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SILIBININ AND RELATED COMPOUNDS ARE DIRECT INHIBITORS OF HEPATITIS C VIRUS RNA-DEPENDENT RNA POLYMERASE

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Short Title: Silibinin is a direct inhibitor of HCV RdRp

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Authors contribution: A.A. and J.-M.P. have brought the study concept and designed the work; A.A., N.A, L.B., C.W., C.P., and R.B. have generated the experimental data; R.-T.P. has provided the silymarin components and all the chemistry knowledge contained in the article ; A.A., and J.-M.P. have analyzed and interpreted the data; A.A. has drafted the manuscript; the manuscript has been critically revised by J.-M.P. who supervised the study.

Conflicts of interest: J.-M. P. is an advisor for Rottapharm | Madaus. R.-T. Pohl is an employee of Rottapharm | Madaus.

Abbreviations: HCV: hepatitis C virus; IFN: interferon; NS3/4A: nonstructural 3/4A; RdRp: RNA-dependent RNA polymerase; NS5B: nonstructural 5B; IC50: inhibitory concentration 50%; FRET: fluorescence resonance energy transfer; EC50: effective

concentration 50%; HCVcc: infectious HCV particles; CC50: cytostatic concentration 50%.

ABSTRACT

Background & aims: Silymarin is a mixture of flavonolignans extracted from the milk thistle. Silymarin contains several molecules, including silibinin A, silibinin B, isosilibinin A, isosilibinin B, silicristin, and silidianin. Intravenous infusion of silibinin induces dose-dependent reduction of hepatitis C virus (HCV) RNA levels. The aim of this study was to test the principal isomers contained in silymarin preparations for their ability to inhibit HCV enzymatic functions and replication in different models.

Methods: The inhibitory activity of silymarin components was tested in HCV RNA-dependent RNA polymerase and NS3/4A protease enzyme assays. Their ability to inhibit replication of an HCV genotype 1b replicon model and the JFH1 infectious HCV model in cell culture was also studied.

Results: Silibinin A, silibinin B, their water-soluble dihydrogen succinate forms and Legalon SIL[®], a commercially available intravenous preparation of silibinin, inhibited HCV RNA-dependent RNA polymerase function, with inhibitory concentrations 50% of the order of 75-100 μ M. Silibinin A and silibinin B also inhibited HCV genotype 1b replicon replication and HCV genotype 2a strain JFH1 replication in cell culture. None of these compounds inhibited HCV protease function.

Conclusions: Silibinin A and silibinin B, as well as Legalon SIL[®], inhibit HCV replicon and JFH1 replication in cell culture. This effect is at least partly explained by the ability of these compounds to inhibit HCV RNA-dependent RNA polymerase activity. Our results provide a basis for the optimization and subsequent development of members of the Flavonoid family as specific HCV antivirals.

Approximately 170 million subjects are chronically infected with hepatitis C virus (HCV) worldwide. Chronic HCV infection leads to cirrhosis and hepatocellular carcinoma and causes more than 300,000 deaths per year.¹ Current treatment is based on a combination of pegylated interferon (IFN)- α and ribavirin, which directly inhibits HCV replication and drives progressive infected cell clearance through intricate and only partly understood mechanisms.^{2, 3} Approximately 80% of patients infected with HCV genotypes 2 or 3, but less than 50% of those infected with HCV genotypes 1 or 4, eradicate infection after pegylated IFN- α and ribavirin therapy.⁴⁻⁶

The need for novel, potent, well-tolerated anti-HCV therapies has stimulated the onset of a number of active drug discovery programs.⁷ The most promising agents on the short- to mid-term are specific inhibitors of HCV enzyme functions. Inhibitors of the nonstructural 3/4A (NS3/4A) serine protease of the virus are the most likely to reach the market within the next couple of years.⁸⁻¹⁰ Nucleoside/nucleotide and non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase (RdRp) are also in development.⁷

The inclusion of both IFN- α and ribavirin in the treatment of chronic hepatitis C was essentially the result of empirical approaches.¹¹ In the search for new anti-HCV therapies, empirical approaches are still used, and several molecules are currently tested in HCV patients after having been developed for other conditions. For instance, this is the case for DEBIO-025 (Debiopharm, Lausanne, Switzerland), a cyclophilin inhibitor initially developed for the treatment of human immunodeficiency virus infection;^{12, 13} nitazoxanide (Romark Laboratories, Tampa, Florida), a broad spectrum anti-parasitic agent;¹⁴ and silibinin, the principal component of silymarin.

Silymarin is a mixture of flavonolignans extracted from the milk thistle (*Silybum marianum*) that has been used for many years as a “hepatoprotector” agent. The

principal components of silymarin include the diastereoisomers silibinin A and silibinin B in a roughly 1:1 ratio, the diastereoisomers isosilibinin A and isosilibinin B, silicristin, and silidianin (Figure 1). These terms are those used in the European Pharmacopoeia, following the World Health Organization “International Non-Proprietary Names of Pharmaceutical Substances“. The United States Pharmacopoeia and several publications use the following terms for the same compounds, respectively: silybin A, silybin B, isosilybin A, isosilybin B, silychristin and silydianin. A few other compounds are present as traces (<5%), such as isosilicristin, dehydrosilibinin, taxifolin, quercetin and deoxy-derivatives (including silandrin, isosilandrin, silimonin, silihermin and others).¹⁵⁻¹⁷ Together, silibinin A and B represent 50-60% of the components of silymarin. Silymarin was recently shown by Polyak *et al.* to inhibit replication of JFH1, an infectious HCV genotype 2a strain, in hepatoma cell culture. The observed antiviral effect was ascribed partly to silymarin induction of intracellular IFN-induced pathways and partly to induction of IFN-independent pathways.¹⁸

Legalon SIL[®] (Madaus GmbH, Cologne, Germany) is an intravenous therapeutic formulation, available as a roughly 1:1 mixture of silibinin A and silibinin B in their water-soluble dihydrogen succinate forms. It is nowadays indicated in many countries for the treatment of death cup (*Amanita phalloides*) intoxication as a specific antidote of amanitin.¹⁹ Recently, Ferenci *et al.* treated patients in whom pegylated IFN- α and ribavirin therapy had previously failed with increasing concentrations of Legalon SIL[®]. These authors observed a significant, dose-dependent reduction of HCV RNA levels upon administration, that was followed in some cases by a relapse after treatment cessation.²⁰

In the present study, the principal flavonolignans contained in silymarin preparations, including silibinin A, silibinin B, their dihydrogen succinate disodium forms, isosilibinin A, isosilibinin B, silicristin, silidianin, and Legalon SIL[®] were tested for their ability to directly inhibit HCV RdRp and NS3/4A protease activities in cell-free enzyme assays, while their antiviral properties were also assessed in cellular models including a subgenomic HCV genotype 1b replicon and the JFH1 infectious HCV model in Huh7 cell lines.

MATERIALS AND METHODS

Preparation of silymarin component isomers and their dihydrogen succinates

The 6 flavonolignans silibinin A, silibinin B, isosilibinin A, isosilibinin B, silicristin and silidianin; their dihydrogen disodium succinate esters; an equal mixture of silibinin A and silibinin B and of their dihydrogen succinates (the water-soluble forms used in the medical preparation); and the commercial product Legalon SIL[®] (the mixture of silibinin A and B dihydrogen disodium succinates used in therapy) were obtained from Madaus GmbH (Cologne, Germany). They were isolated from refined and standardized milk thistle dry extract by means of organic solvents. The purity of the isolated compounds was >98% as assessed by high-performance liquid chromatography. They were then converted into their water-soluble dihydrogen disodium succinate forms. The compounds were dissolved in DMSO as a 100 mM stock and stored at -20 °C. Serial dilutions were made immediately prior to the experiments to reach a constant final DMSO concentration in all reactions of 2% in cell-free assays and JFH1 infectious model and 1% in replicon experiments.

Other compounds

A non-nucleoside HCV RdRp inhibitor belonging to the benzimidazole family, JT-16,²¹ and an α -ketoamide inhibitor of HCV NS3/4A protease, telaprevir,²² were provided by the ViRgil-DrugPharm platform of the European Network of Excellence ViRgil on hepatitis and influenza viruses resistance.

Expression and purification of recombinant HCV NS5B Δ 21 RdRp

The HCV RdRp (non-structural 5B (NS5B) protein) from the J4 genotype 1b reference strain,²³ truncated of its 21 C-terminal amino acids to ensure solubility (NS5B Δ 21) and carrying a hexahistidine tag (His-Tag) at its N-terminus, was expressed in *Escherichia coli* C41(DE3) and purified. Briefly, cultures were grown at 37°C for approximately 1h until the culture reached an optical density of 0.6 at 600 nm. They were then induced with 1 mM isopropyl β -D-thiogalactoside for 4h at 37°C. Cell pellets (1 L) were resuspended in a lysis buffer containing 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 0.1% Triton X100, 0.525 mg/ml lysozyme, 0.1 U/ μ l deoxyribonuclease and CompleteTM Protease Inhibitor Cocktail Tablets (Roche Applied Science, Mannheim, Germany; one tablet for 10 purifications). After sonication, cell lysates were clarified by centrifugation at 10,000 g for 45 min at 4°C. Chromatography was performed on a Ni-NTA column (Qiagen, Valencia, California). The columns were washed with a buffer containing 50 mM NaH₂PO₄ (pH 8.0), 500 mM NaCl, and 20 mM imidazole. The bound protein was eluted in 1 mL fractions with a buffer containing 50 mM NaH₂PO₄ (pH 8.0), 500 mM NaCl, 250 mM imidazole. NS5B Δ 21-enriched fractions were selected using a Bradford colorimetric assay, and NS5B Δ 21 purity was determined by Coomassie-stained SDS-PAGE analysis.

Purified RdRp fractions were pooled and dialyzed against a buffer containing 5 mM Tris (pH 7.5), 0.2 M sodium acetate, 1 mM DTT, 1 mM EDTA, and 10% glycerol. As shown in Figure 2A, NS5B Δ 21 purity was >98%.

In order to assess the effect of amino acid substitutions conferring resistance to other specific HCV RdRp inhibitors, site-directed mutagenesis was performed using the Quick Site-Directed Mutagenesis kit (Stratagene, La Jolla, California). The tested substitutions included S282T, M423T, N316Y, H95Q, and P495L. The constructs were verified by DNA sequencing and the corresponding proteins were purified as described above.

RdRp enzyme assay

An enzyme assay was developed to measure RdRp (NS5B Δ 21) polymerase activity and its inhibition. This assay is based on measurement of the amount of double-stranded RNA synthesized in the presence of NS5B Δ 21, a homopolymeric RNA template (Poly U, GE Healthcare, Chalfont St. Giles, UK) and ATP by means of an intercalating agent (SYBR[®] Green, Applied Biosystems). HCV RdRp assays were performed at room temperature in 96-well microtiter plates in 200 μ l reaction mixes containing 20 mM Hepes (pH 7.3), 5 mM MnCl₂, 0.02 U/ml RNAsin, 50 μ g/ml polyU, 2 mM ATP, 10 mM DTT, 60 mM NaCl, 18.9 μ g/ml bovine serum albumin and 0.135 mg/ml purified NS5B Δ 21. The dynamic range of the enzymatic assay spans recombinant protein concentrations of 8 μ M to 0.05 μ M (data not shown). The assay is specific as telaprevir, a potent HCV NS3/4A protease inhibitor, had no RdRp inhibitory effect at concentrations up to 200 μ M.

RdRp activity in the presence of increasing concentrations of the tested compounds was quantified relative to the control reaction (no inhibitor). Inhibitory

concentrations 50% (IC50s), i.e. the compound concentrations able to inhibit RdRp polymerase activity by 50%, were calculated from the inhibition curves as a function of compound concentration, using a four-parameter logistic regression equation by means of Sigma Plot 10 software (Systat Software, San Jose, California). The reported values are the average of three independent measurements performed in duplicate.

Expression and purification of recombinant NS3/4A protease

A DNA fragment encoding residues Ala1 to Ser181 of the HCV NS3 protease domain was obtained by PCR amplification from the serum of a patient infected with HCV genotype 1b (GenBank accession number AM 423173). The NS3 protease 1-181 sequence was fused to NS3 protease cofactor NS4A (residues 21 to 32) by means of a Gly-Ser-Gly-Ser linker, as previously described.²⁴ The fused NS3/4A sequence was inserted into a pET-21a vector for expression in *Escherichia coli* C41(DE3) of the fully activated recombinant single-chain HCV protease with a C-terminal hexahistidine tag. Briefly, a single colony of freshly transformed cells was grown at 37°C until the culture reached an optical density of 0.6 at 600 nm. It was then induced with 0.5 mM isopropyl β -D-thiogalactoside for 4h at 37 °C. All purification steps were performed at 4°C. Cell pellet was lysed in 10 mL of lysis buffer containing 50 mM Hepes (pH 8.0), 300 mM NaCl, 0.1% n-octyl- β -D-glucopyranoside, 5 mM β -mercaptoethanol, 10% (v/v) glycerol, 1 mg/mL lysozyme and 0.1 U/ μ l desoxyribonuclease I and CompleteTM Protease Inhibitor Cocktail Tablets supplemented with 5 mM imidazole. The sonicated cell lysate was clarified by centrifugation at 16,000 g for 30 min. Chromatography was run on a Ni-NTA column. The columns were washed with 30 column volumes of a buffer containing 50 mM

Hepes (pH 8.0), 300 mM NaCl, 0.1% n-octyl- β -D-glucopyranoside, 5 mM β -mercaptoethanol, 10% (v/v) glycerol, and CompleteTM Protease Inhibitor Cocktail Tablets supplemented with 50 mM imidazole. Bound proteins were eluted in 1 mL fractions of the same buffer containing 250 mM imidazole. Purified recombinant NS3 protease fractions were pooled and dialyzed overnight at 4°C against a buffer containing 5 mM Tris (pH 7.5), 200 mM sodium acetate, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol, and stored at -80°C. As shown in Figure 2B, NS3/4A purity was >98%.

NS3/4A protease enzyme assay

An NS3/4A protease enzyme assay based on fluorescence resonance energy transfer (FRET) was used in 96-well microtiter plates. This assay uses an internally quenched fluorogenic depsipeptide, RET-S1 (DABCYL, Anaspec Inc., San Jose, California). NS3 protease activity was measured according to the manufacturer, with minor modifications. Briefly, the purified NS3/4A protease was pre-incubated with increasing concentrations of the tested compounds in 50 mM Hepes (pH 7.8), 100 mM NaCl, 20% glycerol, 5 mM dithiothreitol at 30°C for 60 min. The reaction was started in a total volume of 100 μ l by the addition of 5 μ M RET-S1 substrate and incubated at 30°C for 20 min. Proteolytic product release was monitored (excitation at 360 nm and emission at 500 nm) in a Mithras LB 940 device (Berthold Technologies, Bad Wildbad, Germany). The dynamic range of the enzymatic assay reported by the manufacturer is from 8.3 pM to 0.5 pM. The assay is specific as JT-16, a potent HCV RdRp inhibitor, had no NS3/4A protease inhibitory effect at concentrations up to 50 μ M. Protease activity in the presence of the compounds was quantified relative to the control reaction (no inhibitor). IC₅₀s were calculated from

the inhibition curves as a function of compound concentration by means of a four-parameter logistic regression equation with Sigma Plot 10 software. The reported values are the average of three independent measurements performed in duplicate.

Assessment of antiviral activity in the replicon model

The genotype 1b bicistronic replicon I389-neo/NS3-3'/5.1 was kindly provided by Dr. Ralf Bartenschlager (University of Heidelberg, Germany). Transfected Huh7 cells were grown in Dulbecco's modified Eagle's Medium Glutamax II (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, 100 µg/mL streptomycin, 0,1 µg/mL fungizone and 600 µg/mL Geneticin (G418, Invitrogen).

HCV replicon harboring cells were seeded at a low density of 5000 cells per well in 96-well plates. The cells were treated with increasing concentrations of the tested compounds in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 1% DMSO without G418 and cultured for 3 days. Total RNA was extracted using the RNeasy 96 kit (Qiagen). HCV RNA levels were measured by means of a quantitative real-time polymerase chain reaction assay using the Taqman technology with HCV-specific primers (sense 5'-CGCCCAAACCAGAATACGA-3' and antisense 5'-AGATAGTACACCCTTTTGCCAGATG-3') and probe (5'-6-FAM-CAATGTGTCAGTCGCG-TAMRA-3') on an ABI 7003 device (Applied Biosystems, Foster City, California). HCV RNA levels were measured by means of Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). The results were normalized to GAPDH gene. Each data point represents the average of at least three replicates in cell culture. HCV RNA level reductions after treatment were assessed by comparing the level of HCV RNA in compound-treated cells to that

of control cells treated with 1% DMSO. Effective concentrations 50% (EC50s), i.e. compound concentrations that reduced HCV replicon RNA levels by 50%, were calculated for each compound using a four-parameter curve fitting method in the Sigma Plot 10 software.

In addition, replicon-containing Huh7 cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and treated with anti-actin and anti-NS5B monoclonal antibodies. Proteins were detected by western blot using goat anti-mouse IgG conjugated with peroxidase (Biorad).

Assessment of antiviral activity in the JFH1 infection model in cell culture

Plasmid pJFH1, containing the full-length cDNA of the JFH1 HCV genotype 2a isolate and the Renilla luciferase gene, was used to generate infectious HCV particles (HCVcc) in Huh7 cell culture, as previously described.²⁵ Huh7 cells were seeded in 24-well plates at a density of 30,000-50,000 cells/well and infected 24h later with 200 μ l of HCVcc for 2h at 37°C. After incubation, the supernatants were removed and JFH1-infected cells were washed with fresh medium. Increasing concentrations of the tested compounds were added in a medium containing 2% DMSO, and cells were incubated at 37°C. At 44 h post-infection, cells were washed once with Dulbecco's PBS (Invitrogen) and 100 μ l Renilla lysis buffer (Promega, Madison, Wisconsin) was added to each well. Lysates were frozen at -80°C. The frozen samples were thawed for reading in one batch and 20 μ l was mixed with luciferase assay substrate as specified by the manufacturer (Promega). Luciferase activity was measured for 10 s in a luminometer.

Assessment of compound cytotoxicity

Huh7 and HEK293 cells were seeded at a density of 2000 and 1000 cells per well, respectively, in 96-well microtiter plates in DMEM glutamax-II-10% FBS. Twenty-four hours later, serial dilutions of the tested compounds were added. Cells were allowed to proliferate for 3 days at 37°C. Cell viability was then assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay.²⁶ The 50% cytostatic concentration (CC50), defined as the compound concentration that inhibited proliferation of exponentially growing cells by 50%, was calculated using a four-parameter logistic equation by means of Sigma Plot 10 software. Each point is the result of three experiments performed in quadruplicate.

RESULTS

Silymarin components directly inhibit HCV NS5B RdRp in an enzyme assay

We developed an *in vitro* enzyme assay based on RNA duplex formation in the presence of NS5B Δ 21. This assay was used to assess the inhibitory effect of the following silymarin components on HCV RdRp: silibinin A and B and their water-soluble dihydrogen succinate forms, isosilibinin A, isosilibinin B, silicristin, silidianin, a mixture of silibinin A plus silibinin B and a mixture of their dihydrogen succinate forms, and the Legalon SIL[®] solution. The NS5B Δ 21 catalytic deficient mutant (NS5B Δ 21-D318A), in which the catalytic aspartate at position 318 has been replaced by an alanine, was used as negative control. Benzimidazole JT-16, a specific non-nucleoside HCV RdRp inhibitor, was used as a positive control. As shown in Table 1, the IC₅₀ of JT-16 was 2.0 \pm 0.9 μ M in our assay. Silibinin A, silibinin

B, the mixture of silibinin A and silibinin B and Legalon SIL[®] all inhibited HCV NS5BΔ21 in a concentration-dependent manner in our assay (Table 1 and Figure 3). Their IC₅₀s were of the same order (range 74.5±5.5 to 97.2±24.1 μM) and there was no difference between the natural extracts and their water-soluble dihydrogen succinate forms (Table 1). Inulin, an excipient contained in Legalon SIL[®], had no effect on HCV RdRp (data not shown). Isosilibinin A and isosilibinin B also inhibited HCV NS5BΔ21 in a concentration-dependent manner, but they were 2-3 times less potent than silibinin A, silibinin B, the mixture of silibinin A and silibinin B or Legalon SIL[®]. In contrast, neither silicristin nor silidianin exerted any inhibitory effect on HCV RdRp, up to a maximum concentration of 500 μM (Table 1). DMSO alone at the concentrations used in the experiments had no effect on RdRp activity (data not shown).

Silymarin components inhibition of HCV RdRp is not altered by amino acid substitutions known to confer resistance to specific RdRp inhibitors

Amino acid substitutions known to confer resistance to different families of specific nucleoside and non-nucleoside inhibitors of HCV RdRp were inserted in the NS5BΔ21 sequence used in our enzyme assay by means of site-directed mutagenesis. They included S282T, M423T, N316Y, H95Q, P495L, which confer resistance to 2'-methyl nucleosides, thiophenes (thumb inhibitors), benzofurans (R200 hinge inhibitor; the wild-type variant used in our assay had an asparagine [N] at position 316), benzothiadiazines (palm inhibitors), and benzimidazoles (thumb-fingertips inhibitors), respectively. As shown in Figure 4, none of the tested substitutions had an effect on silibinin or Legalon SIL[®] RdRp inhibition. In contrast, RdRp susceptibility to JT-16 was significantly reduced in the presence of P495L.

Silymarin components have no inhibitory effect on HCV NS3/4A protease in an enzyme assay

We used an *in vitro* enzyme assay based on FRET to assess the inhibitory effect of the same silymarin components on NS3/4A protease. The IC₅₀ of telaprevir, a potent, specific ketoamide inhibitor of NS3/4A protease, was 0.6±0.2 μM in this assay. In contrast, none of the tested silymarin components showed any inhibitory activity in the NS3/4A protease assay, up to a concentration of 200 μM (data not shown). DMSO at the concentrations used in the experiments had no effect on NS3/4A protease activity.

Silymarin components inhibit HCV genotype 1b replicon replication in Huh7 cells

In order to assess the antiviral activity of silymarin components that inhibit RdRp in a replicative HCV model *in cellulo*, subgenomic HCV genotype 1b replicon-containing Huh7 cells were used.²⁷ They were treated with increasing concentrations of silibinin A, silibinin B, the mixture of silibinin A and silibinin B, Legalon SIL[®], isosilibinin A, isosilibinin B, silidianin and silicristin. As shown in Figure 5 and Table 1, silibinin A, silibinin B, the mixture of silibinin A and silibinin B, Legalon SIL[®], isosilibinin and isosilibinin B inhibited replicon replication in a dose-dependent manner. The EC₅₀s were of the μmolar order, i.e. approximately 100 times lower than their IC₅₀s in the RdRp enzyme assay (Table 1). These EC₅₀s were of the same order as that of telaprevir, a potent inhibitor of HCV NS3/4A protease, in the same model (0.5±0.3 μM). Inhibition of HCV protein expression by the mixture of silibinin A and silibinin B in the replicon-harboring Huh7 cells was confirmed by

western blot analysis (data not shown). Silibinin A was slightly more potent than silibinin B, isosilibinin A and isosilibinin B in this model. Silicristin and silidianin had no antiviral effect in this model, up to a concentration of 100 μ M (Table 1).

Silymarin components inhibit JFH1 replication in Huh7 cells

The antiviral activity of these silymarin components was assessed in the JFH1 infectious HCV genotype 2a system, which has been described elsewhere.^{25, 28, 29} For this, increasing concentrations of silibinin A, silibinin B, the mixture of silibinin A and silibinin B, Legalon SIL[®], isosilibinin A, isosilibinin B, silidianin and silicristin were tested. As shown in Figure 6, silibinin A, silibinin B, the mixture of silibinin A and silibinin B, Legalon SIL[®], isosilibinin A and isosilibinin B inhibited JFH1 replication in a dose-dependent manner. The EC50s were of the same order, approximately one log above those observed with the same compounds in the replicon system (Table 1). Silicristin and silidianin had no antiviral effect in this model, up to a concentration of 100 μ M (Table 1).

Silymarin components are not cytotoxic at inhibitory concentrations

As shown in Table 2, the CC50s of the silymarin components tested in Huh7 cells, replicon-containing Huh7 cells cured by IFN- α treatment and HEK293 cells were always far above their EC50s in the replicon and JFH1 models, indicating that these components are not cytotoxic at inhibitory concentrations. Cytotoxicity experiments were also performed with both silibinin A plus silibinin B and Legalon SIL[®] with higher cell densities, similar to those in the antiviral experiments. The same results as in Table 2 were obtained (data not shown).

DISCUSSION

The current treatment of chronic hepatitis C with pegylated IFN- α and ribavirin eradicates HCV genotype 1, by far the most frequent HCV genotype worldwide, in only 40%-50% of treated patients.^{5, 6, 20} The failure rates are similar for HCV genotype 4, the incidence and prevalence of which is increasing in intravenous drug user populations in industrialized countries.^{30, 31} The need for better HCV therapies is thus urgent. Various approaches have been undertaken, most of which target specific HCV lifecycle steps, such as polyprotein processing or replication.⁷ Ferenci *et al.* recently reported that silibinin, the principal component of silymarin, was able to significantly reduce HCV replication in patients who previously had failed to eradicate HCV on standard combination treatment.²⁰ The rationale for administering silibinin to these patients was that HCV-induced oxidative stress has been suggested to contribute to fibrosis and carcinogenesis, and silibinin is a known anti-oxidative agent. Therefore, the observation of an antiviral effect of silibinin was unexpected. Silibinin was efficient when administered intravenously, as Legalon SIL[®], and the antiviral effect was dose-dependent. The antiviral effect of silibinin appeared to be at least additive to that of pegylated IFN- α and ribavirin when these drugs were added after a week of silibinin monotherapy. HCV RNA became undetectable at week 12 in 7 of 14 patients receiving 15 and 20 mg/kg silibinin as Legalon SIL[®] infusion for 14 days who subsequently continued on pegylated IFN- α and ribavirin only. No changes in oxidative stress parameters were observed during administration.²⁰

In another study, Polyak *et al.* showed that a standardized silymarin extract, MK-001, inhibited infection of Huh7 and Huh7.5.1 cells by the JFH1 HCV strain in a dose-dependent manner.¹⁸ JFH1 replication was also inhibited by MK-001 in these

cell cultures. These authors suggested that the observed antiviral properties of milk thistle extracts were mostly due to a cellular effect, mediated partly by induction of Stat1 phosphorylation, which activates intracellular IFN-induced pathways, and partly by IFN-independent mechanisms.¹⁸

In the present study, we showed that silibinin A and silibinin B inhibit JFH1 replication in cell culture, with EC50s of the order of 20-40 μ M. In addition, we showed that silibinin A, silibinin B, isosilibinin A and isosilibinin B are potent inhibitors of HCV genotype 1b replicon replication in cell culture, with EC50s of the order of 1 μ M. Given the fact that our experiments were performed in the same cell culture system (Huh7), the improved efficacy of silibinin components on the genotype 1b replicon compared to the genotype 2a JFH1 could be genotype-related.

In order to explain the antiviral effect of silibinin and its components on HCV replication, two non-mutually exclusive hypotheses can be raised: (i) silibinin components could induce cellular antiviral effectors; (ii) silibinin components could directly inhibit vital HCV functions. The first hypothesis has been explored by Polyak *et al.* and their work provides arguments in favor of silibinin inducing IFN-dependent and -independent cellular pathways.¹⁸ In the present work, we addressed the second hypothesis by testing the effect of a number of silymarin components and their water-soluble forms on HCV NS3/4A protease and RdRp by means of enzyme assays. None of these compounds inhibited NS3/4A protease function. In contrast, we found that silibinin A, silibinin B, the mixture of silibinin A and silibinin B, their water-soluble dihydrogen succinate forms and Legalon SIL[®] all inhibited RdRp function with IC50s of the order of 75-100 μ M. Isosilibinin A and isosilibinin B also inhibited HCV RdRp but were slightly less potent. In contrast, neither silicristin, nor silidianin had any

effect on HCV RdRp, a finding in keeping with their lack of antiviral effect on the replicon and JFH1 models in Huh7 cells.

As shown in Table 1, the antiviral effect of silibinin was more potent in cell culture systems than in the RdRp enzyme assay. This could be explained by the assay itself or the choice of the genotype 1b reference sequence used in the enzyme assay *versus* the replicon sequence used in our experiments. It is however more likely that RdRp inhibition only partly explains the antiviral effect of silibinin components observed *in vitro* in cell culture systems and *in vivo* in HCV-infected patients, the cellular effects of silibinin also participating in the control of viral replication in infected cells.

Amino acid substitutions known to confer resistance to different families of HCV RdRp inhibitors did not confer reduced susceptibility to silymarin components, suggesting that these compounds have a different target site and/or mechanism of antiviral inhibition. Further studies will be needed in order to identify resistance-associated amino acid substitutions that may indicate the target site of silymarin components on HCV RdRp. Other members of the Flavonoid family have been identified as potent inhibitors of HCV RdRp (Ahmed-Belkacem A. *et al.*, unpublished data). Chemical optimization of these compounds will be needed in order to improve their inhibitory capacity. Analysis of the chemical structure of the different silymarin components shown in Figure 1 provides preliminary information as to the structures that may play a role in the inhibitory effect of these compounds on HCV RdRp function. An intact six-member ring D indeed appears to be essential, since silidianin, which has a five-member ring D, has no anti-RdRp activity. Furthermore, the position of ring E may also be important: isosilibinin A and B indeed have less potent RdRp inhibitory activities than silibinin A and B, while having ring E branched to ring D at

position α instead of β . This information may be useful to improve the antiviral activity of silibinin-related and more generally Flavonoid compounds.

Only the intravenous form of the purified silibinin component has been shown to be effective on HCV replication,²⁰ and the interest for its use in HCV-infected patients must be assessed. Given the IC50s observed in the present work, it is likely that a relatively high exposure is needed to obtain a significant antiviral effect. *In vivo*, intravenous administration of Legalon SIL[®] has been associated with maximum silibinin concentrations (C_{max}) of the order of 17,000 ng/mL, i.e. approximately 50 μ M (Madaus GmbH, data on file). These concentrations are close to the IC50s observed in this study in the enzyme assay, and above the EC50s in the replicon and JFH1 models. They suggest that direct inhibition of HCV RdRp is a mechanism by which silibinin could reduce HCV replication in infected patients. Future clinical trials will be needed to assess which patients could benefit from the administration of silibinin concomitantly to pegylated IFN- α and ribavirin. Second-line treatment in difficult-to-treat patients, previous non-responders to pegylated IFN- α and ribavirin and/or patients with severe liver disease should be explored, given the fact that an intravenous administration is required.

In conclusion, we have shown that silibinin A and silibinin B, as well as Legalon SIL[®], a commercially available intravenous preparation of silibinin, inhibit HCV replicon and JFH1 replication in cell culture. This effect is at least partly explained by the ability of these compounds to inhibit HCV RdRp. Whether silibinin might be useful in combination with pegylated IFN- α and ribavirin in difficult-to-treat populations must be explored. Our results provide a basis for the optimization and subsequent development of members of the Flavonoid family as specific HCV antivirals.

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REFERENCES

1. Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* 2002;36:S21-9.
2. Pawlotsky JM. Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C. *Antiviral Res* 2003;59:1-11.
3. Chevaliez S, Pawlotsky JM. Interferons and their use in persistent viral infections. *Handb Exp Pharmacol* 2009:203-41.
4. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975-82.
5. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958-65.
6. Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004;140:346-55.
7. Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. *Gastroenterology* 2007;132:1979-98.
8. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourliere M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S. Telaprevir and

- peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839-50.
9. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009;360:1827-38.
 10. Kwo P, Lawitz E, J. M, Schiff E, Vierling J, Pound D, Davis M, Galati J, Gordon S, Ravendhran N, Rossaro L, Anderson F, Jacobson I, Rubin R, Koury K, Brass C, Chaudri E, Albrecht J. HCV SPRINT-1 final results: SVR 24 from a phase 2 study of boceprevir plus PegIntron (peginterferon alfa-2b)/ribavirin in treatment-naive subjects with genotype 1 chronic hepatitis C. *J Hepatol* 2009;50:S4.
 11. Pawlotsky JM. Therapy of hepatitis C: from empiricism to eradication. *Hepatology* 2006;43:S207-20.
 12. Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, Pawlowska M, Heathcote JE, Mazzella G, Vandelli C, Nicolas-Metral V, Groscurin P, Liz JS, Scalfaro P, Porchet H, Crabbe R. The cyclophilin inhibitor Debio-025 combined with PEG IFN-alpha2a significantly reduces viral load in treatment-naive hepatitis C patients. *Hepatology* 2009;49:1460-8.
 13. Flisiak R, Horban A, Gallay P, Bobardt M, Selvarajah S, Wiercinska-Drapalo A, Siwak E, Cielniak I, Higersberger J, Kierkus J, Aeschlimann C, Groscurin P, Nicolas-Metral V, Dumont JM, Porchet H, Crabbe R, Scalfaro P. The cyclophilin inhibitor Debio-025 shows potent anti-hepatitis C effect in patients coinfecting with hepatitis C and human immunodeficiency virus. *Hepatology* 2008;47:817-26.

14. Rossignol JF, Elfert A, El-Gohary Y, Keeffe EB. Improved virologic response in chronic hepatitis C genotype 4 treated with nitazoxanide, peginterferon, and ribavirin. *Gastroenterology* 2009;136:856-62.
15. Simanek V, Kren V, Ulrichova J, Vicar J, Cvak L. Silymarin: What is in the name...? An appeal for a change of editorial policy. *Hepatology* 2000;32:442-4.
16. Gazak R, Walterova D, Kren V. Silybin and silymarin: new and emerging applications in medicine. *Curr Med Chem* 2007;14:315-38.
17. Kroll DJ, Shaw HS, Oberlies NH. Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies. *Integr Cancer Ther* 2007;6:110-9.
18. Polyak SJ, Morishima C, Shuhart MC, Wang CC, Liu Y, Lee DY. Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized silymarin. *Gastroenterology* 2007;132:1925-36.
19. Hruby K, Csomos G, Fuhrmann M, Thaler H. Chemotherapy of Amanita phalloides poisoning with intravenous silibinin. *Hum Toxicol* 1983;2:183-95.
20. Ferenci P, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, Schoniger-Hekele M, Holzmann H, Steindl-Munda P. Silibinin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. *Gastroenterology* 2008;135:1561-7.
21. Ishida T, Suzuki T, Hirashima S, Mizutani K, Yoshida A, Ando I, Ikeda S, Adachi T, Hashimoto H. Benzimidazole inhibitors of hepatitis C virus NS5B polymerase: identification of 2-[(4-diarylmethoxy)phenyl]-benzimidazole. *Bioorg Med Chem Lett* 2006;16:1859-63.

22. Summa V. VX-950 (Vertex/Mitsubishi). *Curr Opin Investig Drugs* 2005;6:831-7.
23. Inchauspe G, Zebedee S, Lee DH, Sugitani M, Nasoff M, Prince AM. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc Natl Acad Sci USA* 1991;88:10292-6.
24. Taremi SS, Beyer B, Maher M, Yao N, Prosis W, Weber PC, Malcolm BA. Construction, expression, and characterization of a novel fully activated recombinant single-chain hepatitis C virus protease. *Protein Sci* 1998;7:2143-9.
25. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791-6.
26. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
27. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999;285:110-3.
28. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005;102:9294-9.
29. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005;309:623-6.

30. Payan C, Roudot-Thoraval F, Marcellin P, Bled N, Duverlie G, Fouchard-Hubert I, Trimoulet P, Couzigou P, Cointe D, Chaput C, Henquell C, Abergel A, Pawlotsky JM, Hezode C, Coude M, Blanchi A, Alain S, Loustaud-Ratti V, Chevallier P, Trepo C, Gerolami V, Portal I, Halfon P, Bourliere M, Bogard M, Plouvier E, Laffont C, Agius G, Silvain C, Brodard V, Thieffin G, Buffet-Janvresse C, Riachi G, Grattard F, Bourlet T, Stoll-Keller F, Doffoel M, Izopet J, Barange K, Martinot-Peignoux M, Branger M, Rosenberg A, Sogni P, Chaix ML, Pol S, Thibault V, Opolon P, Charrois A, Serfaty L, Fouqueray B, Grange JD, Lefrere JJ, Lunel-Fabiani F. Changing of hepatitis C virus genotype patterns in France at the beginning of the third millenium: The GEMHEP GenoCII Study. *J Viral Hepat* 2005;12:405-13.
31. Al Ashgar H, Helmy A, Khan MQ, Al Kahtani K, Al Quaiz M, Rezeig M, Kagevi I, Alshehri A, Al Kalbani A, Al Swat K, Dahab S, Elkum N, Al Fadda M. Predictors of sustained virological response to a 48-week course of pegylated interferon alfa-2a and ribavirin in patients infected with hepatitis C virus genotype 4. *Ann Saudi Med* 2009;29:4-14.

FIGURE LEGENDS

Figure 1. Chemical formulae of the 6 principal active components of silymarin tested in this study: silibinin A, silibinin B, isosilibinin A, isosilibinin B, silicristin, and silidianin.

Figure 2. SDS-polyacrylamide gels of (A) purified NS5B Δ 21 and (B) purified NS3/4A proteins (left) and the corresponding densitometry plots (right), generated with ImageJ software. Purity was >98% in both cases.

Figure 3. Inhibition of HCV RdRp activity in an enzyme assay based on RNA duplex formation in the presence of NS5B Δ 21 by silibinin A (A), silibinin B (B), a mixture of silibinin A and silibinin B (C) and Legalon SIL[®] (D). RdRp inhibition is shown as a percent reduction of RdRp activity by increasing concentrations of the compounds as compared to the untreated control.

Figure 4. Effect of amino acid substitutions known to confer resistance to specific nucleoside and non-nucleoside HCV RdRp inhibitors on silibinin A plus B (gray bars) and Legalon SIL[®] (black bars) inhibitory effects on RdRp in the enzyme assay. The following substitutions were tested: P495L (that confers resistance to benzimidazoles, thumb-fingertips inhibitors), M423T (thiophenes, thumb inhibitors), H95Q (benzothiadiazines, palm inhibitors), N316Y (benzofurans, R200 hinge inhibitors), and S282T (to 2'-methyl nucleosides). The fold increases in IC₅₀s are shown. JT-16, a benzimidazole, was used as a control (white bars).

Figure 5. Inhibition of HCV genotype 1b replicon replication in Huh7 cells by silibinin A (A), silibinin B (B), a mixture of silibinin A and silibinin B (C), and Legalon SIL[®] (D). Replicon replication inhibition is shown as a percent reduction of HCV RNA level by increasing concentrations of the compounds as compared to the control.

Figure 6. Inhibition of JFH1 replication in Huh7 cells by silibinin A (A), silibinin B (B), a mixture of silibinin A and silibinin B (C), and Legalon SIL[®] (D). JFH1 replication inhibition is shown as a percent reduction of luciferase activity by increasing concentrations of the compounds as compared to the control.

Table 1. Inhibitory effect of different silymarin components on HCV RdRp activity in an enzyme assay, on HCV replication in a genotype 1b replicon model, and in the JFH1 infectious model in Huh7 cells. NT: not tested.

Compound	HCV NS5B Δ 21 RdRp enzyme assay IC50 (μ M)	Genotype 1b replicon EC50 (μ M)	JFH1 infectious model EC50 (μ M)
Silibinin A	96.4 \pm 15.9	0.4 \pm 0.3	23.4 \pm 7.5
Silibinin A dihydrogen succinate	93.2 \pm 18.3	NT	NT
Silibinin B	97.2 \pm 24.1	1.0 \pm 0.6	39.3 \pm 12.5
Silibinin B dihydrogen succinate	99.3 \pm 28.9	NT	NT
Silibinin A plus silibinin B	82.0 \pm 22.3	0.5 \pm 0.2	25.4 \pm 6.7
Silibinin A dihydrogen succinate plus silibinin B dihydrogen succinate	64.5 \pm 14.4	NT	NT
Legalon SIL [®]	74.5 \pm 5.5	0.6 \pm 0.2	16.8 \pm 10.2
Isosilibinin A	211.6 \pm 34.9	1.5 \pm 0.5	45.8 \pm 5.5
Isosilibinin B	165.5 \pm 17.4	1.3 \pm 0.2	20.9 \pm 6.6
Silicristin	>500	>100	>100
Silidianin	>500	>100	>100
JT-16 (benzimidazole)	2.0 \pm 0.9	5.2 \pm 1.7	5.3 \pm 0.3

Table 2. Cytotoxicity experiments: the 50% cytostatic concentrations (CC50), defined as the compound concentrations that inhibited proliferation of exponentially growing Huh7 cells, cured replicon-containing Huh7 cells and HEK293 cells by 50% as compared to untreated cells, are shown. Each point is the result of three experiments performed in quadruplicate. NT: not tested.

Compound	CC50 (μM)		
	Huh7 cells	<u>Cured replicon-containing Huh7 cells</u>	HEK293 cells
Silibinin A + silibinin B	>200	<u>>200</u>	183 \pm 28
Legalon SIL [®]	>300	<u>>300</u>	>300
Isosilibinin A	>100	<u>NT</u>	>100
Isosilibinin B	168 \pm 5	<u>NT</u>	92 \pm 3
Silidianin	>250	<u>NT</u>	>250
Silicristin	>200	<u>NT</u>	>200