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Anti-herpes virus activity of silibinin, the primary active component of *Silybum marianum*

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ABSTRACT

Silibinin (silybin A and silybin B), the primary active component of *Silybum marianum* has antiviral activity against herpes simplex virus, type 2 (HSV-2). The fifty percent cytotoxic dose (CD_{50}) of silibinin was 378 μg/ml. By plaque reduction assay silibinin had a weak antiviral effect against HSV-2 with a fifty percent inhibitory concentration (IC_{50}) of 100 μg/ml and therapeutic index of 3.8. As a virucide, silibinin was more potent with an IC_{50} of 5 μg/ml and therapeutic index of 76. Further investigations of the mechanisms of action of silibinin against herpes simplex viruses and subsequent silibinin monotherapy or combination therapy with other flavonoids as a topical treatment for genital herpes are warranted.

1. Introduction

Silybum marianum (L.) Gaertn. (Carduus marianus L., Asteraceae), commonly known as milk thistle or marian thistle has been used for gastritis, toxic liver damage, jaundice, chronic viral hepatitis, hepatic cirrhosis, gallbladder colic and diseases of the spleen (Kidd and Head, 2005). The active component of this plant is silymarin (a standardized extract of the seeds), a complex of at least 7 flavonolignans and 1 flavonoid that comprises 65–80% of milk thistle extract (Kroll et al., 2007). Silibinin is the primary active component, a semipurified fraction and was once thought to be a single compound, but is a 1:1 mixture of 2 diastereoisomers, silybin A and silybin B (Kroll et al., 2007). 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 and it is indicated in many countries for the treatment of death cup (*Amanita phalloides*) intoxication as a specific antidote of amanitin (Hruby et al., 1983).

The vast majority of research on *S. marianum* and its components has focussed on hepatoprotection and treatment of chronic viral hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV). Systematic reviews in the recent past suggest that oral *S. marianum* does not improve biochemical markers, improve histology at biopsy, or reduce mortality among patients with chronic liver disease; but reviewers noted that further study was needed (Liu et al., 2003). A double-blinded trial evaluating oral silymarin over one year revealed

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no effect upon HCV viremia, serum alanine aminotransferase, or serum and ultrasound markers for hepatic fibrosis (Tanamly et al., 2004). Fried et al. (2012) conducted a multicenter, double-blind, placebo-controlled trial of oral silymarin and found that it did not significantly reduce serum ALT levels more than placebo in participants with chronic HCV infection unsuccessfully treated with interferon-based therapy.

However, silymarin and silibinin can be administered intravenously (IV) and have been found to have significant activity against HCV and HIV. In one case report, a HIV–HCV co-infected patient was having a poor response to standard therapy, with the addition of IV silibinin therapy resulted in HCV-RNA and HIV-RNA becoming undetectable, and increased CD4+ cell counts after 2 weeks (Payer et al., 2010). In another case report the application of high-dose, intravenous silibinin in combination with ribivirin in an HCV-positive, cirrhotic patient refractory to three courses of interferon-based therapy led to a rapid virologic response as documented by undetectable HCV RNA levels (Biermer and Berg, 2009). An additional case report demonstrated that montherapy with a silibinin IV infusion prevented HCV re-infection after orthotopic liver transplantation (Neumann et al., 2010). Ferenci et al. (2008) treated 16 patients in whom pegylated interferon and ribavirin therapy had previously failed and found a dose dependent decrease in HCV viral load after seven days of monotherapy with Legalon SIL, and viral load decreased further after 7 days combined Legalon SIL, pegylated interferon, and ribavirin combination therapy. The most commonly reported adverse events included mild gastrointestinal symptoms and the sensation of heat after infusion (Ferenci et al., 2008).

Interest in silibinin in our laboratory was sparked by its structural similarity to a number of known antiviral flavonoids. In the literature there seems to be a paucity of research concerning the antiviral effects of silibinin against herpes simplex viruses (HSV). Thus, it was the purpose of this study to examine anti-herpes virus activity of silibinin.

2. Materials and methods

2.1. Cell lines and viruses

For all experiments African green monkey kidney cells (vero) (ATCC, CCL-81) were utilized. The cells were grown and maintained as adherent monolayers in McCoy's 5A medium (Mac 5A, Sigma Chemical) supplemented with 10% newborn calf serum (Hyclone). Herpes simplex virus-2 (HSV-2) (G strain) employed in all experiments were obtained from the American Type Culture Collection (ATCC).

2.2. Antivirals

Silibinin (silybin A and silybin B) and acyclovir were purchased from Sigma Chemical (St. Louis, Missouri). Silibinin was dissolved in 1.0 ml of DMSO and diluted to a 50 ml stock solution of 1.0 mg/ml in Mac-5A (2% DMSO). Of note, DMSO has been demonstrated to be non-cytotoxic up to a concentration of 2% (Sumida et al., 2011).

Table 1 – Cytotoxicity of acyclovir and silibinin determined via MTT assay.

	Absorbance at 550 nm ± SD[a]	% Toxicity ± SD	P-value
Silibinin concentration (μg/ml)			
0	0.62 ± 0.03		
5	0.52 ± 0.07	16 ± 11	0.14
50	0.54 ± 0.06	13 ± 10	0.16
100	0.48 ± 0.06	23 ± 10	0.03
500	0.07 ± 0.005	89 ± 1	<0.01
1000	0.07 ± 0.006	89 ± 1	<0.01
Acyclovir concentration (μg/ml)			
0	0.62 ± 0.03		
5	0.61 ± 0.06	2 ± 10	0.81
50	0.60 ± 0.03	3 ± 5	0.46
100	0.56 ± 0.08	10 ± 14	0.29
500	0.53 ± 0.04	14 ± 6	0.04
1000	0.10 ± 0.07	84 ± 11	<0.001

[a] Standard deviation.

2.3. Cytotoxicity assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was utilized to assess the cytotoxicity of silibinin and acyclovir. 1.0×10^3 Vero cells/well were plated on 96-well flat bottom culture plates (Heo et al., 1990). Wells contained serial dilutions of silibinin or acyclovir (5, 50, 100, 500, and 1000 μg/ml), with respective untreated controls, and were incubated for 24 h. After 24 h, 50 μl of 2 mg/ml MTT (Sigma Chemical) was added to each well and plates were incubated for an additional 4 h at 37 °C. The plates were then washed with 1× phosphate buffered saline (PBS; pH 7.4) and 150 μl of DMSO was utilized to solubilize formazan following the 5 h incubation. Absorbance was measured at 550 nm. Results were expressed as mean absorbance at 550 nm, percent cyto-toxicity, and the fifty percent cytotoxic dose (CD_{50}). When not approximated by assay CD_{50} values were calculated via linear regression (Table 1).

2.4. Plaque reduction assay

The antiviral activity of silibinin was compared to acyclovir, the gold standard for the treatment of HSV infection. Vero cells grown to confluence on Corning 25 cm^2 tissue culture flasks were infected with enough diluted virus to yield 50–100 viral plaques per monolayer. Sililbinin was tested in 0.5, 5, 50, and 125 μg/ml concentrations. The reference antiviral compound was acyclovir and it was serially diluted from 1000 to 0.01 μg/ml. Silibinin was assayed in four separate experiments. The uninfected control groups were mock-infected with serum free Mac-5A. After 1 h incubation, the infected and non-infected controls received Mac-5A whereas cells to be tested with chemicals received the appropriate amount of test chemical diluted in Mac-5A. Plates were incubated until primary plaques were observed. The plates were then fixed with 1% formaldehyde and stained with crystal violet (1% crystal violet in 30% methanol). The number of plaques observed was recorded after drying. Results were expressed as mean plaque forming units per ml (PFU/ml), percent plaque