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SILIBININ INHIBITS ETHANOL METABOLISM AND ETHANOL-DEPENDENT CELL PROLIFERATION IN AN *IN VITRO* MODEL OF HEPATOCELLULAR CARCINOMA

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Abstract

Chronic ethanol consumption is a known risk factor for developing hepatocellular carcinoma (HCC). The use of plant-derived antioxidants is gaining increasing clinical prominence as a potential therapy to ameliorate the effects of ethanol on hepatic disease development and progression. This study demonstrates silibinin, a biologically active flavanoid derived from milk thistle, inhibits cytochrome p450E1 induction, ethanol metabolism and reactive oxygen species generation in HCC cells *in vitro*. These silibinin-mediated effects also inhibit ethanol-dependent increases in HCC cell proliferation in culture.

Keywords

Hepatocellular carcinoma; Ethanol; Cytochrome P450E1; Oxidative stress; Mitogenesis

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most rapidly increasing type of cancer diagnosed in the US and represents a major health burden on a global scale [1]. Chronic ethanol consumption has been identified as a causative factor (carcinogen) for HCC development [2; 3] and acts synergistically with other known HCC risk factors [4; 5]. Increasing experimental and clinical evidence suggest that, following consumption, many of the detrimental hepatic effects of ethanol are attributed to hepatic ethanol metabolism through direct or indirect mechanisms [2; 6; 7]. In the setting of moderate/acute ethanol consumption, the majority of ethanol is metabolized (in hepatocytes) by alcohol dehydrogenase (ADH) to acetaldehyde [2; 7], a highly reactive carcinogen that is in turn rapidly metabolized to acetate by acetaldehyde dehydrogenase (ALDH) [2; 7; 8]. In the

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setting of chronic ethanol consumption, hepatic cytochrome P4502E1 (CYP2E1) is induced [2; 9; 10]. In addition to generating increased levels of acetaldehyde, CYP2E1-dependent ethanol metabolism generates reactive oxygen species (ROS) and increased intracellular oxidative stress. These factors increase the likelihood of genetic damage and alterations in the integrity of signaling pathways that regulate and maintain cell function [10]. Additionally, CYP2E1 induction has been identified as an important factor in the [predominantly hepatic] activation of pro-carcinogens and the influence of other cell types (hepatic and nonhepatic) that contribute toward progressive hepatic disease [7; 11].

The identification of ethanol metabolism and ROS generation/oxidative stress as major components in mediating the effects of ethanol in the liver has led to increased interest in the use of antioxidants to blunt the deleterious effects of ethanol on hepatic function. S-adenosyl-L-methionine (SAME), a precursor in the synthesis of the endogenous hepatic antioxidant glutathione (GSH), has been reported to exert hepatoprotective effects in a wide range of animal models of hepatic disease [12; 13], including those associated with alcoholic liver disease [13; 14; 15]. However, the use of SAME in clinical trials has been controversial and the results less conclusive [16]. This has led to renewed interest in alternative therapies based on naturally occurring, plant-derived compounds including those such as silibinin, a biologically active flavinoligand derived from the milk thistle plant (*S. marianum*) [17; 18; 19].

The aims of the current study were to determine the effects of silibinin on ethanol metabolism and enzyme expression in HCC cells, and whether ethanol and/or silibinin act to alter the rate of HCC cell proliferation *in vitro*. In doing so we also identify underlying mechanism(s) by which silibinin may act to slow the rate of HCC progression in the absence or presence of ethanol.

2. MATERIALS AND METHODS

2.1. Materials

Fetal bovine serum (FBS), MEM α cell culture medium, TRIZOL[®] reagent, the Image-iT[®] LIVE Green-ROS detection assay kit, and the MTT proliferation assay kit were purchased from Invitrogen (Carlsbad, CA). Antibodies against ADH, ALDH, total/active (phosphorylated) extracellular signal regulated kinase 1/2 (ERK/pERK) and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody specific against CYP2E1 was purchased from Millipore (Temecula, CA). The IMPROM II[™] transcription system (used for first strand cDNA synthesis) and GoTaq green master mix (for PCR amplification) were purchased from Promega (Madison, WI). The EnzyChrom[™] ethanol detection assay was purchased from BioAssay Systems (Hayward, CA). The Lipid Peroxidation Assay kit was purchased from Calbiochem/EMD Biosciences (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture conditions and treatments

The rat H4IIE hepatoma cell line (ATCC, Bethesda, MD) was cultured in MEM α medium supplemented with FBS (10% v/v) to 75–80% confluence as previously reported [20]. At this point cells were made quiescent by replacing the culture medium with low serum MEM α (LSM; 0.1% (v/v) FBS) for 36 hours. For cells exposed to ethanol, an aliquot of culture medium was removed and 200-proof ethanol (diluted in PBS) was added such that the final culture medium concentration (0–75mmol/L) was achieved. In experiments employing silibinin a stock solution (10mM) was prepared by dissolving silibinin in dimethyl sulfoxide (DMSO) and cells were pre-treated (2 hours, 10 μ M) prior to ethanol

treatment (0–75mmol/L). Control experiments were performed in which vehicle alone (DMSO; 0.01% (v/v)) was added in place of silibinin.

2.3. Preparation of Cell Lysates and Immunoblotting

Following treatment, cells were washed with PBS (4°C) and lysates prepared using RIPA buffer (ADH, ALDH and CYP2E1 analysis) or MAPK lysis buffer [21]. In both instances lysates were aliquoted and stored at –80°C and protein levels equalized prior to analysis. Protein expression was determined by Western blot whereby cell lysates were resolved by 15% SDS-PAGE as previously performed [21]. Primary antibodies were diluted 1:1000 in 5% (w/v) nonfat dry milk dissolved in 0.05% (v/v) Tween-20/Tris-buffered saline (TBS). Signal intensity was determined using NIH-ImageJ and equal protein loading confirmed by stripping/re-probing membranes using a β -actin antibody.

2.4. mRNA analysis

Following H4IIE cell treatment total RNA was extracted using TRIZOL as per the manufacturer's instructions. To perform RT-PCR, 1 μ g of total RNA was reverse transcribed by Improm IITM (Promega) using random hexamers to generate complementary DNA (cDNA) (per manufacturer's protocol); cDNA was precipitated and resuspended in nuclease-free water to be used in PCR reactions. Briefly, 100ng cDNA was used in each reaction with the following gene-specific primers: CYP2E1 forward primer (fp) 5'CCTACATGGATGCTGTGGTG 3' and reverse primer (rp) 5'CTGGAAACTCATGGCTGTCA 3'; β -actin; fp: 5'GAGCTATGAGCTGCCTGACG 3', and rp: 5'GGATGTCAACGTCACACTTC 3'. PCR reactions consisted of 95°C for 2 minutes followed by 35 cycles of 95°C, 55°C and 72°C for 45 seconds each with a final elongation at 72°C for 2 minutes. Amplified products were resolved using a 1.5% (w/v) agarose gel and stained with ethidium bromide before conversion to digital images. The level of each PCR product was semi-quantitatively determined using Quantity One software.

2.5. Ethanol assay

Aliquots (100 μ l) of culture medium were sequentially removed (0–24 hours) and stored at –80°C prior to analysis. Ethanol concentration was determined using an EnzyChromTM ethanol assay according to manufacturer's protocol.

2.6. Cell proliferation

Cell proliferation was initially assessed by performing sequential cell counts over a 96 hour period as previously described [22]. Briefly, cells were made quiescent in LSM for 24 hours then pre-treated with silibinin (10 μ M) or vehicle (DMSO; 0.01% (v/v)) for 2 hours. At the end of this period the culture medium was removed and replaced with MEM α containing 1% (v/v) FBS alone or 1% (v/v) FBS, 10 μ M silibinin and/or 25mmol/L ethanol. Cells were then detached and counted at 24 hour intervals using a hemocytometer. Cell numbers averaged for 5 independent experiments. In studies lasting >24 hours, culture medium was removed and replaced at 24 hour intervals with fresh medium containing ethanol and/or silibinin. To confirm cell count data were due to proliferation *per se*, a parallel series of experiments were performed using a MTT reduction/proliferation assay. Briefly, cells were counted and plated at 10⁵ cells/well onto 96-well plates. Cells were then either pre-treated with silibinin (10 μ M) or vehicle (DMSO; 0.01% (v/v)) for 90 minutes followed by ethanol (25mmol/L), or treated with ethanol alone (25mmol/L). The MTT assay was then performed according to the manufacturer's instructions.

2.7. Analysis of lipid peroxidation and reactive oxygen species (ROS)/oxidative stress

Lipid peroxidation was determined using the Lipid Peroxidation Assay kit as per the manufacturer's instructions. Changes in cellular oxidative stress/ROS generation were determined using an Image-iT® LIVE Green Reactive Oxygen Species Assay in conjunction with Hoechst 33342 (for cell/nuclear localization) as per the manufacturer's instructions. Using this approach cells were either plated to single-well cover slips for microscopic analysis or into 96-well plates for quantitative analysis (at 485nm excitation-530nm emission). Untreated cells were assigned an arbitrary value of 1 and relative ROS production expressed as %carboxy-DCF fluorescence $\pm$ SEM.

2.8. Effect of silibinin on CYP2E1 activity

We next sought to assess whether the effects of silibinin on CYP2E1 responsiveness to ethanol treatment in HCC cells were due to direct or indirect mechanisms. To perform these studies human HepG2 cells (ATCC, Bethesda, MD) and HepG2 cells that had undergone mock transfection (C37-HepG2) or transfection to express constitutively active CYP2E1 (E47-HepG2) were used (N.B. The C37 and E47 cell lines were a kind gift from the laboratory of Dr A. Cederbaum, Mount Sinai School of Medicine, New York, NY). Cells were grown to 75–80% confluence and made quiescent in LSM prior to the addition of ethanol (25 mmol/L). 100 μ l aliquots of culture medium were sequentially removed (0–24 hours) and stored at -80°C prior to analysis for ethanol concentration using the EnzyChrom™ ethanol assay. To determine the contribution of CYP2E1 activity during these events, we next measured the rate of hydroxylation of *p*-nitrophenol to 4-nitrocatechol in H4IIE and HepG2-E47 cells in the presence or absence of silibinin following ethanol treatment [23]. Briefly, cells were grown to 70% confluence and treated with silibinin and/or ethanol as previously described. After 24 hours of treatment cells were washed with PBS (4°C), collected in KH_2PO_4 (pH 6.8, 4°C), sonicated, and stored at -80°C prior to analysis. Following total protein determination and correction (to 25mg/ml), reaction mixture (100 μ M *p*-nitrophenol and 1mM NADPH) was added to sample for a total reaction volume of 1.0 ml and incubated for 10 minutes at 37°C . The reaction was then stopped by the addition of 300 μ l 60% (*v/v*) 1N perchloric acid and samples centrifuged at $10,000 \times g$ for 5 minutes. The supernatant was then removed, 100 μ l of 10M NaOH added and the samples read on a spectrophotometer at 540nm. Activity was then calculated against a standard curve of known 4-nitrocatechol concentrations.

2.9. Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using a Student t-test or one-way ANOVA with Tukey's or Dunnett's multiple comparison post tests (as appropriate) between control and treatment groups. $p < 0.05$ was considered significant.

3. RESULTS

Effect of ethanol on ethanol metabolizing enzyme expression in the absence and presence of silibinin

Western blot analysis of ADH and ALDH expression in H4IIE cells identified no significant difference in expression of either enzyme following ethanol treatment (0–75mmol/L, 24 hours) in the absence or presence of silibinin (10 μ M) (Figure 1 a and b, $n=4$ separate experiments). In contrast exposure to ethanol caused a dose-dependent increase in CYP2E1 protein expression, a significant increase being detected at 10mmol/L increasing to a maximum at 25mmol/L (Figure 1a, $n=4$ separate experiments, $p < 0.05$ *versus* untreated). Of note, at higher doses of ethanol (50 and 75mmol/L), CYP2E1 expression was lower than that observed at 25mmol/L. To address the possibility of ethanol exerting a toxic effect at

higher doses, we performed a trypan blue exclusion assay. These studies demonstrated a significant decrease in cells excluding trypan blue at ethanol concentrations of 50mmol/L ($73.49 \pm 2.23\%$) and 75mmol/L ($66.05 \pm 2.89\%$) compared to cells treated with 25mmol/L ethanol (93.10 ± 2.86). Pretreatment of cells with silibinin (10 μ M, 2Hrs) followed by ethanol (10 or 25mmol/L), significantly inhibited ethanol-induced increases in CYP2E1 protein expression (Figure 1b, n=4 separate experiments, $p<0.05$).

In light of data demonstrating the effects of ethanol on CYP2E1 protein expression in the absence and presence of silibinin, we next sought to determine whether these changes were also manifest at the mRNA level. RT-PCR analysis using primers specific against rat CYP2E1 mRNA demonstrated significantly increased CYP2E1 mRNA expression following treatment of H4IIE cells with ethanol (25mmol/L) as compared to untreated cells (Figure 1c, n=4 separate experiments, $p<0.05$). Furthermore, pretreatment of H4IIE cells with silibinin (10 μ M, 2Hrs) abrogated ethanol-dependent increases in CYP2E1 mRNA expression (Figure 1c, n=3 separate experiments).

Having demonstrated basal CYP2E1 expression in the H4IIE cell line *in vitro*, we next sought to identify levels of CYP2E1 protein expression in HCC tumors formed following parenchymal injection of this cell line *in vivo* [22]. These data demonstrated that H4IIE-HCC tumors formed *in vivo* continue to express CYP2E1 protein (Supplementary Data).

To determine the functional significance of the effects of silibinin on CYP2E1 expression and ethanol metabolism, we analyzed culture medium ethanol content in the absence or presence of silibinin pretreatment (10 μ M, 2Hrs). These data demonstrate significant ethanol metabolism in H4IIE cells over a 24 Hr time period (Figure 2a, n=4 separate experiments, samples analyzed in triplicate, $p<0.05$ *versus* ethanol in medium alone [no cells]). Pretreatment of cells with silibinin significantly inhibited the rate of ethanol metabolism in H4IIE cells such that at the end of the 24 Hr period $28.1 \pm 1.7\%$ percent of the initial ethanol added remained in silibinin pre-treated cells versus $16.4 \pm 1.1\%$ in cells treated with ethanol alone (Figure 2b, n=4 separate experiments, samples analyzed in triplicate, $p<0.01$).

H4IIE cell proliferation following ethanol exposure in the absence or presence of silibinin

H4IIE cell proliferation was determined for cells cultured in 1% FBS (v/v) culture medium in the absence and presence of 25mmol/L ethanol with or without silibinin pretreatment (10 μ M, 2Hrs) over a 96 Hr period. No significant difference in cell number was detected in any of the experimental groups (1% FBS (v/v) versus 1% FBS + ethanol (25mmol/L), DMSO (0.01% (v/v)), silibinin (10 μ M) or silibinin (10 μ M) + ethanol (25mmol/L)) 24 Hrs after initiation of the experimental procedures (data not shown). However, by 48 Hrs all of the treatment groups demonstrated significantly increased cell number as compared to initial cell counts at time 0. By 96 hours, the number of cells counted in the 1% (v/v) FBS + ethanol group (4.79 ± 0.26 fold change versus day 0, Figure 3a) was significantly greater than cells treated with culture medium containing 1% (v/v) FBS alone (4.05 ± 0.31 fold change versus day 0, n=5 independent experiments, $p<0.05$ Figure 3a). No significant difference in cell numbers were measured between culture medium containing 1% (v/v) FBS, and that containing 1% (v/v) FBS and silibinin (10 μ M) or DMSO (0.01% (v/v)), Figure 3a). However, pretreatment of cells with silibinin significantly inhibited the effect of ethanol on FBS mediated cell proliferation to a level not significantly different to that of either control (1% FBS alone) or silibinin or vehicle treated cells (Figure 3a, n=5 independent experiments, $p<0.05$ ethanol treated cells versus silibinin + ethanol treated cells).

To confirm that the effects of ethanol on cell proliferation as assayed by cell counting were due to proliferation *per se*, we next performed an MTT proliferation assay. These data demonstrated that neither silibinin (10 μ M) nor vehicle (DMSO; 0.01% (v/v)) significantly

altered proliferation as compared to untreated cells (Figure 3b, n=4 separate experiments assayed in duplicate). In contrast, treatment with ethanol (25mmol/L) significantly increased proliferation as compared to untreated cells (3.03 ± 0.35 fold (ethanol treated) versus 1.28 ± 0.22 fold (control), n=4 separate experiments assayed in duplicate, $p < 0.05$, Figure 3b). Pretreatment of H4IIE cells with silibinin (10 μ M) significantly blunted the effect of ethanol alone on cell proliferation (2.04 ± 0.24 fold (silibinin + ethanol) versus 3.03 ± 0.35 fold (ethanol alone), n=4 separate experiments assayed in duplicate, $p < 0.05$, Figure 3b).

Effect of ethanol and silibinin on ERK 1/2 activity in H4IIE cells

The ERK-MAPK signaling pathway has been identified as a central pathway in mediating cell proliferation in H4IIE (and other) cells [24, 25]. Western blot analysis of H4IIE cells treated with ethanol demonstrated active ERK1/2 (pERK 1/2) increased significantly 10 minutes after addition (Figure 4 a and b, 4.46 ± 0.56 fold increase as compared to untreated cells, n=5 separate experiments, $p < 0.05$). Pretreatment of cells with silibinin (10 μ M) failed to significantly alter the magnitude or time course of ERK1/2 activity following treatment with ethanol as compared to ethanol alone (Figure 4 a and b, n=5 separate experiments).

Effect of ethanol and silibinin on lipid peroxidation and reactive oxygen species in H4IIE cells

Malondialdehyde (MDA) levels were significantly higher in cells exposed to 10 or 25mmol/L ethanol *versus* untreated cells (Figure 5a, n= 4 separate experiments, $p < 0.05$).

Pretreatment of cells with silibinin followed by ethanol (10 or 25mmol/L) significantly inhibited increases in MDA levels measured in cells treated with ethanol alone to levels not significantly different to untreated cells (Figure 5a, n=4 separate experiments, $p < 0.05$ ethanol treated *versus* silibinin + ethanol). Silibinin alone did not significantly alter basal MDA levels in untreated cells (Figure 5a).

To further evaluate the effect of silibinin on ethanol-dependent increases in oxidative stress, we next used the carboxy- H_2 DCFDA assay as a marker of intracellular peroxide levels. Using a microscopic analysis approach, relatively low fluorescence/ROS was detected in untreated cells and those treated with silibinin alone (Figure 5b). Conversely, a dramatic increase in fluorescence/ROS was observed following ethanol treatment, an effect that was abrogated by pretreatment with silibinin (Figure 5b). To perform quantitative analysis of these data, parallel experiments were performed using cells seeded to 96-well plates. These data confirmed that ethanol (25mmol/L) treatment led to a 3.55 ± 0.68 fold increase in fluorescence/ROS production compared to untreated cells (Figure 5c, n=4 separate experiments, $p < 0.05$). Furthermore, pretreatment of cells with silibinin abolished the effects of ethanol on fluorescence/ROS production to levels not significantly different to untreated cells or, cells treated with silibinin alone (Figure 5c, n=4 separate experiments, $p < 0.05$ silibinin+ethanol *versus* ethanol only). Treatment of cells with vehicle (DMSO, 0.01% (v/v)) did not significantly alter fluorescence/ROS production compared to untreated cells (data not shown).

Direct effect of silibinin on CYP2E1 activity and ethanol metabolism

To determine whether silibinin affects CYP2E1 induction, as compared to activity, we next employed the human HepG2 cell line in conjunction with HepG2 cells transfected to express CYP2E1 (E47-HepG2), an empty vector control (C37-HepG2) or the previously described H4IIE rat HCC cell line. These data demonstrated significant ethanol metabolism by the HepG2 and C37-HepG2 cell lines over a 24 Hr period, an effect that was not significantly different in the presence of silibinin (10 μ M) (Figure 6a and b). Conversely, while the rate of ethanol metabolism was significantly accelerated in E47-HepG2 (CYP2E1 expressing) cells, this effect was abrogated by pretreatment with silibinin to a level not significantly different

to untransfected/C37-HepG2 cells (Figure 6 c, $p < 0.05$ ethanol metabolism in E47-HepG2 *versus* HepG2 and C37-HepG2 cells).

To confirm the specific role of CYP2E1 during ethanol metabolism, we next determined CYP2E1 activity in H4IIE and E47-HepG2 cells by analyzing *p*-nitrophenol hydroxylation (to 4-nitrocatechol) in untreated cells, and cells treated with ethanol (25mmol/L) in the absence or presence of silibinin (10 μ M). These data demonstrated ethanol alone significantly increases CYP2E1 activity in H4IIE and E47-HepG2 cells compared to untreated H4IIE cells (Figure 6d, $n=4$ separate experiments, $p < 0.05$). Pre-treatment of cells with silibinin (10 μ M) significantly inhibited the effect of ethanol on CYP2E1 activity in both H4IIE and E47-HepG2 cells (Figure 6d, $n=4$ separate experiments, $p < 0.05$ ethanol alone *versus* silibinin + ethanol).

4. DISCUSSION

Ethanol has been identified as a causative agent in the development of several cancers including HCC [2; 3]. The effects of ethanol on liver function and hepatic disease initiation and progression can be both direct and indirect in nature [2; 7; 26]. However, considerable evidence suggests many of the intrahepatic effects of ethanol are due to metabolism in the hepatocyte and the associated increases in acetaldehyde production and oxidative stress [2; 7]. The identification of oxidative stress as a major factor in the progression of liver disease towards cirrhosis and HCC has led to intense interest in the therapeutic use of antioxidants [6; 13; 15; 18].

The current study utilizes the rat H4IIE HCC cell line. Previous studies by our group show that this cell line expresses ADH and ALDH, is capable of metabolizing ethanol, and that this metabolism can be significantly inhibited using 4-methylpyrazole [21]. In the current study we demonstrate that exposure to ethanol at doses similar to blood ethanol content observed in the setting of moderate ethanol consumption (10–25mmol/L), leads to a significant increase in CYP2E1 expression in the absence of changes in ADH or ALDH expression *in vitro*. Furthermore, CYP2E1 induction and the rate of ethanol metabolism are significantly inhibited by the pretreatment of cells with silibinin (10 μ M). These data correspond to the inhibition of ethanol-dependent increases in cell proliferation in the H4IIE cell line *in vitro*. This evidence would suggest that, even in the setting of moderate ethanol consumption, HCC cells that express CYP2E1 may be subject to increased CYP2E1 induction and that these changes may significantly affect the rate of tumor progression. Indeed, this may be of particular importance in considering the “two hit” model of hepatocarcinogenesis in which transformation and progression may be regulated by independent pathophysiological stimuli. However, it is important to highlight that the effects observed *in vitro* may not be similarly manifest in the *in vivo* setting in which the non-tumorigenic liver mass will, more than likely, play a more significant role in ethanol metabolism. Similarly, the concentrations and time of exposure to ethanol that the tumor mass is likely to experience *in vivo* will differ to that of a static *in vitro* culture employing purified HCC cell populations.

Previous studies demonstrate that silibinin inhibits human HCC cell proliferation *in vitro* [27; 28] and does so via inhibition of ERK-MAPK signaling in the (human) HepG2 cell line [27]. In contrast, we failed to detect a significant effect of silibinin on cell proliferation or ERK-MAPK activity profiles in H4IIE cells treated with silibinin alone. However, studies by Momeny et al [27] and Lah et al [28] demonstrated significant inhibitory effects of silibinin at doses of 50–75 μ M and 120–240 μ M respectively, whereas our studies employed silibinin at 10 μ M final culture medium concentration. Indeed, both of these studies failed to observe significant effects of silibinin on proliferation and/or ERK-MAPK

activity at this dose [27; 28]. While our data raise the possibility that the dose of silibinin used in the current studies was too low to affect ERK-MAPK signaling and/or proliferation, we were cognizant of not using higher doses in vitro due to data regarding the bioavailability of silibinin following oral ingestion in animal models and humans in vivo [29; 30]. In addition to the potential effects of silibinin on an ERK-MAPK signaling cascade other signaling mechanisms have been identified whereby silibinin may act to inhibit net cell accumulation. Using the human HepG2 and Hep3B cell line Varghese *et al* report silybin treatment inhibits the expression of a range of cyclins and cyclin-dependent kinases and, in the case of the Hep3B line, inhibits regulators of proteins involved in transition between the G2-M phases [31; 32]. Conversely, silibinin has also been reported to exert pro-apoptotic effects (albeit at higher doses than those that inhibit ERK-MAPK signaling) in other cancer cells [18; 33] an effect that, in combination with inhibition of cell cycle progression, would result in decreased net cell accumulation [18].

As with many complimentary/alternative medicines the efficacy and mechanisms of action whereby the active constituents of milk thistle exert their effects remain controversial. Several recent reviews suggest that the clinical benefits of milk thistle for a range of hepatic (and other) disease states is difficult to assess due to a lack of high-quality, adequately conducted/reported clinical trials [19; 34]. Similarly, increasing evidence indicates that, much like ethanol consumption, the systemic effects of silibinin following ingestion may play as important a role in influencing the hepatoprotective effects as the direct actions in the liver [32; 34; 35]. The antioxidant/free radical scavenging capacity of milk thistle derivatives, including silibinin, appears to be directly related to the flavinoligand chemical structure(s) [18]. Currently no evidence has been presented to indicate silibinin acts to alter the rate of GSH synthesis although, the presence of silibinin has been demonstrated to reduce GSH depletion in isolated hepatocytes exposed to allyl alcohol [36]. Indeed, these data are supported by our findings that pre-treatment of cells with silibinin effectively decreases ROS/oxidative stress in H4IIE cells associated with ethanol metabolism. Specifically, data from our studies suggests that the ability to reduce oxidative stress is due to the ability to inhibit CYP2E1 induction/activity rather than affecting ADH-dependent ethanol metabolism. These data are consistent with ethanol metabolism studies that report the majority of hepatic oxidative stress associated with chronic ethanol consumption is due to CYP2E1 induction and the generation of ROS, including hydroxyethyl radicals [6; 10]. However, while our studies using E47-HepG2 cells (transfected to express constitutively active CYP2E1) raise the possibility that silibinin may interact directly with the CYP2E1 enzyme to inhibit activity, these data contradict previous studies by Míguez *et al* that failed to provide evidence for the involvement of CYP2E1 as an underlying mechanism for the effects of silymarin [36].

The effects of ethanol on hepatocyte integrity, and liver function as a whole, can be both direct and indirect in nature [2; 7; 9]. Similarly, oral ingestion of silibinin can exert indirect and direct effects, the net result of which are hepatoprotective. For example, chronic ethanol consumption is documented to stimulate a hepatic immune response due, at least in part, to changes in the balance of the bacterial flora of the GI-tract and increased permeability of the GI tract to lipopolysaccharide resulting in Kupffer cell activation [37]. In this regard silibinin has been demonstrated to inhibit the transcription and DNA binding activity of NF- κ B [38; 39], a key factor in regulating and coordinating the hepatic inflammatory response [40]. In addition to modulating the immune response silibinin, and other flavinoligands, have been reported to modulate steroid hormone receptor-dependent gene expression [41; 42]. This may be of particular significance in alcoholic liver disease given the relationship between the liver and the regulation of sex hormone levels/activity and the effects of chronic ethanol consumption on hepatic sex hormone regulation and signaling [43; 44]. Similarly, the progression of hepatic foci to HCC and subsequent tumor expansion is dependent on

neoangiogenesis and, silibinin is reported to inhibit angiogenesis due to inhibition of vascular endothelial growth factor VEGF-dependent signaling [45].

In conclusion, data presented in our study demonstrate silibinin inhibits CYP2E1 induction/ethanol metabolism and cell proliferation in a rat HCC cell line in vitro and does so at doses lower than those described in other (human) HCC cell lines. Our data suggest that the underlying mechanisms by which silibinin exerts these effects are due to decreased CYP2E1-dependent ROS generation. While these data provide an insight into possible mechanisms whereby silibinin may slow the rate of HCC progression the wide spread systemic effects of both ethanol and silibinin are such that further studies are required to determine whether these mechanisms are equally important in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Ethanol stimulates cytochrome P4502E1 expression in H4IIE cells, an effect inhibited by silibinin pretreatment

a) Representative immunoblots demonstrating the effect of increasing doses of ethanol (EtOH) on the expression of cytochrome P4502E1 (CYP2E1), alcohol dehydrogenase (ADH), and acetaldehyde dehydrogenase (ALDH) in the H4IIE HCC cell line 24 hours after EtOH addition (0–75mmol/L). Equal protein loading was confirmed using an antibody specific against β -actin. The effect of EtOH on CYP2E1 expression was then analyzed by optical integrated volume for repeat experiments and data expressed as fold change *versus* untreated (0mmol/L EtOH). $n=4$ separate experiments, $*p<0.05$ *versus* untreated cells. **b)** Representative immunoblots demonstrating the effect of EtOH (10 and 25mmol/L) on the expression of CYP2E1, ADH, ALDH in the H4IIE HCC cell line in the presence and absence of silibinin (10 μ M). Equal protein loading was confirmed using an antibody specific against β -actin. The effect of EtOH in the absence and presence of silibinin (10 μ M) on CYP2E1 expression was then analyzed by optical integrated volume for repeat experiments and data expressed as fold change *versus* untreated (0mmol/L EtOH). $n=4$ separate experiments, $*p<0.05$ *versus* untreated cells, $\#p<0.05$ EtOH + Sil *versus* EtOH alone. **c)** Representative RT-PCR analysis demonstrating the effect of EtOH (10 and 25mmol/L) on the expression of CYP2E1 mRNA in the presence and absence of silibinin (10 μ M). CYP2E1 mRNA expression was then analyzed by optical integrated volume for repeat experiments and data expressed as fold change *versus* untreated (0mmol/L EtOH). $n=3$ separate experiments, $*p<0.05$ *versus* untreated cells, $\#p<0.05$ EtOH + Sil *versus* EtOH alone

FIGURE 2. H4IIE cells metabolize ethanol, an effect inhibited by silibinin pretreatment
a) Ethanol (EtOH) content in cell culture medium was determined in the absence (○—○) or presence (△—△) of silibinin (Sil; 10μM) over a 24 Hr period following the initial addition of 25mmol EtOH to H4IIE cell cultures. n=4 separate experiments analyzed in duplicate, *p<0.05 EtOH + Sil *versus* EtOH alone. To ensure changes in culture medium EtOH concentration were not due to evaporation, parallel experiments were performed in which EtOH containing (25mmol) culture medium was placed in cell culture flasks in the absence of H4IIE HCC cells (C; □—□). **b)** The percentage of EtOH remaining in culture medium following the addition of 25mmol/L EtOH to H4IIE cells in the absence or presence of silibinin. n=4 separate experiments, *p<0.05.

FIGURE 3. Silibinin inhibits ethanol-stimulated cell proliferation in H4IIE HCC cells

a) H4IIE HCC cells were cultured in medium containing 1% FBS in the presence or absence of silibinin (Sil; 10µM) for 96 Hrs. Following cell counts the number of cells was calculated as a fold change *versus* the number of cells at day 0. n=5 separate experiments, *p<0.05 *versus* control (C; 1% FBS alone), #p<0.05 EtOH (25mmol/L) *versus* EtOH (25mmol/L) + Sil (10µM). To ensure that DMSO (vehicle for dissolving Sil) did not affect proliferation parallel experiments were performed in which cells were treated with 1% FBS + 0.01% (v/v) DMSO (V). **b)** H4IIE HCC cells were cultured in medium in the presence or absence of silibinin pretreatment (Sil; 10µM, 2 Hrs) prior to exposure to ethanol (EtOH, 0, 10 or 25 mmol/L) and an MTT proliferation assay performed. Data are expressed as fold change *versus* control (untreated; C). n=4 separate experiments analyzed in duplicate, *p<0.05 *versus* control (C), #p<0.05 EtOH (25mmol/L) + Sil (10µM) *versus* EtOH (25mmol/L) only. To ensure that DMSO (vehicle for dissolving Sil) did not affect proliferation parallel experiments were performed in which cells were treated with 0.01% (v/v) DMSO (V).

FIGURE 4. Silibinin does not affect ethanol-dependent ERK1/2 activation in H4IIE HCC cells
a) Representative immunoblots demonstrating the effect of ethanol (EtOH; 25mmol/L) on the activation (phosphorylation) of ERK1/2 in the absence (upper panel) or presence (lower panel) of silibinin (10µM). **b)** Following analysis of pERK1/2 expression, membranes were stripped and probed for total ERK1 expression. The ratio of pERK1/2:ERK1 expression was then analyzed by optical integrated volume for repeat experiments and data expressed as fold change *versus* untreated. n=5 separate experiments, *p<0.05 *versus* untreated cells.

FIGURE 5. Silibinin inhibits ethanol-dependent oxidative stress in H4IIE HCC cells

a) Malondialdehyde (MDA) levels were determined in H4IIE HCC cells following treatment with ethanol (EtOH, 10 and 25 mmol/L) in the absence (–) and presence (+) of silibinin (Sil; 10 μM). $n=4$ separate experiments, $*p<0.05$ EtOH versus no EtOH, $\#p<0.05$ EtOH + Sil versus EtOH - Sil. **b)** Representative immunofluorescent cytochemistry images of H4IIE HCC cells treated with EtOH (25 mmol/L) in the absence or presence of silibinin (Sil; 10 μM) analyzed for ROS/oxide generation using a carboxy- H_2 DCFDA assay. ROS/oxide was detected as green fluorescence. Cells were counterstained with Hoescht 33342 (blue) for nuclear localization. **c)** Quantitative analysis of the carboxy- H_2 DCFDA assay was performed in parallel studies using a 96-well plate assay as described in the Methods. $n=4$ separate experiments analyzed in duplicate, $*p<0.05$ versus control (C; untreated), $\#p<0.05$ EtOH treated (25 mmol/L) versus EtOH (25 mmol/L) + Sil (10 μM) treated.

FIGURE 6. Silibinin inhibits ethanol metabolism in human HepG2 HCC cells transfected to express CYP2E1 and CYP2E1 activity in HCC cells

a) Human HepG2 HCC cells were treated with ethanol (EtOH; 25mmol/L) in the absence (▲—▲) or presence (■—■) of silibinin (Sil; 10μM) for 24 Hrs and EtOH content in the culture medium determined. **b)** Human HepG2 HCC cells transfected with empty vector (C37-HepG2 cells) were treated with EtOH (25mmol/L) in the absence (▲—▲) or presence (■—■) of Sil (10μM) for 24 Hrs and EtOH content in the culture medium determined. **c)** Human HepG2 HCC transfected to express CYP2E1 (E47-HepG2 cells) were treated with ethanol (EtOH; 25mmol/L) in the absence (▲—▲) or presence (■—■) of silibinin (Sil; 10μM) for 24 Hrs and EtOH content in the culture medium determined. n=4 separate experiments analyzed in duplicate, *p<0.05 *versus* time 0, #p<0.05 cells treated with Sil (10μM) and EtOH *versus* EtOH alone. **d)** Rat H4IIE and human E47-HepG2 cells were cultured in the presence or absence of ethanol (EtOH; 25mmol/L) and/or silibinin pretreatment (Sil; 10μM) and CYP2E1 activity determined by measurement of *p*-nitrophenol hydroxylation to 4-nitrocatechol. n=4 separate experiments, *p<0.05 *versus* untreated H4IIE cells, #p<0.05 cells treated with Sil (10μM) and EtOH *versus* EtOH alone.