DISCUSSION

Silymarin has been used for a long time as an alternative medication to treat acute and chronic liver diseases [20, 22, 28, 41, 43, 44]. Even today, it is one of the most widely used herbal medications [41]. Many studies suggest that Silymarin has a broad spectrum of hepatoprotective effects [41a]. But despite its broad use, the exact molecular mechanism by which Silymarin confers hepatoprotection is not known. Recent findings indicate that a standardized preparation of Silymarin showed anti-inflammatory actions via inhibition of NF-κB induced transcription in human liver cell cultures, inhibition of inflammatory cytokine induction in human peripheral blood mononuclear cells, and direct antiviral effects against HCV infection [29]. The data presented in this work also show that Silymarin possesses antiviral effects on HCV core expression and stimulatory effects on the antioxidant enzyme HO-1.

The standard treatment for patients with CHC is pegylated IFN-α plus ribavirin, although the rate of sustained viral response or cure is far from ideal, and there is substantial toxicity associated with these treatments. All of these factors suggest that it is important to study the effects of alternative medications such as Silymarin, for the treatment of liver diseases, especially chronic hepatitis C infections.

With recent advances including the HCV replicon system, renewed effort has been directed towards developing drugs that inhibit viral replication. Previous HCV molecular studies had been hampered due to a lack of an efficient cell culture system which could produce an infectious virion. A new system, based on a unique full length HCV genome, has been developed, and this new system is a potentially powerful tool which could mimic the HCV's *in vivo* behavior (replication pathogenesis and persistence). This full length replicon system (JFH1) may give us insight into the *in vivo* behavior of the virus. However if the goal is to analyze a specific response of a given HCV protein it may be preferable to do the experiment in replicons expressing only the protein of interest. In this study two different cell lines containing replicons from HCV were analyzed, CNS3 cells which contain the structural proteins and part on the

nonstructural proteins of the HCV, and 9-13 cells which express only nonstructural HCV proteins (NS3-NS5).

In this study, Silymarin at concentrations of 100 and 200 μM was not toxic to the CNS3, 9-13 or Huh7 cell lines. Although 300 μM was not evidently toxic to the cells, it seemed to be an inhibitor of cell growth, possibly by the same mechanism as has been shown in previous studies on the inhibition of cell growth by Silymarin [41b, 42].
Although it was shown that a standardized preparation of Silymarin was effective at lower doses (20.7 and 41.9 μmoles/L) [29] than the doses we used, it is important to note that different molecular forms of Silymarin, different replicons and different cell lines will likely have different effects on cellular and HCV mediated responses.

Polyak *et al* [29], recently showed that a standardized Silymarin preparation has a direct antiviral effect against HCV. This is the first paper to show that Silymarin has antiviral and anti-infective effects against HCV, and it will be used as a basis for comparison with the results described in this thesis.

The antiviral effects of Silymarin are shown by the decrease of HCV core mRNA and protein in CNS3 cells. Silymarin concentrations used to treat the cells all decreased HCV core levels without any obvious toxic or inhibitory effects, with exception of 300 μM Silymarin which was not used subsequently for this reason. The replication rate for CNS3 cells is lower than the rates of the other cell lines used (9-13 and Huh7 cells), and this is one reason why these cells were treated for a longer time. It was very interesting to observe that interferon-α , which is considered a strong positive control to reduce viral load, did not decrease either HCV core mRNA or protein as expected. Instead, the levels of HCV core were very similar to the levels in cells treated with Silymarin. Unexpectedly, the lower IFN concentration (10 U/mL) was the more effective at decreasing the HCV core expression than the higher concentration (50 U/mL).
We used 50 U/mL IFN to treat CNS3 cells and next measured HCV core protein as described in Methods. Western blots showed that similar to the results obtained for HCV core mRNA, IFN did not down-regulate HCV core protein, even in combination with

Silymarin. This lack of IFN antiviral effect may be due to the presence of HCV structural proteins (core-E1-E2) which impairs IFN-α -mediated antiviral activity, also HCV core protein has significant effects at the cellular level [43, 44, 45, 46, 47]. Reported evidence shows that HCV core protein impairs IFN-α-induced signal transduction via induction of SOCS3 expression and inhibits nuclear translocation of STAT1 [43]; that the expression of viral proteins, in particular of the HCV core protein, results in IRF-1 repression [44]; that HCV core protein inhibits IFN-α–induced transcription of antiviral genes by decreasing binding of ISGF3 to the ISRE [45]; that expression of the HCV core protein alone or with other HCV proteins modulate transacting factors of Jak/Stat signaling pathway [46] ; and that HCV core protein is sufficient for immunosuppression, prolonged viremia, and increased mortality in mice models [47]. Thus, the evidence presented may be the reason for IFN inefficiency on HCV expression.

It has been proposed that IFN-α binds to cellular receptors which dimerize and cause the activation of Janus-activated and tyrosine kinases (JAK) which subsequently phosphorylate the cytoplasmic signal transducers and activators of transcription (STAT) protein. This JAK/STAT pathway activates the transcription of multiple interferon-stimulated genes (ISG) which encode proteins that interfere with virus replication, protein synthesis and assembly [48]. It is possible that Silymarin could activate the JAK-STAT pathway in a manner analogous to that of IFN.

The study by Polyak *et al* used a different HCV replicon system (JFH1), a different cell line (Huh7.5) and different concentrations of Silymarin (20.7 μmoles/L) and IFN (100 U/ml). However they also showed that HCV core protein is down-regulated to a similar extent using their standardized Silymarin compared with IFN for 48 h. It was shown that the standardized Silymarin increased the phosphorylation levels of Stat1 Y701 and Stat1 S727, but not the total Stat1 or Stat2 [29]. To gain insight into whether Silymarin-mediated down-regulation of HCV core in CNS3 cells could be related with the JAK-STAT pathway, we also measured Stat1 and Stat1 phosphorylation on tyrosine (Y701) protein levels in CNS3 and 9-13 cells, but no changes were observed either in total Stat1 or Stat1 (Y701) protein levels were detected either in CNS3 or in 9-13 cells. This suggests that Silymarin down-regulation of HCV core is not related with the Jak-Stat

signaling pathway. Thus, more investigation must be done on the possible mechanism of Silymarin down-regulation of HCV core in CNS3 cells.

Polyak *et al*. reported that HCV NS5A protein expression is down-regulated by the standardized Silymarin in Huh7.5.1 transfected with JFH1. In contrast with their results, we did not observe any down-regulation on HCV NS5A by Silymarin in treated 9-13 cells, either in the mRNA levels or the protein levels. These differences may be due to; 1) The different replicons used in each study. We used cells expressing only the nonstructural HCV proteins (9-13), while they used a full length HCV genome (JFH1). Different replicons can give rise to different responses and the presence of one protein can modulate the activity of the other proteins. Moreover, they used Huh7.5.1 as the cells containing the replicon while we used Huh7 cells; 2) The JFH1 system is based on an HCV 2a genotype while the 9-13 replicon is based on the HCV 1b genotype. Different genotype of HCV show differences of 20-30% in their sequences, they differ in their response to treatment and the symptoms they produce; 3) The different Silymarin formulations used. They treated the cells with a standardized Silymarin which they synthesized, whereas we used a commercial Silymarin preparation which may contain different chemical properties that may lead to different cellular responses.

The lack of any antiviral effects of Silymarin on HCV NS5A mRNA or protein levels may be due to the ability of NS5A to produce a resistance effect on the cells, thereby inactivating the possible mechanism by which Silymarin produces an antiviral effect in CNS3 cells.

HCV NS5A can serve as an inhibitor of IFN-α-induced antiviral activity, and thus play a role in development of resistance to IFN treatment in patients with chronic hepatitis C [49, 50]. In contrast other studies show that cell clones carrying replicons with HCV NS5A sequences from IFN responder and nonresponder patients, did not reveal significant differences on HCV RNA replication to IFN- α treatment, so this data provide no evidence that NS5A protein contributes to resistance of HCV replication to IFN- α [51]. Results presented here shows that in contrast to CNS3 cells, IFN treatment to 9-13 cells showed a significant down-regulation of the HCV NS5A mRNA and protein. Also

in contrast with the HCV core down-regulation from CNS3 cells, Silymarin treatment did not decrease HCV NS5A expression. The reasons why Silymarin is down-regulating HCV core but not HCV NS5A in these cell lines is a subject which needs further investigation.

In the three cell lines used in these studies (CNS3, 9-13 and wild type Huh-7 cells), Silymarin up-regulated significantly HO-1 gene expression. The maximum up-regulation depends on the cell line, the time of exposure, and the Silymarin concentrations used. In CNS3 and 9-13 cells, HO-1 gene expression was not up-regulated by IFN treatment, and the levels of HO-1 were the same or lower than the controls. However, when cells were treated with the combination of Silymarin and interferon, a significant increase in HO-1 mRNA was observed with both cell lines. It is possible that this increase is more due to Silymarin treatment than to the interferon. In contrast to the results seen with CNS3 and 9-13 cells, interferon treatment of Huh-7 cells up-regulated HO-1 mRNA, as did the mixed treatment of Silymarin and IFN. These findings raise the question as to whether HCV expression in human hepatoma cells represses the interferon-mediated induction of cellular stress response elements. In other words, why does IFN treatment not have the same effect on HO-1 gene expression in cells expressing HCV replicons as it does in wild-type Huh-7 cells? The answers to these questions could be the subject of additional studies.

Many reports have demonstrated that the heme derived metabolites generated by HO-1 catalysis have potent antioxidant activity. It is known that HO-1 is a highly inducible enzyme which is transcriptionally regulated by a large number of chemical and physical factors.

The results presented in this thesis showed no clear relation between these transcription factors and the Silymarin-mediated up-regulation of HO-1. Two transcription factors, Bach1 and Nrf2, in particular, have been shown to be important to the heme mediated induction of HO-1 expression [30, 31, 52, 53]. Therefore, other mechanism would have to be investigated to understand how Silymarin induces the expression of the antioxidant enzyme HO-1.

Quercetin, a compound that is structurally similar to Silymarin, is a common polyphenolic compound found in plants. Quercetin induces HO-1 in a time and dose-dependent way, and it acts at both transcriptional and translational levels. This induction occurs via up-regulation of the p38 mitogen-activated protein kinase (p38MAPK) [54]. This could be a possible mechanism by which Silymarin induces HO-1 gene expression, but further analysis would have to be done to evaluate this issue.

Oxidative stress plays an important role in various diseases, including viral infection and chronic inflammation. HCV gene expression in particular can increase the levels of reactive oxygen species (ROS) [55, 31]. Thus, it is important to analyze the effects that Silymarin has on the induction of HO-1, a well known antioxidant enzyme.