies have led to the establishment of infectious culture systems. This system,[27] derived from a genotype 2a genome isolated from a Japanese patient with fulminant hepatitis (JFH-1),[28] produces virus particles that are transmissible to naïve Huh7 cells, and are also infectious in the chimpanzee model of HCV infection.[27,29,30] Further refinements have led to the development of a subclone of Huh7 cells, called Huh7.5.1, which are highly permissive to JFH-1 virus infection.[30] Thus, the HCV replicon system, particularly the JFH-1 infectious culture system, is a powerful tool to characterize antiviral potential of botanicals.

Inflammatory responses are initiated upon virus infection and culminate in the release of inflammatory cytokines and chemokines, which are often induced by nuclear factor kappa B (NFκB).[31] The chief objectives of this response are to recruit inflammatory leukocytes, limit virus replication and spread, and ultimately eradicate the infection. However, prolonged expression of pro-inflammatory cytokines and chemokines in the context of chronic viral infection may be detrimental to the host. For example, induction of tumor necrosis factor-alpha (TNF-α), CXCL-6, and interleukin 8 (CXCL-8) have been reported in patients with chronic hepatitis C,[32–36] and are implicated in the pathogenesis of HCV-induced liver diseases including fibrosis[37–40] and antiviral resistance.[41]

In the current study, we evaluated the anti-inflammatory and antiviral actions of a rigorously standardized extract of Silymarin (MK-001). We focused our analyses on NF-κB-dependent signaling responses and HCV infection in human liver cell cultures, and inflammatory cytokine expression by human peripheral blood mononuclear cells (PBMC).

Materials and Methods

Patients

Overall, 3 of the 6 subjects were male, and the median age was 42.5 years (range, 37–52). Four of 6 subjects were HCV-infected (all genotype 1, median HCV RNA level 11,891,922 IU/mL (range, 402,708–46,573,010). Liver biopsy data were available on 3 HCV-infected subjects. The necroinflammatory activity (grade) and degree of fibrosis (stage) of the liver disease were scored semiquantitatively, each on a scale of 0–4, using the Batts and Ludwig method.[42] All had at least grade 1 inflammation. Fibrosis stage was scored as 1, 1–2, and 2 for each of the 3 subjects, respectively. This study was approved by the institutional review boards at the University of Washington. Informed consent was obtained from all study participants.

Cells, Viruses, and Botanicals

Human hepatoma Huh7 cells were grown in Huh7 medium that contained DMEM, 10% fetal bovine serum, 1× penicillin, streptomycin, fungizone, 10 mmol/L L-glutamine, and 1× nonessential amino acids (all reagents were from Invitrogen, Carlsbad, CA). Huh7.5.1 cells were obtained from Francis Chisari[30] and were cultured in Huh7 medium. All cell lines were checked for mycoplasma using MycoAlert assay (Cambrex Bio Science, Rockland, ME) and found to be mycoplasma-free.

JFH-1 viral stock preparation, cell infection, and titration was performed exactly as described.[27,30,43]

Preparation of Standardized Extracts of **Silybum marianum** *(MK-001)*

The seeds of *Silybum marianum* collected at the agricultural station in Northern China (Hen-Long-Jiang) was authenticated by Dr Chen Shi–Lin at the Institute of Medicinal Plant Development in Beijing, China, and assigned a batch number (MK-0100804). Although the efficiency of solvent extraction[44,45] and the effects of defatted and nondefatted seeds have been investigated,[46] extraction with organic solvent is superior.[47] Therefore, defatted seed meal of *Silybum marianum* was extracted with aqueous acetone. The extract was concentrated to remove acetone, and then washed by hexane to wash away hydrophobic impurities. The remaining concentrate was treated with 1% NaCl solution to remove water-soluble impurities. The precipitate and solid obtained through spray drying were combined together to form crude Silymarin. The crude Silymarin was washed with aqueous ethanol and then dried completely to give the refined yellowish powder (MK-001). This MK-001was standardized with a significant reduction of polar non-flavonolignans impurities compared to commercial products. MK-001 and the purified fractions of MK-001 were solubilized in DMSO at 50 mg/mL.

Commercial Preparations of Silymarin

To compare anti-HCV action of MK-001 with other preparations of Silymarin, we tested Ultrathistle (Natural Wellness, Montgomery, NY) and Silybinin (Indena SpA, Milan, Italy), kindly provided by Dr Leanna Standish (Bastyr University). Capsule contents were solubilized in 100% EtOH at 150 mg/mL and vortexed for 1 minute. Samples were heated at 75°C for 5 minutes, insoluble excipients were removed by a 10-second spin in a microcentrifuge, and supernatants were transferred to new tubes.

Plasmids

Generation of JFH-1 RNA and transfection of cells was performed as described.[27] To measure NF-κB-dependent transcription, we used 2 different luciferase reporter genes. NF-κB-luc (Stratagene, La Jolla, CA) contains consensus DNA sequences for NF-κB binding. We also measured CXCL-8 transcription, because CXCL-8 is a well-known NF-κB responsive gene.[48] In this case, the luciferase gene was placed under control of the full-length CXCL-8 promoter and referred to as −1481-luc. This plasmid was obtained from Naofumi Mukaida.[49]

Reporter Gene Assays

Reporter gene assays were performed as described.[50] Briefly, the day before transfection, 3×10^4 cells were plated in black, clear-bottomed, 96-well tissue culture plates. Endotoxin-free plasmid DNA was purified (Endofree kit, Qiagen, Valencia, CA), and was introduced into cells with lipofectamine 2000 according to manufacturer's recommendations (Invitrogen). For reporter gene studies, unless otherwise indicated, 100 ng of the luciferase gene under control of promoter construct of interest was transfected into cells in quadruplicate. Eighteen hours later, stimuli such as rhTNF-α (15 ng/mL; Pierce Biotechnology, Rockford, IL) was added. Four hours later, luciferase activity was measured on cell lysates using the Britelite assay system (Perkin-Elmer, Boston, MA).

Cellular Toxicity Assay

We used the luminescence ATP detection assay system (ATPlite, Perkin-Elmer) as described by the manufacturer. Huh7 or Huh7.5.1 were grown overnight in black 96-well view plates (1×10^4 cells per well). After a 24-hour incubation with the compound the wells were washed twice with 0.2 mL of phosphate-buffered saline followed by addition of 0.1 mL of phosphate-bufered saline and 50 μL of lysis solution (provided with the kit) to each well. The microplate was shaken for 5 minutes at 600 rpm on an orbital shaker to allow cell lysis and ATP stabilization. Fifty microliters of the substrate solution were then added, and the microplate was shaken for 5 minutes at 600 rpm. Luminescence was measured on a TopCount NXT microplate scintillation and luminescence counter (Packard; Perkin-Elmer) after a 10-minute dark adaptation.

Cytotoxicity for PBMC was tested after 24-hour incubation with MK-001 at various concentrations using Live/Dead Fixable Dead Cell Stain Kit (Molecular Probes, Eugene, OR), α-CD3-FITC (BD Biosciences, San Jose, CA). After fixing cells at a final concentration of 1% paraformaldehyde, samples were analyzed on a BD LSR II (BD Biosciences) at the University of Washington Department of Immunology Cell Analysis Facility (Seattle, WA) with data analysis performed using FlowJo software for Macintosh (Tree Star, Inc., Ashland, OR).

Western Blot Analysis

Protein lysates were quantitated (BCA Protein Assay, Pierce) and equal amounts of total protein (10–20 μg) was separated on 4%–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Stat proteins were detected using polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). NS5A was detected using a polyclonal antibody to NS5A (Chiron, Emeryville, CA), while the HCV core protein was detected with a monoclonal antibody (Affinity Bioreagents, Golden, CO). GAPDH was detected with polyclonal antiserum (Santa Cruz Biotechnology). Blot images were scanned and protein pixel intensity measured using Image J as described.[43]

HCV RNA Quantitation

HCV RNA was quantitated by real time reverse-transcriptase polymerase chain reaction, as previously described.[51]

T-Cell Analyses

PBMC were freshly isolated from whole blood using standard Ficoll-Hypaque centrifugation and cultured for 24 hours in the presence of plate-bound anti-CD3 (10 mg/mL) and MK-001 (20 μg/mL) or DMSO (0.5%) control. Supernatants were tested for TNF-α levels using a standard enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) at the Fred Hutchinson Cancer Research Center Cytokine Analysis Facility (Seattle, WA).

Statistics

Differences between means of quadruplicate luciferase readings were compared using a Student's *t* test. A *P* value of $<.05$ was considered significant.

Results

MK-001: A Standardized Extract of Silymarin

Biologic authentication of milk thistle is based on macroscopic and microscopic examinations of the plant by light and electronic microscopy, followed by chemical authentication by high-performance liquid chromatograph and/or liquid chromatograph-mass spectrometry fingerprinting. Using these methods, a standardized preparation of Silymarin, called MK-001, was recently developed.[22] MK-001 contains the highest content (92%) of flavonolignans and every component (>2%) in MK-001 has been completely identified and characterized.[23]

Cytotoxicity of MK-001 in Human Hepatoma Huh7 Cells and PBMC

Silybinin has been shown to inhibit cell growth,[12–14,52] especially in tumor cell lines. As a result, before initiating studies on MK-001, we determined the appropriate dose of the extract to use in our cell cultures, because we wanted to examine the effect of MK-001 on signal transduction independently of cytotoxic effects. Figure 1 shows the effect of different doses on ATP production, a sensitive marker of cell viability, in Huh7 human hepatoma cells. The data demonstrate that MK-001 at concentrations of 10–20 μg/mL had no effect on Huh7 viability, whereas concentrations of 40 and 100 μg/mL caused significant cytotoxicity ($P = .05$ and $P = .007$, respectively). These data were further verified by morphologic evaluation of the cells and standard trypan blue viability measurements, which demonstrated that MK-001 between 0 and 20 μg/mL had no inhibitory effects on cell growth and viability (data not shown).

Cytotoxic effects of MK-001 on human PBMC were analyzed after a 24-hour incubation of freshly isolated cells with 20, 40, 80, and 200 μg/mL of MK-001. Cell viability was evaluated using a viability dye and flow cytometric analysis, as described in the Materials and Methods section. At a dose of 20 μg/mL, a 1.28% or 0.73% negligible increase in cell death was found compared to DMSO controls for all cells and CD3+ T cells, respectively (data not shown). Therefore, all subsequent experiments in hepatoma cells and PBMC were performed between 0 and 20 μg/mL of MK-001.

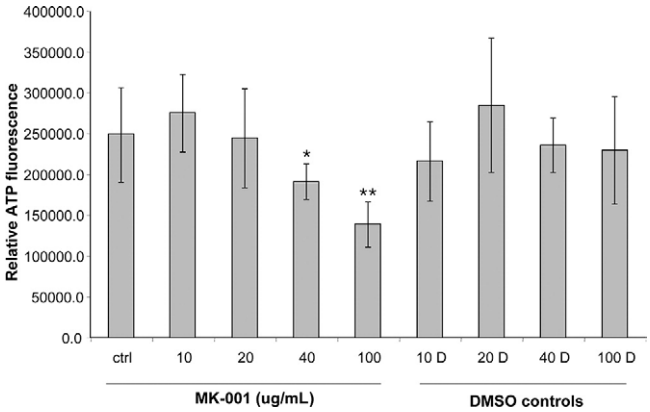

Figure 1. MK-001 cytotoxicity. Huh7 cells were incubated with the indicated concentrations of MK-001 for 72 hours, and cell viability measured by ATPlite assay. *Error bars* represent standard deviation of 4 replicates per condition. DMSO controls represent cultures that were treated with the same volume of DMSO as the corresponding MK-001 samples. *Asterisks* above 40 and 100 μg/mL concentrations indicate significant suppression of Huh7 cell growth. *$P = .05$; **$P = .007$.

MK-001 Exhibits Anti-Inflammatory Effects at 2 Levels: Inhibition of TNF-α Secretion and Inhibition of NF-κB-Dependent Transcription

To examine the effect of Silymarin on inflammatory cytokine release, human T cells were stimulated with plate-bound anti-CD3 in the presence and absence of MK-001. As shown in Figure 2*A*, PBMC from 2 healthy donors exhibited markedly reduced TNF-α secretion in the presence of MK-001. MK-001 had no effect when tested in the absence of anti-CD3 stimulation. Furthermore, as shown in Figure 2*B*, 4 HCV-infected subjects also showed pronounced decreases in the secretion of TNF-α upon treatment with MK-001 (mean fold change = 6.5, range: 1.7–11.7). Although the PBMC samples tested were not depleted of monocytes, additional experiments using isolated monocyte fractions stimulated with anti-CD3 as well as intracellular staining did not demonstrate a significant contribution of monocyte-derived TNF-α to the overall TNF-α response to anti-CD3 stimulation (data not shown).

Because TNF-α signals through NF-κB, the effect of MK-001 on TNF-α activation of NF-κB transcription in Huh7 human hepatoma cells was evaluated using luciferase reporter gene assays. Figure 3*A* shows that MK-001 dose dependently inhibited TNF-α induction of NF-κB transcription in Huh7 cells. Furthermore, MK-001 also inhibited TNF-α activation of CXCL-8 transcription (Figure 3*B*), an NF-κB target gene,[53] in Huh7 cells. Cumulatively, the data demonstrate that MK-001 inhibits pro-inflamma-

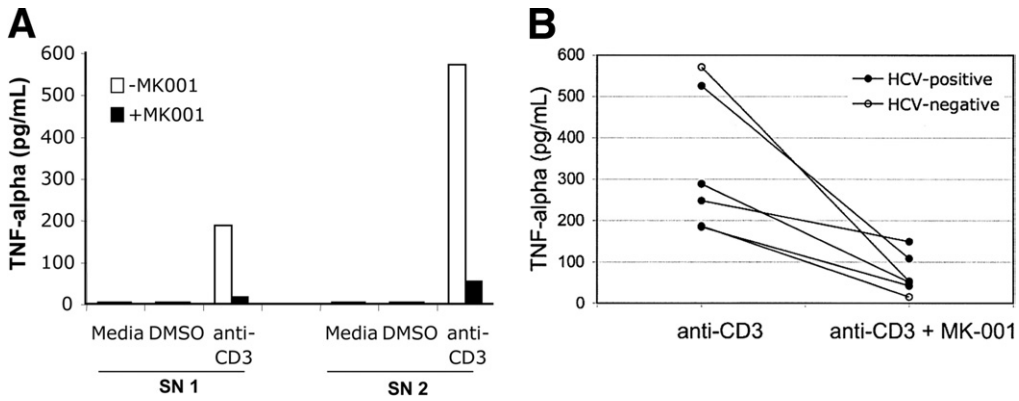

Figure 2. MK-001 abrogates CD3 induced TNF-α secretion by human PBMC in vitro. (*A*) Peripheral blood mononuclear cells (100,000/well) from 2 healthy HCV-seronegative (SN) subjects were stimulated with plate-bound anti-CD3 or negative controls (DMSO or media) in the presence and absence of MK-001 (20 μg/mL). Supernatants collected 48 hours after stimulation were tested by enzyme-linked immunosorbent assay for the presence of TNF-α. (*B*) inhibition of TNF-α secretion by MK-001 in 4 additional HCV-infected patients. The SN patient data from panel *A* are also depicted in this panel. Demographic and clinical laboratory data on these subjects are described in Materials and Methods.

tory TNF-α secretion by human T cells tested freshly ex vivo, as well as TNF-α-induced transcriptional responses mediated by NF-κB in human liver cells. Thus, MK-001 has both immunomodulatory and anti-inflammatory properties in our model system.

MK-001 Inhibits HCV Infection

To determine the effect of MK-001 on HCV infection, Huh7.5.1 cells were treated with various doses of MK-001 and infected with JFH-1 virus[27] for 72 hours. Viral NS5A and core protein expression was monitored by Western blot analysis. As shown in Figure 4*A*, pretreatment of Huh7.5.1 cells with MK-001 dose dependently inhibited HCV infection. The data indicate a prophylactic effect for MK-001 against HCV infection.

To investigate potential therapeutic effects of MK-001, Huh7.5.1 cells were first infected with the JFH-1 virus for 24 hours before adding MK-001 or IFN for an additional 48 hours. As shown in Figure 4*B*, MK-001 displayed pronounced antiviral effects on established HCV infection. In fact, treatment with 20 μg/mL of MK-001 inhibited HCV protein expression to an almost similar extent as IFN-α. These results were not unique to Huh7.5.1 cells, which are highly permissive to HCV infection due to a mutation in the dsRNA sensing protein, retinoic acid inducible gene-I.[54] Indeed, as shown in Figure 4*C*, MK-001 also dose-dependently inhibited established JFH-1 infection of Huh7 cells. In this case, the cells were infected for 96–120 hours because the cells are less permissive to HCV infection and display slower replication kinetics. MK-001 also inhibited HCV RNA expression in both Huh7.5.1 and Huh7 cells (Figure 4*D*), indicating that the compound inhibits both HCV RNA and protein expression.

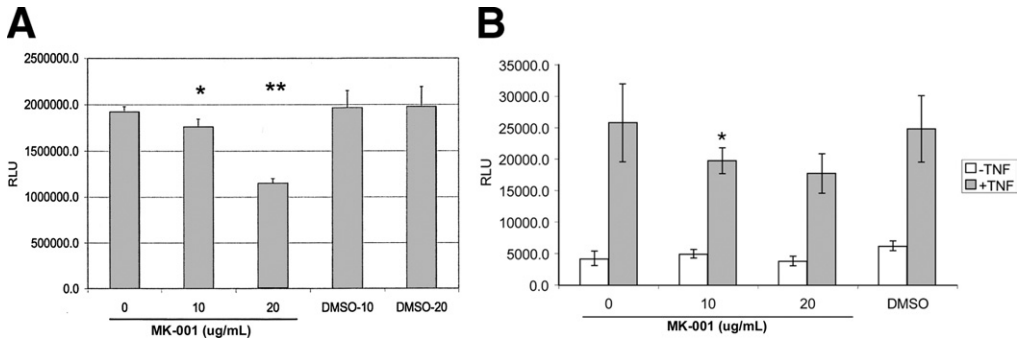

Figure 3. MK-001 inhibits NF-κB-dependent inflammatory signal transduction. (*A*) MK-001 inhibits NF-κB-dependent transcription. Huh7 cells were transfected with an NF-κB–luciferase reporter plasmid. Transfection complexes were removed 3 hours later and cells incubated overnight with the indicated concentrations of MK-001. The next day, TNF-α (10 ng/mL) was added to cells and luciferase activity was determined 4 hours later on quadruplicate cultures by Britelite assay. DMSO-10 and DMSO-20 represent controls for the 10 and 20 μg/mL MK-001 doses and contain the equivalent volume of solvent only. *Asterisks* indicate significant reduction in NF-kB reporter gene activity. *$P = .05$; **$P < .001$. (*B*) MK-001 inhibits TNF-α induced activation of the CXCL-8 promoter. Huh7 cells were transfected with the full-length CXCL-8 promoter-luciferase reporter plasmid (−1481-luc), and upon removal of the transfection mixture, treated overnight with MK-001. Eighteen hours later, cells were treated with or without 15 ng/mL of TNF-α. Luciferase activity was determined 4 hours later. *Asterisks* indicate significant reduction in TNF-α induced CXCL-8 reporter gene activity. *$P = .05$; **$P = .03$.

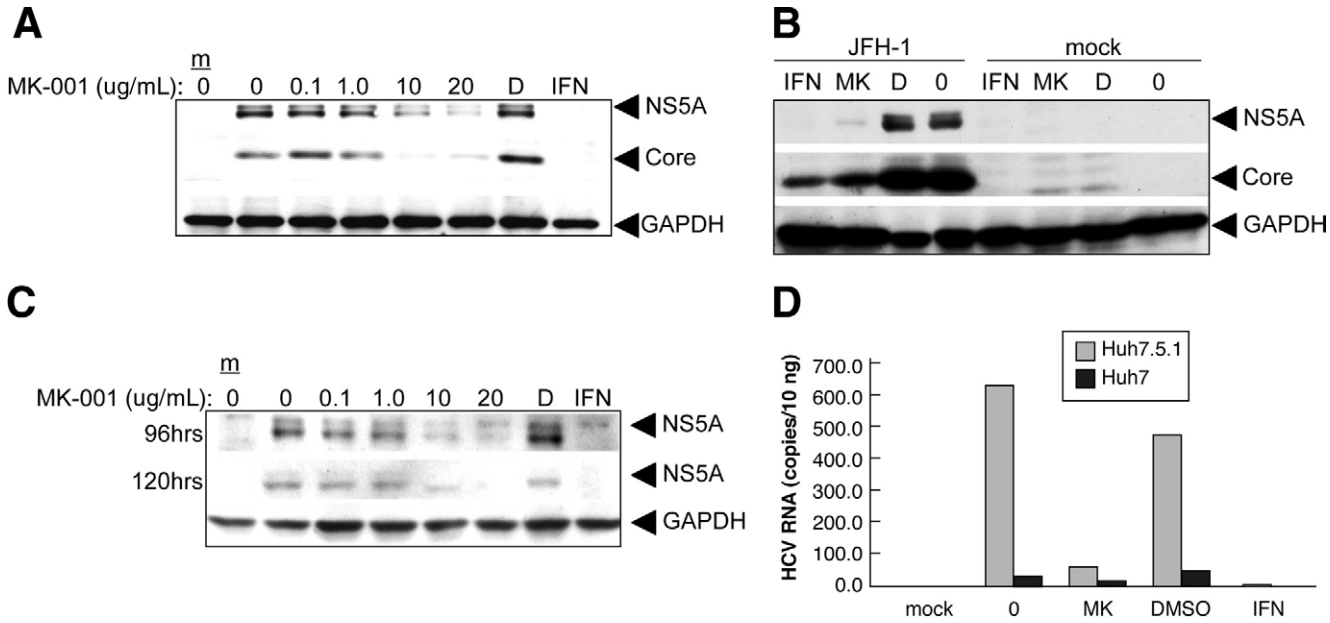

Figure 4. MK-001 inhibits HCV infection. (*A*) Prophylactic effect of MK-001. Huh7.5.1 cells were grown overnight in the presence of the indicated concentrations of MK-001 or DMSO or IFN-α at 500 U/mL, and infected with JFH-1 infectious virus at a multiplicity of infection (MOI) of 0.01 for 72 hours. Whole-cell protein extracts were separated by SDS-PAGE and levels of HCV NS5A (*top bands*), Core (*middle bands*), and control GAPDH proteins (*lower bands*) were detected by Western blot analysis. (*B*) Therapeutic effect of MK-001. Huh7.5.1 cells were infected as described above for 24 hours before treatment with MK-001 (20 μg/mL), DMSO, or IFN-α (100 U/mL) for 48 hours before proteins were extracted and blotted as described above. (*C*) MK-001 inhibits HCV infection of Huh7 cells. Huh7 cells were infected at a MOI of 0.01 for 24 hours with JFH-1, then treated at the indicated therapeutic concentrations of MK-001, DMSO, or 100 U/mL IFN. Protein extracts were harvested at 96 and 120 hours and the HCV NS5A protein detected by Western blot. D = DMSO, MK = MK-001. (*D*) MK-001 inhibits HCV RNA expression in human liver cell cultures. Huh7.5.1 and Huh7 cells were incubated overnight with 20 μg/mL MK-001, DMSO control, or 100 U/mL of IFN-α, and cells were infected with JFH-1 virus at an MOI of 0.01. Total RNA was extracted from cells 72 hours later and HCV RNA detected by real-time reverse transcriptase polymerase chain reaction.

Other Preparations of Milk Thistle Display Anti-HCV Activities

To determine if the anti-HCV activity of MK-001 was specific to this rigorously standardized herbal preparation, we performed viral infectivity studies in the presence of 20 μg/mL of MK-001, and 2 commercially available preparations, Ultrathistle and Silybinin. As shown in Figure 5, MK-001 and the 2 commerical preparations all inhibited HCV infection. However, MK-001 elicted more potent antiviral action than the 2 commercial preparations. The data suggest that anti-HCV activity is a natural component of the milk thistle herb, but there is variation among different preparations of this plant.

MK-001 Enhances Signaling Through the Jak-Stat Pathway

To gain insight into the mechanism for MK-001 inhibition of HCV infection, we examined the phosphorylation status of Stat1 and Stat2 cellular proteins, which is a prerequisite for IFN signaling and antiviral actions.[55] As shown in Figures 6*A* and *B*, in the absence of IFN, MK-001 modestly increased basal Stat1 tyrosine and serine phosphorylation in Huh7 cells, and had no effect on Stat2 tyrosine phosphorylation. In the presence of IFN, MK-001 induction of Stat1 tyrosine and serine phosphorylation was still present, although the increases in phosphorylation were less evident. MK-001 clearly enhanced IFN induced Stat2 tyrosine phosphorylation. Furthermore, combining MK-001 with IFN-α caused a greater therapeutic inhibition of HCV core and NS5A protein expression than when IFN-α was given alone (Figure 6*C*). This was particularly evident at physiologic IFN-α concentrations of 1 and 10 units per milliliter. As a control for IFN-induced gene expression, we probed for the total amounts of Stat1 protein, which was dose de-

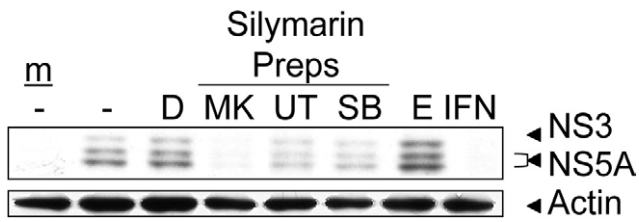

Figure 5. Anti-HCV activity is a general feature of Silymarin extracts. Huh7.5.1 cells were incubated with 20 μg/mL of MK-001 (MK), Ultrathistle (UT), or Silybinin (SB) prior to infection with JFH-1 at an MOI of 0.01. Control cultures were treated with DMSO (D), ethanol (E), or IFN (I). Whole-cell protein extracts were separated by SDS-PAGE and levels of HCV NS3 and NS5A, and control Actin proteins (*lower bands*) were detected by Western blot analysis.

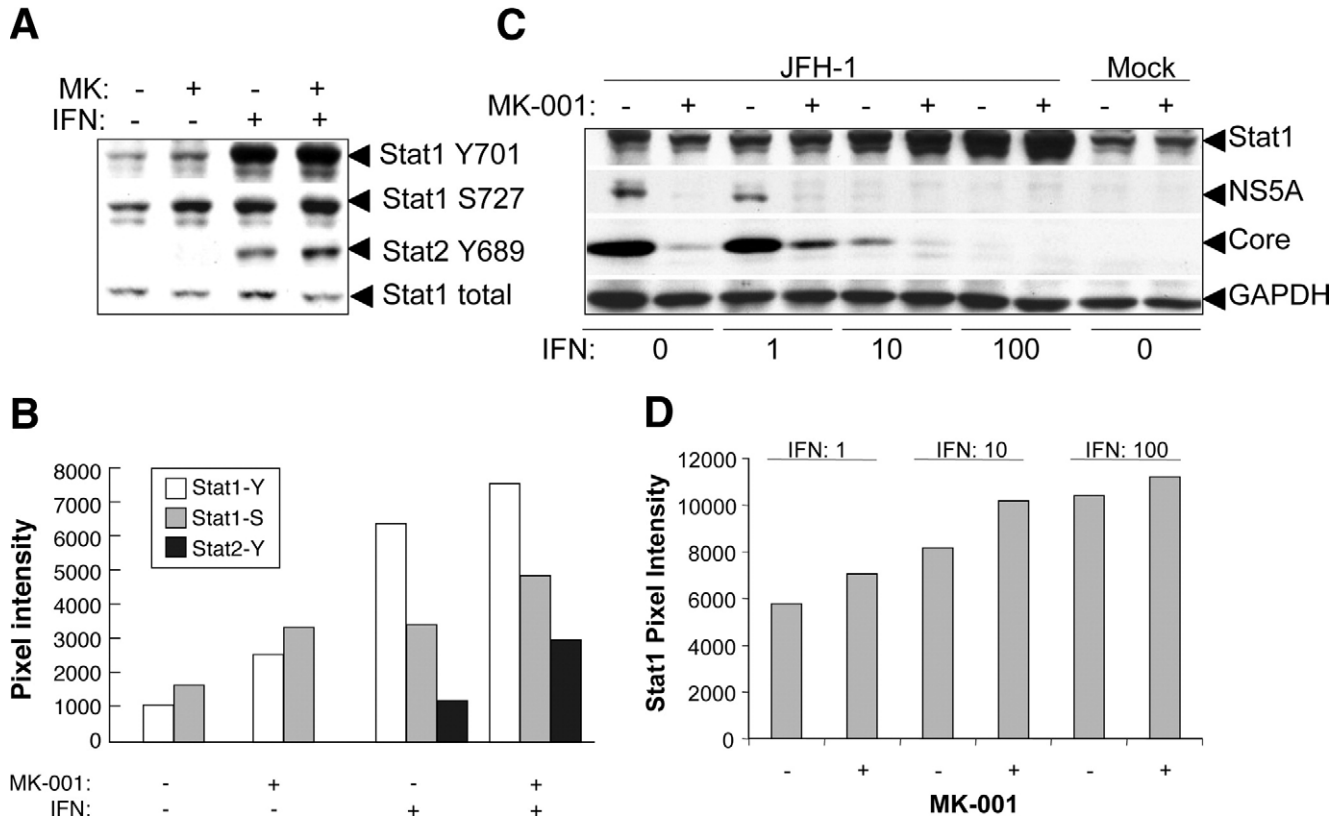

Figure 6. MK-001 enhances signaling through the Jak–Stat pathway. (*A*) Huh7 cells were treated with 20 μg/mL MK-001 or not treated in the presence and absence of 500 U/mL of IFN, and 30 minutes later, phosphorylation of Stat1 on tyrosine (Y701) and serine (S727) and Stat2 on tyrosine (Y689) was assessed on whole-cell extracts using phospho-specific antibodies. (*B*) Quantitation of pixel intensity of Western blot images from panel *A*, showing that MK-001 enhances basal and IFN-induced phosphorylation of Stat1 on serine and tyrosine residues, and IFN-induced Stat2 phosphorylation on tyrosine residues. (*C*) Huh7.5.1 cells were infected with JFH-1 at a multiplicty of infection of 0.01 for 24 hours before treatment with 20 μg/mL MK-001 in the presence and absence of 0, 1, 10, and 100 U/mL IFN-α. Protein extracts were harvested 48 hours later and Stat1, NS5A, core, and GAPDH proteins were detected by Western blot. (*D*) Quantitation of Stat1 pixel intensity of Western blot images from panel *C*, showing that MK-001 enhances IFN-induced up-regulation of Stat1 expression.

pendently increased by IFN-α, as expected. Moreover, in IFN-α doses of 1 and 10 U/mL, there was a small yet consistent enhancement of Stat1 levels in the presence of MK-001. Thus, although MK-001 had modest effects on the Jak-Stat pathway by itself, it appeared to enhance signaling through the Jak-Stat pathway, which correlated with augmentation of the antiviral efficacy of exogenously added IFN.

Characterization of the Components of MK-001 That Mediate Anti-Inflammatory and Antiviral Actions

To determine the active components in MK-001, the standardized extract was purified by high-performance liquid chromatography into different fractions as described.[22,23] The individual fractions were then solubilized in DMSO, and tested for inhibition of TNF-α induced CXCL-8 transcription. As shown in Figure 7*A*, fractions 2, 7, 8, 9, 11, and 16 inhibited TNF-α induced CXCL-8 transcription, with fractions 9, 11, and 16 being the most potent. The data demonstrate that MK-001 contains potent anti-inflammatory molecules. Figure 7*B* shows the effects of the different fractions on JFH-1 infection. Cells were infected for 24 hours, then treated with 20 μg/mL of each fraction for 48 hours and HCV protein expression was assessed by Western blot. As shown in the figure, many fractions inhibited HCV infection therapeutically, as demonstrated by reduction of HCV core and NS5A protein expression. However, fractions 2, 9, 11, and 16 displayed the most potent anti-HCV activity. These fractions contain silybin A, silybin B, and isosylibin A and isosilybin B. There was a general correlation between anti-inflammatory and antiviral activities of the different MK-001 fractions. The only exception was fraction 5, which did not affect TNF-α induction of CXCL-8 transcription, yet it inhibited HCV infection. Furthermore, blots were probed to determine the phosphorylation status of the Stat1 protein, an indicator of activation of the Jak–Stat pathway, to assess the potential involvement of Jak–Stat signaling in antiviral activity. As shown in the Figure 7*B*, most fractions did not induce Stat1 tyrosine phosphorylation, except for fraction 5, which caused induction of Stat 1 phosphorylation. As a

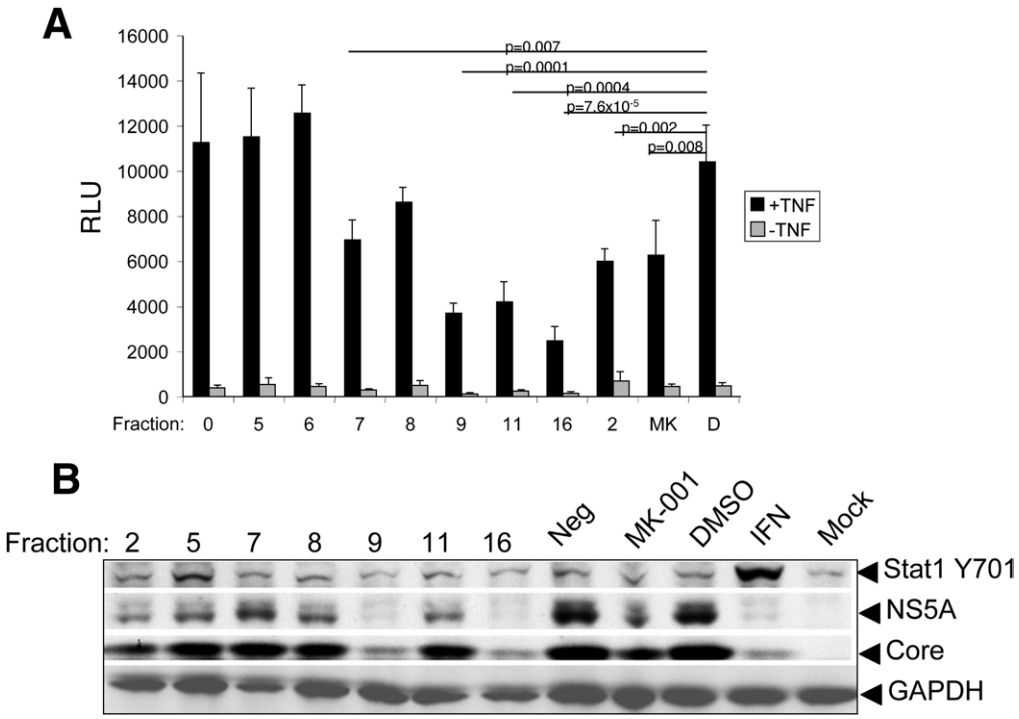

Figure 7. Characterization of the anti-inflammatory and antiviral components of MK-001. MK-001 was biochemically fractionated into its various components as described.[22,23] (*A*) Huh7 cells were treated overnight with MK-001 or the various fractions (all at 10 μg/mL) or DMSO (D) as a control, then transfected with the full-length CXCL-8 promoter–luciferase reporter plasmid. Twenty hours later, cells were treated with or without 15 ng/mL of TNF-α. Luciferase activity was determined 4 hours later. Significant reductions in TNF-α induced CXCL-8 transcription are indicated relative to the DMSO control. *P* values were derived from *t* tests of quadruplicate readings for each condition. (*B*) Huh7.5.1 cells were infected for 24 hours with JFH-1 at an MOI of 0.01 before the different fractions were added to cells at 20 μg/mL. As controls, cells were treated with DMSO or IFN-α (100 U/mL), or mock infected. HCV and cellular protein expression was assessed by Western blot 48 hours later. *Arrows* indicate the location of the Stat1 phosphorylation on conserved tyrosine residue 701, and HCV NS5A and core proteins. D = DMSO, MK = MK-001, Neg = negative control; cells not treated with any compound.

control, cells were treated with IFN-α, which induced robust phosphorylation of Stat1 Y701 phosphorylation. The data suggest that some components of MK-001 inhibit HCV infection via stimulation of the Jak–Stat pathway. However, a major mechanism for inhibition of HCV infection by MK-001 may occur independently of IFN action.

Discussion

Despite global use, the detailed molecular mechanisms of Silymarin-induced hepatoprotection are not known. In the current report, we show for the first time that a rigorously standardized Silymarin (MK-001) displayed anti-inflammatory actions via inhibition of NF-κB induced transcription in human liver cell cultures, inhibition of inflammatory cytokine induction in human PBMC, and direct antiviral effects against HCV infection. Thus, our data are in accord with the view that Silymarin is thought to possess antioxidant and anti-inflammatory effects.[15] Moreover, Silymarin has been shown to inhibit the growth of endothelial,[13] lung tumor,[14] prostate cancer,[12] and human hepatoma HepG2 and Hep3B cells.[52] The antiproliferative actions of Silymarin also converge on inhibition of signaling pathways that regulate the cell cycle including Akt and cyclin-dependent kinases. Thus, Silymarin may be useful as an anticancer agent for many types of human tumors.[11,56] Silymarin has also been shown to inhibit basal and TNF-α induced activation of NF-κB, rendering prostate cancer cells sensitive to TNF-α induced apoptosis.[57] Thus, our results are also in agreement with previous studies demonstrating anti-NF-κB effects. However, it is important to emphasize that the antiproliferative and anti-NF-κB actions of Silymarin have been described chiefly with 1 component of Silymarin extract, Silibinin, at relatively high doses (100–300 μmol/L or ca. 50–150 μg/mL).[52] Moreover, silymarin is known to contain silybin A, silybin B, isosilybin A, and isosilybin B.[22] Thus, it is possible that the antiproliferative, anti-inflammatory, immunomodulatory, and antiviral effects of Silymarin may be dependent on the dose and the molecular form of Silymarin and other molecules present in MK-001. In the current report, we found that MK-001, at doses between 10 and 20 μg/mL, inhibits NF-κB and CXCL-8 transcription and HCV infection without inhibiting cell growth. Future studies are warranted to validate the findings based on reporter assays with endogenous NF-κB, CXCL-8, and related inflammatory cytokine levels and activities.

Exactly how MK-001 inhibits NF-κB-dependent inflammatory responses is presently not known. It is known that virus infection,[58] including HCV specifically,[59–69] induces oxidative stress and inflammation. Oxidative stress is defined by the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from cellular enzymes such as mitochondrial NADPH and cytoplasmic nitric oxide synthetase in excess of the "buffering" capacity of the cellular antioxidant response, mediated by molecules such as superoxide dismutase, heme oxygenase I, and glutathione. Types of ROS and RNS include superoxide, peroxide, hydroxyl, and nitric oxide. Unchecked oxidative stress, such as in the case of chronic diseases or viral infections, can modify proteins and lipids, damage DNA, and alter mitochondrial membrane potential. Deregulation of ROS and RNS production are thus implicated in the pathogenesis of both viral and nonviral diseases.[70–73] Moreover, ROS and RNS play sensory and regulatory roles in that they activate signal transduction pathways. Because NF-κB is the best-characterized redox-responsive transcription factor,[74] it is therefore possible that Silymarin elicits anti-inflammatory actions by inhibiting NF-κB via antioxidant actions. It is also possible that Silymarin modulates cellular membranes and/or membrane receptor functions. Indeed, Silymarin has been shown to modulate the stability of hepatic membranes[75] and inhibit the binding of epidermal growth factor to its receptor.[76] Thus, Silymarin induced membrane alterations should also be considered in the hepatoprotective effects of this botanical.

We provided evidence to suggest that the antiviral effect of the standardized Silymarin used in this study may be due in part to stimulation of the Jak–Stat pathway and induction of an IFN antiviral response. Mechanistically, this could involve Silymarin effects on hepatocyte membranes and/or the IFN-α receptor, or inhibition of negative regulators of the Jak–Stat pathway such as SH2-containing phosphatases, the protein inhibitors of activated STATs, or the suppressors of cytokine signaling proteins.[77] However, virologic studies with biochemically fractionated Silymarin extract suggested that IFN- or Jak–Stat-independent mechanisms may be more closely tied with Silymarin's anti-HCV effects. Possible mechanisms could involve TLR7,[78] IRF-3,[79,80] and p38 MAPK pathways,[81] all of which can modulate antiviral responses independently of the classic IFN → Jak–Stat → interferon-stimulated gene paradigm. Additional studies are currently underway to evaluate these issues.

The components of MK-001 that elicited potent anti-inflammatory and anti-HCV effects were silybin A, silybin B, isosilybin A, and isosilybin B. These results are in agreement with previous observations that isosilybin A and B display the most potent suppression of prostate cancer cell growth.[82] Moreover, these compounds also exert differential effects on primary human hepatocytes.[83] Thus, in addition to considering rigorously standardized Silymarin for use in clinical trials, an evaluation of the potential clinical utility of different molecular forms of Silymarin might also be warranted.

This is the first report to demonstrate a direct antiviral effect of highly standardized preparation of Silymarin against bona fide HCV infection. We also found that 2 commercial preparations of Silymarin also displayed antiviral activity, indicating that antiviral action is not unique to MK-001, but possibly a general feature of the herb itself. Nonetheless, our viral replication studies are at odds with clinical studies that found no effect of Silymarin on HCV replication in vivo.[84] Possible reasons for this could be the fact that the clinical trials used different preparations of Silymarin that likely differ in standardization procedures and activities, that Silymarin is often administered with other herbs, that HCV RNA quantitation in patient serum is based on measurement of viral RNA and not infectious virus, and that the chemistry and pharmacokinetics of Silymarin and its components may change when Silymarin is given orally, the usual route of administration, and thus plasma/liver levels may not approach levels tested in vitro. However, meta analyses of clinical trials of Silymarin have shown that Silymarin reduces liver enzyme (ALT) elevations associated with hepatitis.[9,84] Treatment of 50 HCV patients with a mix of 7 antioxidants including Silymarin also reduced ALT levels in 44% of patients, and reduced viral load in 25% of patients.[85] However, many studies have found no significant clinical effects of Silymarin, despite the fact that the botanical is well tolerated and safe.[86,87] Another possible reason for the discrepancies between our in vitro studies and clinical experiences with Silymarin may relate to limitations with our model system. For example, the infectious culture system we employed is based on highly adapted human hepatoma cell lines that may not reflect hepatocyte physiology in vivo. Moreover, the JFH-1 strain is derived from a patient with genotype 2a infection. Additional in vitro studies on recently described chimeric[88] or genotype 1 culture systems[89] will help to resolve this issue.

Herbalists and indigenous healers throughout the world use botanical medicines for the prevention and treatment of liver disease. Moreover, millions of people have consumed Silymarin for centuries to promote vitality and liver function. Published clinical trials that examine the efficacy of Silymarin are rare in chronic hepatitis C.[87,90,91] Thus, what are clearly lacking are well-designed clinical trials studying the effects of botanicals on chronic hepatitis C.[8] With future clinical and mechanism of action studies with rigorously standardized botanical preparations, it is anticipated that complementary and alternative medicine therapies will continue to gain increasing acceptance in Western medical practices, to reduce the morbidity and mortality of HCV-associated liver diseases.

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