Multiple Effects of Silymarin on the Hepatitis C Virus Lifecycle

By: Jessica Wagoner, Amina Negash, Olivia J. Kane, Laura E. Martinez, Yaakov Nahmias, Nigel Bourne, David M. Owen, Joe Grove, Claire Brimacombe, Jane A. McKeating, Eve-Isabelle Pécheur, Tyler N. Graf, Nicholas H. Oberlies, Volker Lohmann, Feng Cao, John E. Tavis, and Stephen J. Polyak

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Abstract:

Silymarin, an extract from milk thistle (*Silybum marianum*), and its purified flavonolignans have been recently shown to inhibit hepatitis C virus (HCV) infection, both *in vitro* and *in vivo*. In the current study, we further characterized silymarin's antiviral actions. Silymarin had antiviral effects against hepatitis C virus cell culture (HCVcc) infection that included inhibition of virus entry, RNA and protein expression, and infectious virus production. Silymarin did not block HCVcc binding to cells but inhibited the entry of several viral pseudoparticles (pp), and fusion of HCVpp with liposomes. Silymarin but not silibinin inhibited genotype 2a NS5B RNA-dependent RNA polymerase (RdRp) activity at concentrations 5 to 10 times higher than required for anti-HCVcc effects. Furthermore, silymarin had inefficient activity on the genotype 1b BK and four 1b RDRPs derived from HCV-infected patients. Moreover, silymarin did not inhibit HCV replication in five independent genotype 1a, 1b, and 2a replicon cell lines that did not produce infectious virus. Silymarin inhibited microsomal triglyceride transfer protein activity, apolipoprotein B secretion, and infectious virion production into culture supernatants. Silymarin also blocked cell-to-cell spread of virus. *Conclusion:* Although inhibition of *in vitro* NS5B polymerase activity is demonstrable, the mechanisms of silymarin's antiviral action appear to include blocking of virus entry and transmission, possibly by targeting the host cell.

Article:

Chronic hepatitis C is a serious global medical problem necessitating novel, effective, inexpensive, and less toxic treatments. Hepatitis C virus (HCV) infects an estimated 130 million people throughout the world, leading to a half million deaths per year due to liver disease.[1]

Pegylated interferon (IFN) plus ribavirin therapy is the current treatment for the patient with chronic hepatitis C.[2] However, 50% of treated patients do not clear viremia during treatment, which is costly and has significant side effects. As a result, many patients use natural products to supplement or circumvent IFN-based regimens, with silymarin being the most common botanical medicine.[3]

Silymarin is an extract from the plant *Silybum marianum*, which consists of at least seven flavonolignans and the flavonoid taxifolin.[4] Silibinin is a partially purified mixture of two flavonolignans, silybin A and silybin B. Silymarin has been used to treat a range of liver disorders, including hepatitis, cirrhosis, and poisoning from wild mushrooms.[5] Recently, we showed silymarin inhibits HCV infection of Huh7 and Huh7.5.1 cells,[6] and Ferenci's group showed that intravenous silibinin administration reduces viral loads in previous nonresponders to IFN therapy.[7] Therefore, in the current study, we determined the stages in the HCV life cycle that are blocked by silymarin.

MATERIALS AND METHODS

Reagents

Human hepatoma Huh7 cells were grown in Huh7 medium as described.[6] HepG2 cells and Huh7.5.1 cells[8] were cultured in Huh7 medium. Blazing Blight 7 (BB7) and Full Length-NEO (FL-NEO) cells are Huh7 cell lines that contain subgenomic and genomic length genotype 1b replicons, respectively.[9] JFH-1 subgenomic genotype 2a replicon cell lines in Huh7 or Huh7.5 backgrounds were generated by transfecting *in vitro* transcribed subgenomic replicon (SGR) SGR-JFH1 replicon RNA into Huh7 or Huh7.5 and selecting with 800 μg/mL G418. Single colonies expressing the highest level of HCV protein were designated SGR7 and SGR7.5. Luc-ubi-neo/ET is a subgenomic genotype 1b-derived replicon cell line.[10] The HCV genotype 1a H77s subgenomic replicon cell line has been described previously.[11] All replicon lines were maintained in Huh7 medium containing 400 μg/mL G418. Primary human hepatocytes were provided by Dr. Stephen Strom, University of Pittsburgh, and maintained in hepatocyte culture medium (Lonza, Walkersville, MD).

JFH-1 viral stock preparation, cell infection, and titration was performed as described.[6] Silymarin from US Pharmacopeia (Rockville, MD) was used in all experiments except for the microsomal triglyceride transfer protein (MTP) (Fig. 4A) and apolipoprotein B (apoB) (Fig. 4E) assays, where silymarin from Sigma was used. Sigma silymarin contains similar levels of the major flavonolignans to USP silymarin, as described by Wen et al.[12] Stocks were prepared in dimethylsulfoxide (DMSO) at a concentration of 50 mg/mL. Silibinin was purified from silymarin as described.[13]

Egg yolk phosphatidylcholine, cholesterol, and Triton X-100 were purchased from Sigma. Octadecyl rhodamine B chloride (R_{18}) was from Fluoprobes.

Addition of Compounds to Cultures

Silymarin was further diluted in DMSO before use, and the final concentration of DMSO in culture media was always less than 0.5%. Silymarin treatment involved exposing cells to a single administration of silymarin for various times. DMSO was included as a solvent control. To focus on the effects of silymarin on infectious virus production, in some experiments cells were first infected at a multiplicity of infection (MOI) of 0.01, and cells were passaged at 72 hours after infection, followed by addition of silymarin 24 hours after passaging, or 96 hours after infection. Under these conditions, the culture was fully infected (Supporting Fig. S1).

Roferon, Intron-A, or Pegasys was used as a positive control for antiviral effects. Roferon (Roche, Palo Alto, CA) was used at 100 IU/mL, Pegasys was used at 10 ng/mL, and Intron-A

was used at various concentrations. BMS-200150, a small molecule inhibitor of MTP, was provided by Pablo Gastaminza and Francis Chisari.

Western Blot Analysis

Western blots were performed as described.[6] Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), apoB, Stat1, NS5A in FL-NEO and BB7 cells, and core proteins were detected using commercial antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; BioDesign/Meridian Life Science, Saco, ME; Affinity BioReagents, Golden, CO). NS5A in JFH-1-infected cells was detected with serum from patients infected with HCV genotype 2a.

HCV RNA Quantitation

HCV RNA was quantitated by real time reverse transcription polymerase chain reaction, as previously described.[14]

HCV Luciferase Replicon Assay

Luc-ubi-neo cells were plated and grown for 24 hours. Medium was removed, and the cells washed once and treated subsequently with given concentrations of silymarin or DMSO in triplicate. After 3 days of incubation at 37°C, cell culture medium was removed and cells were lysed by freeze-thawing in buffer and luciferase assays were performed.

HCV-1a Replicon Assay

Antiviral studies to determine the impact of silymarin treatment on HCV RNA levels with the genotype 1a replicon were conducted using real time reverse transcription polymerase chain reaction and assay conditions previously described.[15]

HCV NS5B Polymerase Assays

NS5BΔC21 C-terminally fused to a hexa-histidine tag was expressed and purified for HCV JFH1 and for the genotype 1b isolates as described.[16, 17] All measurements were done in triplicate, and the half maximal inhibitory concentration (IC50) values were calculated with GraphPad Prism.

Viral Pseudoparticle Entry Assays

Pseudoviruses were generated as previously described.[18] Huh-7.5 cells were pretreated for 1 hour with 40, 80, and 160 μM silymarin (SM) or equivalent volume of DMSO, diluted in media. Cells were inoculated with an equal volume of pseudoparticles, bringing the final concentration of SM to 20, 40, and 80 μM. Seventy-two hours postinfection, the medium was removed and cells lysed with cell lysis buffer (Promega, Madison, WI). Luciferase activity was then measured. Specific infectivity was calculated by subtracting the mean Env-pp signal from the HCVpp, Murine Leukemia Virus pseudoparticle (MLVpp), or Vesicular Stomatitis Virus pseudoparticle (VSVpp) signals. Relative infectivity was then calculated as a percentage of untreated control cell infection; in other words, the mean luciferase value of the replicate untreated cells was defined as 100%.

Fusion Assay

Fusion between HCVpp and liposomes was assayed as already described.[19] Briefly, liposomes composed of phosphatidylcholine, cholesterol, and octadecyl rhodamine B chloride (R_{18}) (65:30:5 mol%) were added at a 15-μM final concentration to a cuvette at 37°C containing

HCVpp in phosphate-buffered saline at pH 7.2. After equilibration, diluted HCl was added to pH 5.0 final and lipid mixing measured as the dequenching of R_{18} (excitation 560 nm, emission 590 nm), resulting in an increase in the fluorescence signal. Silymarin was preincubated with HCVpp and liposomes for 3 minutes at 37°C, and lipid mixing measured after medium acidification.

Infectious Virion Production

Culture supernatants were harvested at defined time points postinfection, and supernatants were clarified by centrifugation. Intracellular virus titers were determined after treatment with Brefeldin A, which has been shown to block HCV release by causing intracellular accumulation of virions. For this, treated cells were scraped into phosphate-buffered saline and lysed by freeze-thawing as described.[20] All supernatants were stored at −80°C before dilution and titering on naïve Huh7.5.1 cells using standard focus-forming unit assays as previously described.[8]

Cell-to-Cell Spread

Cell-to-cell spread of virus was measured as previously described.[21] Briefly, unlabeled naïve "target" cells were incubated with HCV H77/JFH infected "producer" cells that were labeled with 5-chloromethylfluorescein diacetate (Molecular Probes, Invitrogen, Carlsbad, CA). A neutralizing antibody was added to the co-culture to abrogate the infectivity and transmission of cell-free virus particles within the culture media, allowing us to quantify antibody insensitive viral transmission. Infection was quantified by staining the co-cultures with an anti-NS5A antibody (9E10) and appropriate secondary antibody, followed by flow cytometry.

apoB Enzyme-Linked Immunosorbent Assay

Huh7.5.1 cells were grown overnight in Huh7 media. The media was removed, cells were washed, and fresh medium with the appropriate compounds was added to cells. Culture supernatants were harvested at 24 and 48 hours later, clarified by centrifugation, and stored at −80°C. Apolipoprotein B was measured by enzyme-linked immunosorbent assay (ELISA) using manufacturer's protocol (ALerCHEK, Portland, ME).

MTP Assay

MTP activity was measured using a commercially available fluorescence assay using a commercial kit (Roar Biomedical Inc., New York, NY) as described.[22]

Statistics

Differences between means of readings were compared using a Student *t* test. A *P*-value of <0.05 was considered significant.

RESULTS

We previously showed that silymarin inhibits HCV RNA and protein expression in the HCVcc system with JFH-1 virus.[6] Figure 1A demonstrates that, in addition to wild-type JFH-1 virus, silymarin also blocks replication of HCVcc chimeras, including constructs that contain the H77 (genotype 1a) and J6 (genotype 2a) structural genes in the JFH-1 nonstructural gene backbone. Inhibition of HCVcc was 50% for H77/JFH and 75% for J6/JFH chimeras. Thus, silymarin has antiviral actions against multiple HCVcc infectious systems. To determine whether silymarin could inhibit binding of HCV virions to cells, we performed virus-cell binding studies at 4°C under conditions in which virus binds to but does not enter cells.[23] As shown in Fig. 1B, when

silymarin was present only during virus-binding, there was little effect on HCV replication. However, if silymarin was added to cells immediately after binding and for the duration of the infection, HCV protein expression was severely impaired. The same effect was observed if silymarin was present during binding and for the duration of the experiment. Next, to determine whether silymarin blocked virus entry, we tested the effect of silymarin on viral pseudoparticle entry including HCVpp, VSVpp, and MLVpp. Figure 1C demonstrates that silymarin inhibited the entry of all three pseudotyped viruses. We then examined the effect of silymarin on the fusion of HCVpp with fluorescent liposomes, which examines the effects of compounds on lipid mixing and membrane fusion.[24] As shown in Fig. 1D, silymarin drastically inhibited HCVpp-mediated fusion by 80% at 10 μM silymarin, whereas 20 μM led to a 90% reduction in fusion. DMSO, the solvent control, did not affect fusion. The IC50 of silymarin for membrane fusion inhibition was estimated at 5 μM, far below the doses of silymarin known to confer cytotoxicity in Huh7.5.1 cells (>80 μM, Supporting Fig. S2, Panel E). The data suggest that silymarin does not affect binding but inhibits the entry of HCV at the fusion stage.

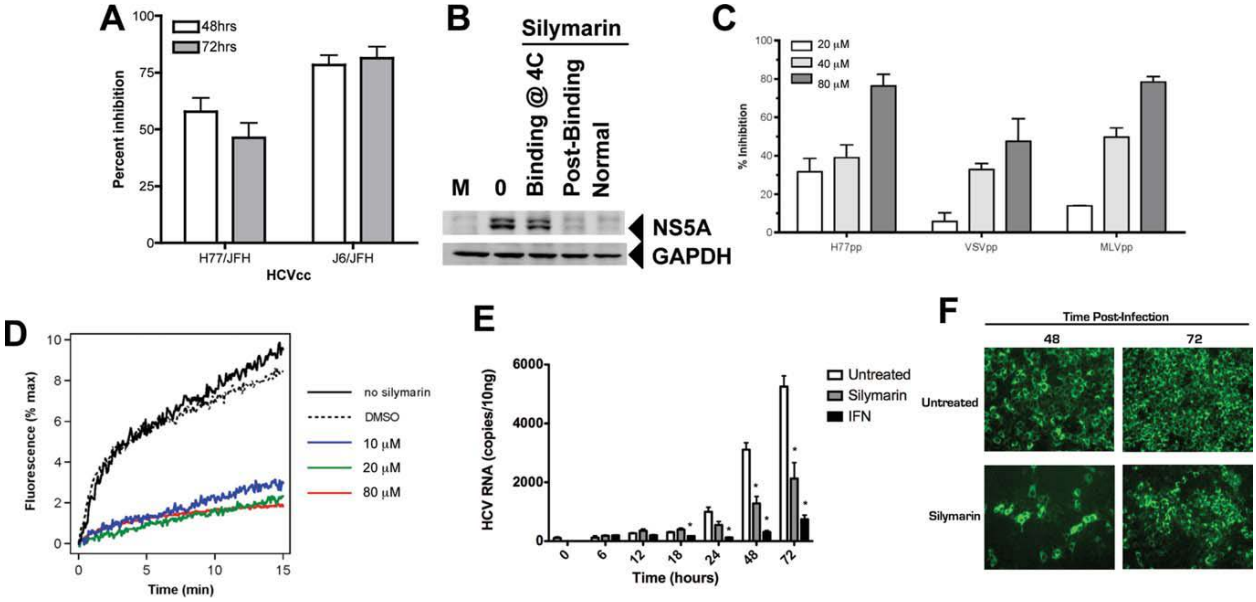

Figure 1: Antiviral effects of silymarin against HCVcc. (A) Silymarin inhibits virus infection in multiple HCVcc systems. Huh-7.5 cells were incubated with 80 μM silymarin or DMSO for 1 hour at 37°C before inoculation with either H77/JFH or J6/JFH HCVcc viruses. The infection was allowed to proceed in the presence of the compounds for 48 and 72 hours before virus was quantitated by staining for NS5A-positive foci. Percent inhibition reflects inhibition of NS5A-positive foci by silymarin relative to the DMSO control. (B) Silymarin does not block virus binding. Huh7.5.1 cells were incubated at 4°C for 5 hours in the presence of HCVcc (JFH-1, MOI of 0.01) with or without 40 μM silymarin. Cells were then washed extensively at 4°C and then cells were incubated for 72 hours at 37°C to allow the HCV lifecycle to continue in the presence or absence of additional silymarin. "M" denotes mock infected cells. "0" denotes cells that were infected but not treated with anything. "Binding @ 4C" denotes silymarin was only present during the 5-hour adsorption period. "Post-binding" denotes that silymarin was added after the 5-hour adsorption period and for the duration of the infection. "Normal" denotes that silymarin was present during the 5-hour adsorption and for the duration of the infection. (C) Silymarin inhibits pseudoparticle entry. Huh-7.5 cells were treated with silymarin (SM) or an equivalent volume of DMSO for 1 hour before infection with HCV, VSV, or MLV pseudoparticles (pp). Seventy-two hours postinfection, the medium was removed and luciferase activity was measured on cell lysates. (D) Silymarin blocks HCVpp-mediated lipid mixing. HCVpp in phosphate-buffered saline at pH 7.2 were incubated or not with indicated concentrations of silymarin in DMSO, for 3 minutes at 37°C, in the presence of phosphatidylcholine:cholesterol:R_{18} liposomes. Acidification to pH 5.0 was performed by adding

diluted HCl to the cuvette, and R_{18} dequenching was assayed for 15 minutes at excitation and emission wavelengths of 560 and 590 nm, respectively. Maximal dequenching was obtained after addition of 0.1% final Triton X-100 to the cuvette. Black, no silymarin; blue, 10 μM; green, 20 μM; and red, 80 μM; silymarin, respectively. Dotted curve, lipid mixing in the presence of 1% final DMSO (highest concentration used in the assay). (E) Silymarin inhibits HCV RNA production. Huh7.5.1 cells were infected at an MOI of 0.01 with JFH-1, and 24 hours later, silymarin (40 μM) or IFN (10 units/mL) was added to cells, and thereafter, total RNA was isolated from cells at the indicated time points. HCV RNA was quantitated by real-time reverse transcription polymerase chain reaction. Asterisks indicate that silymarin or IFN reduction of viral loads is significantly different from untreated cells ($P < 0.01$). (F) Silymarin reduces infectious virus production into culture supernatants. Huh7.5.1 cells were infected at an MOI of 0.01 with JFH-1 in the presence of 40 μM silymarin or DMSO, and supernatants were harvested 48 and 72 hours postinfection and titered by focus-forming unit assay on naïve Huh7.5.1 cells.

Next, we examined the kinetics of inhibition of HCV RNA production. In this experiment, we first infected cells for 24 hours, followed by silymarin administration, or IFN-α as a positive control. As shown in Fig. 1E, relative to untreated cells, silymarin caused a significant ($P < 0.01$) reduction in JFH-1 RNA production at 48 and 72 hours after treatment. IFN treatment also reduced viral loads. However, significant suppression ($P < 0.01$) of HCV RNA production by IFN started at 18 hours posttreatment and was maintained until 72 hours of treatment. Thus, the kinetics of silymarin mediated suppression of HCV RNA replication were delayed as compared with IFN.

As shown in Fig. 1F, silymarin reduced infectious virus yields (measured as focus/mL) by fivefold and twofold at 48 and 72 hours postinfection from Huh7.5.1 cells (and in Huh7 cells; data not shown). We can rule out the possibility of carryover silymarin from the initial culture because the supernatants were diluted 1:5 to 1:1000 before testing on naïve cells. Altogether, the data show that silymarin does not affect virus binding to cells but inhibits virus entry and fusion, HCV protein and RNA synthesis, and production of progeny viruses in culture supernatants.

Inhibition of HCV RNA and protein expression by silymarin could be attributable to direct inhibition of viral enzymes, as recently shown for NS5B polymerase activity.[25] Therefore, we tested whether silymarin and silibinin block HCV NS5B polymerase activity. Recombinant NS5B protein from JFH-1 (genotype 2a) lacking the C-terminal 21 amino acids was expressed in *Escherichia coli* and purified.[16] As shown in Fig. 2, silymarin was able to inhibit JFH-1 NS5B polymerase activity, with an IC50 for silymarin at approximately 300 μM. Silibinin had minimal effects on JFH-1 polymerase, but only at very high doses (IC50 > 400 μM), which were at least fivefold to 10-fold higher than effective antiviral doses *in vitro*.[6] At the doses required for inhibition of *in vitro* NS5B polymerase activity, silymarin used in this study was toxic to cultured Huh7[6] and Huh7.5.1 cells (Supporting Fig. S2).

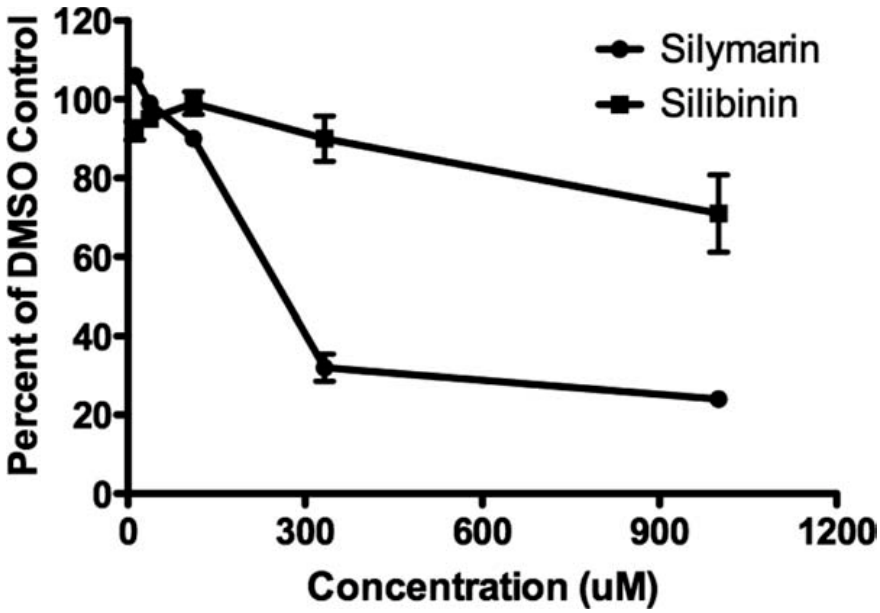

Figure 2: Silymarin inhibits JFH-1 polymerase at high dose. HCV NS5B polymerase from isolate JFH-1 (genotype 2a) was incubated with the indicated concentrations of silymarin or silibinin, respectively, in the presence of α[^{32}P]GTP and polyC. Incorporation of radioactivity was quantified by trichloroacetic acid precipitation and liquid scintillation counting. Silymarin and to a lesser degree, silibinin, inhibited JFH-1 polymerase.

We next tested silymarin on RNA-dependent RNA polymerase (RdRp) activity of the genotype 1b BK strain and four patient-derived 1b RdRps from patients in the Virahep-C clinical study.[26] The RNA polymerase activities of the patient-derived enzymes were variable (16%-104% relative to the well-characterized BK enzyme; Table 1). Silymarin inhibited all five RdRps, with IC50 values ranging from 27.7 to 162 μM. However, in four of the five cases, the inhibitory activity of silymarin rapidly plateaued, with maximal inhibition levels of 42.6% to 82.8% relative to the activity in the absence of silymarin (Supporting Fig. S3). The fifth enzyme (#242) had an inhibition profile that could not be fit to a single-phase exponential decay curve, but its maximal inhibition by silymarin was only 43% and its apparent IC50 was greater than 1000 μM. Therefore, the IC50 values for most of these subtype 1b RdRps were respectable below the plateau level, but the enzymes were poorly inhibited by silymarin at the concentrations employed in the cell culture experiments.

Table 1: Inhibition Profiles of Genotype 1b RdRps by Silymarin

	Polymerase				
Polymerase Function	**BK**	**#234**	**#242**	**#245**	**#103**
Relative RdRp activity	100	15.8	104	99	38.3
IC_{50} (μM)	27.7	45.4	>1000	162	162
Maximal inhibition (%)	48.5	42.6	43.0	53.3	82.8

Polymerase assays were performed and analyzed as described in Materials and Methods. IC_{50}, half-maximal inhibitory concentration.

If silymarin truly inhibits NS5B polymerase activity, it should be able to inhibit HCV replication in replicon cell lines that do not produce infectious virus. Figure 3A-C depicts the effects of various doses of silymarin on HCV protein and RNA expression in genotype 1b BB7 subgenomic and FL-NEO genomic replicon cell lines. Silymarin did not significantly inhibit viral protein expression in either cell line when assessed by western blot (Fig. 3A) or by

immunofluorescence (Fig. 3B). Silymarin did not inhibit HCV RNA expression in either cell line (Fig. 3C). HCV replication was also not inhibited by silymarin in Luc-ubi-neo/ET cells, an independent genotype 1b replicon (Fig. 3D), or in a subgenomic genotype 1a replicon cell line (Fig. 3E). In contrast, treatment with IFN-α caused robust suppression of HCV RNA production from the HCV-1a replicon. We tested concentrations of silymarin up to 1000 μM but failed to see any suppression of HCV RNA from the 1a replicon that was independent of cytotoxicity, measured as GAPDH messenger RNA levels (Supporting Fig. S4). NS5A protein expression was not affected by silymarin in JFH-1-derived genotype 2a SGR7 (Fig. 3F) or SGR7.5 replicon cell lines (data not shown). Furthermore, extended treatment of FL-NEO replicon cells (or BB7 cells; data not shown) for 13 days did not affect the levels of HCV NS5A protein (Supporting Fig. S5). Therefore, silymarin had no antiviral activity against replicon cell lines that did not produce infectious virus. The data in Figs. 2 and 3 suggest that silymarin inhibition of NS5B polymerase activity is not a significant component of silymarin's anti-HCV activity in the HCVcc system.

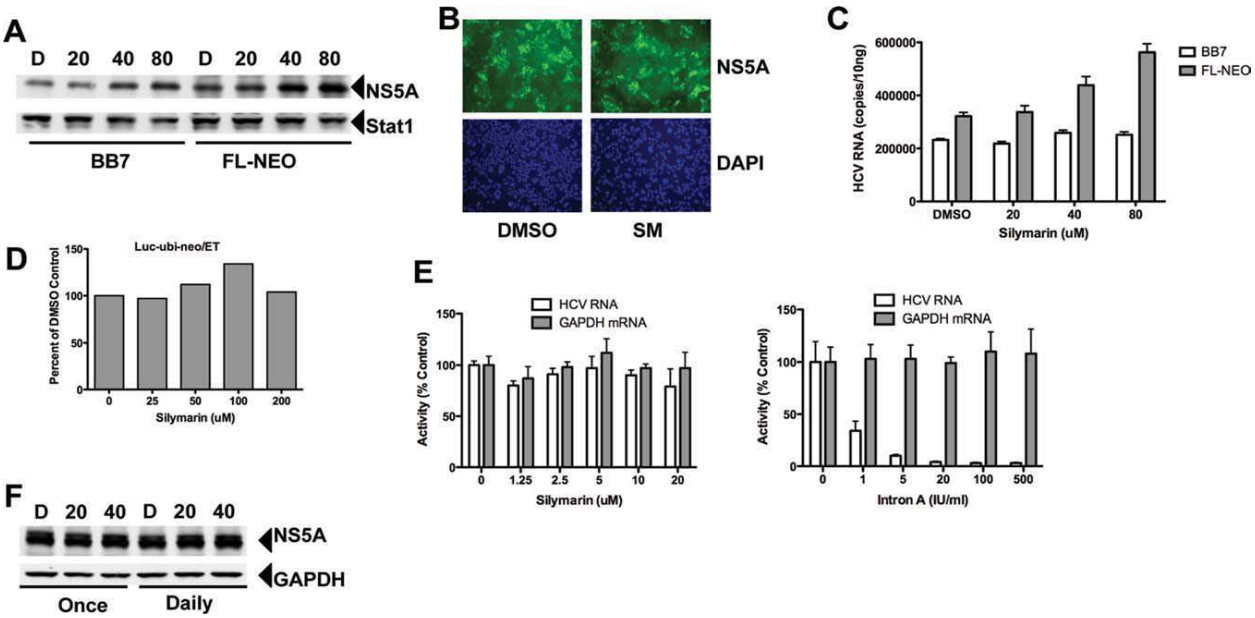

Figure 3: Silymarin does not block HCV replication in HCV replicon cell lines. Cells were treated with the indicated doses of silymarin and incubated for 72 hours before replication was assessed. (A) Protein expression in subgenomic BB7 and genomic FL-NEO HCV-1b replicons after treatment with 20 to 80 μM of silymarin. D, DMSO. Positions of HCV NS5A and cellular Stat1 proteins are indicated. (B) Intracellular NS5A protein expression in DMSO and silymarin treated FL-NEO cells. Cells were treated as described previously, and NS5A protein detected by immunofluorescence as described in the Materials and Methods. NS5A-positive cells are depicted in green, and the blue cells represent nuclei counterstained by 4',6-diamidino-2-phenylindole. (C) HCV RNA levels in FL-NEO and BB7 cells. (D) Effect of silymarin against Luc-ubi-neo/ET cells, a Con1-based genotype 1b replicon subgenomic replicon. Values represent percent change in luciferase light units relative to DMSO control. (E) Effect on HCV-1a subgenomic replicon. Left panel shows HCV and GAPDH RNA levels after silymarin treatment, whereas the right panel shows RNA levels after treatment with Intron-A (recombinant IFN-α). (F) Effect of silymarin on subgenomic genotype 2a JFH-1-derived Huh7 replicon cell line. Cells were treated with 20 or 40 μM silymarin once or each day for 3 days. Positions of HCV NS5A and cellular GAPDH proteins are indicated.

HCV assembles at lipid droplets,[27, 28] and the virus is thought to exit the infected liver cell by hitching a ride on the apolipoprotein assembly and secretion pathway, in particular MTP-dependent very-low-density lipoprotein release.[20, 29, 30] Because silymarin blocked infectious virus production (Fig. 1), we determined whether silymarin also inhibits MTP activity and apoB

secretion. In these studies, silymarin was added to cells that were either fully infected (96 hours postinfection) or chronically infected for 14 days. Thus, the experimental design effectively eliminated antiviral effects involving blockade of virus entry and instead allowed us to focus on the effects of silymarin on production of progeny viruses. Silymarin inhibited MTP activity in a dose-dependent manner in 14-day chronically infected cells by 25% ± 15% and in noninfected cells by 66% ± 1% at 80 μM (Fig. 4A). Naringenin, shown recently to block MTP-dependent virus release,[22] also blocked MTP activity. Silymarin inhibition of MTP activity correlated with reduced apoB secretion in both mock and JFH-1-infected Huh7.5.1 cells (Fig. 4B). The small molecule inhibitor of MTP, BMS-200150, served as a positive control for inhibition of apoB secretion. Silymarin inhibition of MTP activity and apoB secretion correlated with a reduction in *de novo* virion production from fully infected cultures treated for 5 hours (Fig. 4C). Importantly, the reduction in infectious virus production was not attributable to a reduction in intracellular replication, because NS5A protein levels were not affected by the 5-hour treatments with DMSO, silymarin, or BMS-200150 (Fig. 4D). Furthermore, the effect on apoB secretion was not unique to Huh7 cells, because silymarin also caused dose-dependent suppression of apoB secretion from primary human hepatocytes (Fig. 4E) and HepG2 cells, as measured by ELISA and western blot (Fig. 4F). When we examined intracellular infectious virus as a measure of virus assembly, the general secretion inhibitor Brefeldin A caused accumulation of intracellular infectious virus, which was inhibited by the MTP inhibitor BMS-200150, as described by Gastaminza et al.[20] However, silymarin had no effect on Brefeldin A-induced accumulation of infectious virus (Supporting Fig. S6). Collectively, the data demonstrate that silymarin blocks MTP-dependent apoB secretion and infectious virion production into culture supernatants, but does not appear to block virus assembly. We then determined whether silymarin blocks other pathways of virus transmission.

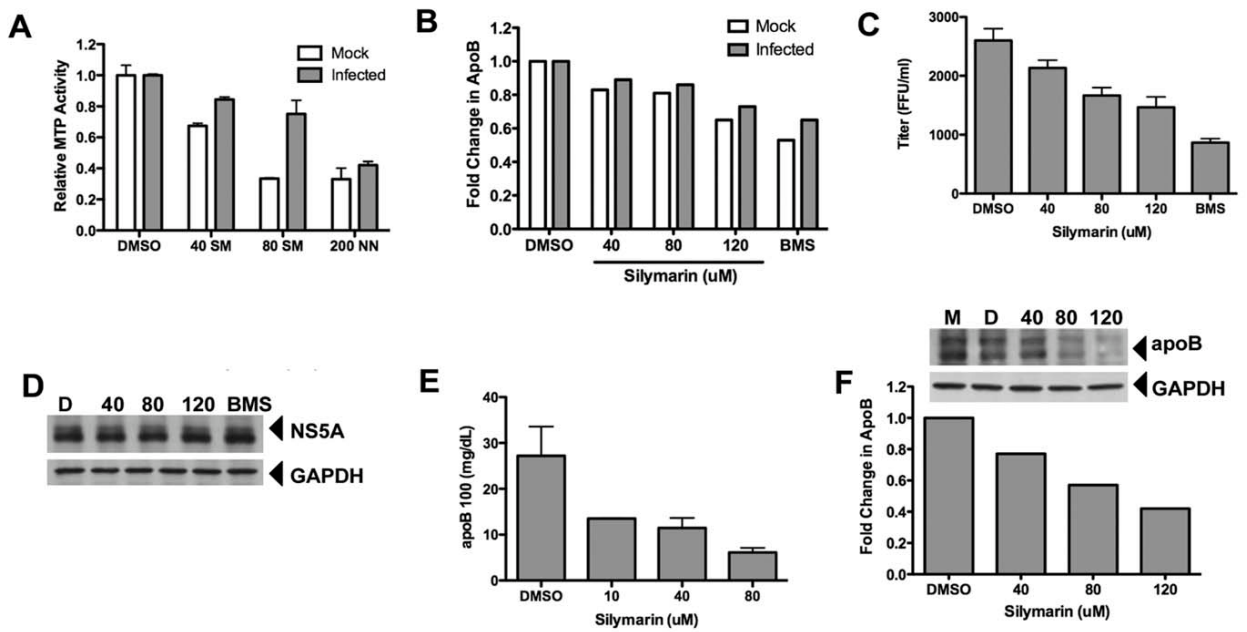

Figure 4: Silymarin inhibits microsomal triglyceride transfer protein (MTP) activity, apolipoprotein B (apoB) secretion, and infectious virus production. (A) Silymarin inhibits MTP activity in Huh7.5.1 cells. Chronically infected Huh7.5.1 cells (14 days postinfection), and uninfected cells were treated with the indicated micromolar doses of silymarin (SM) or as a positive control, 200 μg/mL naringenin (NN), and 24 hours later MTP activity was measured as described in Materials and Methods. (B) Silymarin inhibits apoB secretion from mock and HCV-

infected cells. Huh7.5.1 cells were infected or mock-infected with JFH-1 at an MOI of 0.01 for 96 hours before treatment with fresh medium containing DMSO, 10 μM BMS-200150 (a small molecule inhibitor of MTP), or silymarin at the indicated micromolar doses for 5 hours. Culture supernatants were harvested and apoB measured by ELISA. (C) *De novo* infectious virion production into culture supernatants is blocked by silymarin. Supernatants from panel B were diluted 1:20, and infectious titers were determined by focus-forming unit assay on naïve Huh7.5.1 cells. (D) Treatment of infected cells for 5 hours does not inhibit intracellular HCV replication. Protein lysates were harvested from cultures described in panel B, and equal amounts of total protein were blotted for NS5A. The positions of HCV NS5A and loading control GAPDH are indicated. D is the DMSO control, 40, 80, and 120 are the doses of silymarin in μM. (E) Silymarin blocks apoB secretion from primary human hepatocytes. Cells were treated with the indicated concentrations of SM for 24 hours before supernatants were harvested, and apoB was measured by ELISA. (F) Silymarin blocks apoB secretion in HepG2 cells. Cells were treated with the indicated micromolar concentrations of silymarin for 5 hours before supernatants were harvested and apoB measured by ELISA. Inset, Silymarin blocks intracellular apoB levels. Cell lysates from HepG2 cells treated in panel F were probed for apoB by western blot. ApoB100 and loading control GAPDH are indicated with arrows. M is the media control, D is the DMSO control.

It has been recently shown that, in addition to releasing virus particles into culture medium, HCV is capable of direct cell-to-cell transmission.[21] To examine effects of silymarin on this antibody-insensitive route of transmission, we used a novel assay in which fluorescently labeled infected producer cells were mixed with unlabeled naïve cells, and HCV NS5A protein expression was detected using antibodies labeled in the red spectrum. Silymarin reduced both total and cell-to-cell transmission (Fig. 5A). We also observed equal suppression of both total and cell-to-cell transmission (Fig. 5B), suggesting that silymarin does not discriminate between routes of virus transmission.

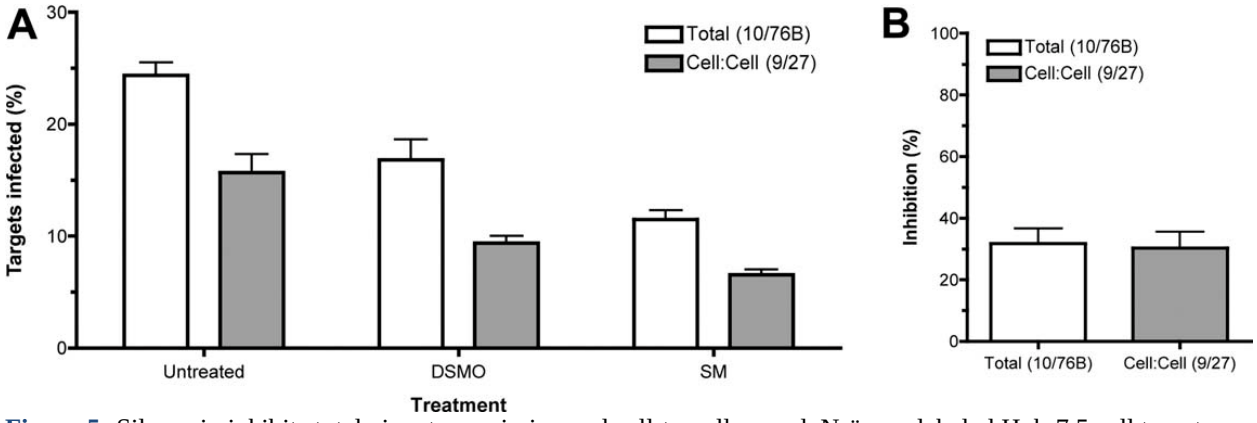

Figure 5: Silymarin inhibits total virus transmission and cell-to-cell spread. Naïve unlabeled Huh-7.5 cell targets were preincubated with 80 μM silymarin or equivalent volume of DMSO for 1 hour at 37°C. The pretreated cells were then co-cultured with 5-chloromethylfluorescein diacetate-labeled H77/JFH-infected Huh-7.5 producers for 48 hours. The cultures were performed in the presence of either monoclonal antibody 9/27, an anti-E2 (HVR) neutralizing antibody, or an anti-HIV gp120 irrelevant control, monoclonal antibody 10/76B. Each treatment was performed in duplicate. The co-cultures were harvested, stained, and analyzed by flow cytometry, and the percentage of infected cells was calculated. Cell culture media from each co-culture was tested for the presence of HCVcc particles. Released H77/JFH virus were readily detectable in media containing control antibody, and no infectious particles were detected in the media containing monoclonal antibody 9/27, indicating that extracellular spread of virus was not occurring, and only cell-cell spread was operative. (A) Silymarin (SM) reduces both total and cell:cell transmission. (B) Silymarin inhibits both total and cell:cell transmission in comparable manner, suggesting that it does not discriminate between the alternative routes.

DISCUSSION

Despite global use for millenia, the detailed molecular mechanisms of silymarin-induced hepatoprotection are not known. In recent studies,[6, 31] we have shown that silymarin displays antiviral, anti-inflammatory, and immunomodulatory functions. These activities, together with antioxidant functions of silymarin,[32] could effectively constitute the hepatoprotection observed in many animal models of liver disease.[33-35] Using HCVpp, HCVcc, and liposome mixing experiments, we demonstrate that silymarin inhibits virus entry and fusion, RNA and protein synthesis, and infectious virus production into culture supernatants and cell-to-cell spread. Silymarin but not silibinin inhibited NS5B polymerase activity. The lack of activity of silibinin in our study contrasts with a recent study showing that silybin A, silybin B, and their water-soluble dihydrogen succinate forms found in Legalon-SIL, a commercial preparation of silibinin, inhibited HCV polymerase function, with IC50s in the 75 to 100 μM range.[25] The water-soluble versions of silybin A and silybin B found in Legalon-SIL contain two succinate moieties that increase the molecular weight of the compound by over 244 atomic mass units, from 482 to 726. Thus, the water-soluble molecules are quite different chemically from the natural compounds, which are insoluble in water, and as a result the metabolism and biological effects of the compounds may differ. However, in our study, silymarin did not inhibit HCV RNA and protein expression in multiple independent replicon cell lines that did not produce infectious progeny viruses, in agreement with a recent study showing that silymarin did not inhibit HCV NS5A protein or RNA expression in a subgenomic replicon cell line.[36] The data suggest that blockade of polymerase activity is not a major antiviral mechanism, at least in the HCVcc system. Instead, we provided evidence to suggest that inhibition of virus entry and virus transmission contribute to the antiviral effects of silymarin.

Indeed, silymarin blocked the entry of three different enveloped pseudoviruses and also potently inhibited the fusion of liposome membranes. Silymarin flavonolignans belong to the family of phytoestrogens and are composed of a phenylbenzopyrone structure.[4] The structures of these molecules are relatively hydrophobic, so it is possible that silymarin may act by incorporating into lipid membranes of both viruses and target cells, or at least may display partition into lipid bilayers, similar to other plant flavonoids.[37] This would lead to the stabilization of membranes by silymarin, which would in turn become less prone to fusion. This behavior is reminiscent of arbidol, a broad-spectrum antiviral inhibiting HCV entry, membrane fusion, and replication.[24] This hypothesis is further corroborated by our observations that silymarin blocks cell entry of pseudotyped particles of other enveloped viruses such as VSVpp and MLVpp. Future studies will focus on further dissecting these mechanisms.

We also showed that silymarin inhibits MTP activity, apoB secretion, and production of infectious virus particles. In support of this argument and in agreement with the results obtained in the current report, the flavonoid taxifolin, which is present in silymarin, has been shown to block MTP activity and apoB secretion.[38] Silymarin has also been shown to alter lipid profiles,[39] so it is possible that the botanical may block virus transmission by targeting multiple components of lipid metabolism.

Silymarin does many things to cells, including modulation of signal transduction,[40] the redox state,[41] T-cell function,[6, 31] and nuclear factor kappa B.[42] These studies suggest that direct effects of silymarin on cell functions are responsible for the prevention of liver disease in many animal

models.[33-35] We therefore hypothesize that silymarin's blockade of virus entry and transmission occurs by targeting the host cell. Studies are in progress to identify the cellular target(s) of silymarin that may explain the many activities elicited by this botanical.

Our demonstration of anti-HCV actions of silymarin[6] was initially at odds with clinical studies that found no effect of silymarin on HCV replication *in vivo*.[43] However, daily intravenous administration of a soluble form of silibinin inhibits HCV viral loads by three to four logs in 1 to 2 weeks in previous IFN nonresponder patients.[7] This important study illustrates the clear differences in outcome based on route of administration and the type of silymarin-derived preparation being tested. Further clinical and *in vitro* studies are required to evaluate silymarin's hepatoprotective effects, metabolism, and bioavailability. Moreover, because it is now clear that patients with chronic hepatitis C self-prescribe botanicals, especially silymarin,[3] regardless of whether they receive standard of care therapy with pegylated IFN plus ribavirin, it will be important to design clinical trials that evaluate the effects and interactions of silymarin, given orally and intravenously, either by itself or with antivirals for HCV, including new specifically targeted antiviral therapy for HCV therapies, on reduction of viral load and improvement in liver function or prevention of liver disease. Because of its multiple actions on cells and hypothesized modulation of cellular targets, silymarin and silymarin-derived compounds also may prove relevant for liver diseases of nonviral origin.

ABBREVIATIONS

apoB, apolipoprotein B; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCV, hepatitis C virus; HCVcc, hepatitis C virus cell culture; HCVpp, hepatitis C virus pseudoparticle; IC50, half maximal inhibitory concentration; IFN, interferon; JFH-1, Japanese Fulminant Hepatitis; MTP: microsomal triglyceride transfer protein; NS5B: nonstructural 5B; R_{18}*, octadecyl rhodamine B chloride; RdRp: RNA dependent RNA polymerase; SM, silymarin.*

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