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MECHANISM FOR THE PROTECTIVE EFFECTS OF SILYMARIN AGAINST CARBON TETRACHLORIDE-INDUCED LIPID PEROXIDATION AND HEPATOTOXICITY IN MICE

EVIDENCE THAT SILYMARIN ACTS BOTH AS AN INHIBITOR OF METABOLIC ACTIVATION AND AS A CHAIN-BREAKING ANTIOXIDANT

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Abstract—Administration of silymarin (800 mg/kg i.p.) 30 min before carbon tetrachloride (18 μL/kg i.p.) did not modify total hepatic levels of CCl_4 and metabolites in mice, but decreased by 40% the *in vivo* covalent binding of CCl_4 metabolites to hepatic lipids at 2 hr. This pretreatment decreased by 60% the exhalation of ethane during the first hour after CCl_4, and decreased by 50% the incidence of liver cell necrosis. *In vitro*, silymarin (800 μg/mL) decreased by 50 to 70% various monooxygenase activities, and decreased by 20% the covalent binding of CCl_4 metabolites to microsomal proteins. Silymarin (800 μg/mL) decreased by 70% *in vitro* lipid peroxidation mediated by CCl_4 metabolites, and decreased by 90% peroxidation mediated by NADPH alone. Silibinin, one of the three isomers composing silymarin, also decreased carbon tetrachloride-induced lipid peroxidation; this effect, however, was less than that of silymarin *in vitro*, and was more transient *in vivo*. Pretreatment with silibinin (800 mg/kg i.p.) 30 min before CCl_4 (18 μL/kg i.p.) did not improve SGPT activity or liver histology at 24 hr. We conclude that silymarin prevents carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice, firstly, by decreasing the metabolic activation of CCl_4, and, secondly, by acting as a chain-breaking antioxidant.

Silymarin is the collective name for an extract from the milk thistle *Silybum marianum* (L.) Gartneri. Silymarin is composed mainly of three isomers, namely sibilinin (also called silybinin, silybin or silibin), silidianin (silydianin) and silichristin (silychristin). *In vivo*, silymarin has been shown to protect experimenal animals against various hepatotoxins, including allylalcohol [1], carbon tetrachloride [1–4], galactosamine [1], phalloidin [5] and thioacetamide [6]. *In vitro*, silymarin has been shown to prevent lipid peroxidation induced by Fe^{2+} [7, 8], *t*-butyl hydroperoxide [8], and *N*-ethylmaleimide [9].

Silymarin has been used in Europe for over two decades in the treatment of alcoholic liver disease. Although a randomized clinical trial failed to demonstrate beneficial effects in alcoholic hepatitis after 3 months of treatment [10], several other trials have concluded to beneficial effects [11–13]. In patients with mild alcoholic liver disease receiving silymarin, liver function tests improved more rapidly than in those receiving a placebo [11, 12]. A recent randomized controlled multicenter trial showed that silymarin administration, 420 mg daily for several years, significantly reduced mortality in patients with alcoholic liver cirrhosis [13]. Another clinical indication of such drugs may be the prevention of hepatitis due to the ingestion of the toxic mushroom *Amanita phalloides* [5]. Administration of silibinin dihemisuccinate within the following hours has been reported to prevent hepatocellular necrosis in such subjects [5].

Increasing evidence for a possible efficacy of silymarin in humans has renewed interest in delineating the mechanisms of action of this plant preparation. The protective effects against the mushroom toxins, phalloidin and α-amanitin [14] have been ascribed to reduced hepatocytic uptake of phalloidin [15], and to protection against the inhibitory effects of α-amanitin on RNA-polymerase [16]. The mechanism, however, for the protective effect of silymarin against the hepatotoxicity of carbon tetrachloride remains unknown. A short letter has reported decreased amounts of diene conjugates in rats pretreated by silymarin before the administration of carbon tetrachloride [17]. The mechanism for the latter effect has not been studied further.

The hepatotoxic effect of carbon tetrachloride is thought to result from its reductive dehalogenation by cytochrome P-450 into the trichloromethyl free radical [18]. This radical quickly adds molecular oxygen to form the trichloromethyl peroxyl radical.

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Abstraction of hydrogen atoms from unsaturated lipids by such radicals create carbon-centered lipid radicals [18]. These lipid radicals quickly add molecular oxygen to form lipid peroxyl radicals, thereby initiating the process of lipid peroxidation. Unless scavenged by vitamin E or other radical scavengers, these lipid peroxyl radicals in turn abstract hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation.

Theoretically, silymarin may act in this model either by decreasing the metabolic activation of carbon tetrachloride, or by scavenging free radicals, or by a combination of these two effects. In the present study, we have determined the effects of silymarin and silibinin on the metabolic activation of carbon tetrachloride and on carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice.

MATERIALS AND METHODS

Animals. Male Crl:CD^R-1(ICR)BR Swiss mice were purchased from Charles River (Saint-Aubin-lès-Elbeuf, France). Mice were fed with a normal standard diet (M 25 biscuits, Extra Labo, Piétrement, Provins, France) given *ad lib.*

Chemicals and treatments. Silibinin dihemisuccinate was a kind gift from Dr Madaus and Co., Köln, F.R.G. Silymarin was kindly given by Roger Bellon laboratories, Neuilly-sur-Seine, France.

[^{14}C]Carbon tetrachloride (103.6 mBq/mmol i.e. 2.8 mCi/mmol) was purchased from New England Nuclear (Boston, MA).

Silibinin (200–800 mg/kg) was administered i.p. in 0.154 M NaCl. Silymarin (200–800 mg/kg) was administered i.p. after dissolution in 0.1 N NaOH. Carbon tetrachloride (12–18 μL/kg) was administered i.p. in 0.2 mL of corn oil.

Serum transaminases. Some mice were given carbon tetrachloride (12, 14 or 18 μL/kg i.p. in 0.2 mL of corn oil) 30 min after the administration of silibinin or silymarin (800 mg/kg i.p.). Twenty-four hours later, blood was drawn from the retroorbital sinus and serum glutamic pyruvic transaminase (SGPT) activity was measured according to Reitman and Frankel [19]. In some mice, SGPT activity was also determined 48, 72 or 96 hr after the administration of carbon tetrachloride (18 μL/kg i.p.). Other mice received various doses of either silibinin or silymarin (200, 400 or 800 mg/kg i.p.) 30 min before the administration of carbon tetrachloride (18 μL/kg).

Liver histology. Mice received silibinin or silymarin (800 mg/kg i.p.) 30 min before the administration of carbon tetrachloride (18 μL/kg i.p.) Twenty-four hours later the mice were killed. The liver was removed. A liver fragment was placed in Bouin's fluid, embedded in paraffin and stained with hematoxyllin and eosin.

In vivo *lipid peroxidation. In vivo* lipid peroxidation was assessed by measurement of ethane exhalation. Mice received either silibinin or silymarin (800 mg/kg i.p.) followed, 30 min later, by carbon tetrachloride (18 μL/kg i.p.).

Five mice were placed in a closed chamber (2.1 L), where CO_2 and H_2O were trapped, and the partial pressure of O_2 was maintained constant. Aliquots (5 mL) from the chamber's atmosphere were withdrawn, and injected in a gas–liquid chromatography apparatus (Packard 437) equipped with a flame ionization detector. The column consisted in a 100 × 0.16 cm i.d. stainless steel tube packed with carbosieve G. The column was maintained isothermally at 170°. The detector was set at 200°.

Hepatic levels and in vivo *covalent binding.* [^{14}C]Carbon tetrachloride (18 μL/kg; 75 μCi/kg) was administered i.p. 30 min after the administration of either silibinin (800 mg/kg i.p.) or silymarin (800 mg/kg i.p.). Two hours later, mice were killed by cervical dislocation. The livers were excised, rinsed in ice-cold 0.154 M NaCl, minced and homogenized in 3 volumes of 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. An aliquot (50 μL) of the liver homogenate was counted to determine total levels of [^{14}C]CCl_4 and metabolites present in the liver at that time.

The amount of ^{14}C-labelled material irreversibly bound to hepatic lipids was measured as previously described by Labbe *et al.* [20]. Briefly, total lipids were extracted from the whole liver homogenate by adding 5 mL of a chloroform/methanol mixture (2/1; v/v) containing nordihydroguaiaretic acid (1 μg/mL). After centrifugation, 2.5 mL of the lower chloroform phase containing lipids was removed, placed in preweighed tubes and evaporated. Lipids were successively washed by adding 1 mL of various solvents, followed by evaporation (chloroform, ethyl ether, carbon tetrachloride and ethyl ether). After weighing, lipids were dissolved in choroform at a concentration of 10 mg/mL, and an aliquot was counted for ^{14}C activity.

The amount of ^{14}C-labelled material irreversibly bound to liver proteins was measured as described by Hoellinger *et al.* [21]. Briefly, proteins were precipitated with 10% perchloric acid, and washed three times with ethylacetate. The pellets were then extracted with perchloric acid (twice), acetone, ethanol, and finally with 0.1 N sodium–potassium phosphate buffer. Proteins were hydrolysed overnight with 1 mL of 1 N NaOH at ambient temperature. An aliquot was then counted after acidification with 12 N HCl. Another aliquot was taken for protein determination according to the method of Lowry *et al.* [22].

Monooxygenase activities. Mice were killed by cervical dislocation and the liver was rapidly removed, rinsed in ice-cold 0.154 M NaCl solution, minced and homogenized in 3 volumes of 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer pH 7.4. Homogenates were centrifuged at 10,000 *g* for 10 min. The pellets were discarded, and the supernatants were centrifuged at 100,000 *g* for 60 min. Aminopyrine *N*-demethylase was determined according to Mazel [23], benzo(*a*)pyrene hydroxylase according to Kuntzman *et al.* [24], hexobarbital hydroxylase according to Kupfer and Rosenfeld [25], and 7-ethoxycoumarin *O*-deethylase according to Greenlee and Poland [26]. In these assays, the concentration of the substrates was 0.25 mM, while the concentration of silibinin or silymarin was 800 μg/mL (1.66 mM). In some experiments, the 7-ethoxycoumarin *O*-deethylase assay was performed with

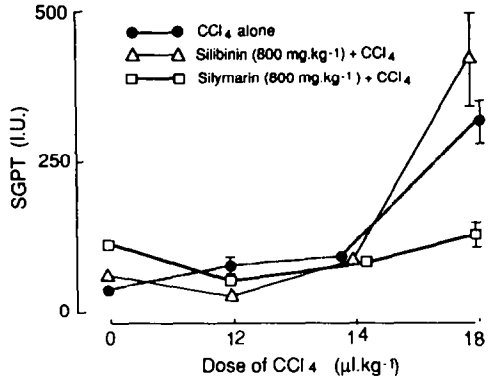

Fig. 1. Effect of a constant dose of either silibinin or silymarin on SGPT activity 24 hr after administration of various doses of carbon tetrachloride. Silibinin (800 mg/kg i.p.) or silymarin (800 mg/kg i.p.) was administered 30 min before various doses of carbon tetrachloride (12, 14 or 18 µL/kg i.p.). Results are means ± SE for 10–15 mice. The asterisk indicates a significant difference from mice receiving carbon tetrachloride alone, $P < 0.01$.

different concentrations of 7-ethoxycoumarin and different concentrations of silibinin.

In vitro *covalent binding*. [^{14}C]Carbon tetrachloride (18 nL/mL; i.e. 0.185 mM; 0.5 µCi/mL) was incubated in 1 mL of 0.07 M KCl, 0.13 M sodium–potassium phosphate buffer pH 7.4 containing hepatic microsomes (5 mg protein/mL), and a NADPH-generating system: NADP (1 mM), glucose 6-phosphate (6 mM), glucose 6-phosphate dehydrogenase (3 units) and magnesium chloride (6 mM). A zero time aliquot (0.5 mL) was taken and placed in 2 mL of ice-cold perchloric acid (10%). The remaining volume was incubated for 10 min at 37°. The amount of ^{14}C-labelled material irreversibly bound to microsomal proteins was determined according to Hoellinger *et al.* [21], as described above for *in vivo* covalent binding studies.

In vitro *lipid peroxidation*. In a first series of experiments, NADPH-induced lipid peroxidation was measured with hepatic microsomes (5 mg protein) incubated in 3.5 mL of sodium–potassium phosphate buffer pH 7.4, containing 0.2 mM NADPH, in the presence or absence of silibinin or silymarin (800 µg/ml). Peroxidation was assayed by the formation of thiobarbituric acid-reactants as previously described [27]. This first assay measures peroxidation mediated by the reduction of endogenous iron to the ferrous state [27].

In a second series of experiments, EDTA (0.05 mM) was added to block this iron-catalysed peroxidation, and to determine lipid peroxidation specifically mediated by the addition of carbon tetrachloride (18 nL/mL) to the incubation mixture [27].

Statistical analysis. Results are means ± SE. Data were compared by analysis of variance and Dunnett's *t*-test or Scheffé's test.

The presence or absence of liver cell necrosis were tested with a chi-square test in a contingency table.

RESULTS

Serum transaminases

In a first series of experiments, mice received various doses of carbon tetrachloride (12, 14 or 18 µL/kg i.p.) given 30 min after the administration of a constant dose (800 mg/kg) of either silibinin or silymarin. SGPT activity was slightly increased 24 hr after the administration of 12 or 14 µL/kg of carbon tetrachloride given alone, but was markedly increased after 18 µL/kg (Fig. 1). Pretreatment of mice with silibinin did not modify this dose–effect relationship (Fig. 1). In contrast, pretreatment with silymarin afforded protection against the hepatotoxic effects of 18 µL/kg of carbon tetrachloride (Fig. 1).

In a second series of experiments, mice were treated with various doses of either silibinin or silymarin (200, 400 or 800 mg/kg i.p.) given 30 min before the administration of a constant dose of carbon tetrachloride (18 µL/kg i.p.). Pretreatment with the two higher doses of silymarin (400 and 800 mg/kg) significantly decreased SGPT activity measured 24 hr after the administration of carbon tetrachloride

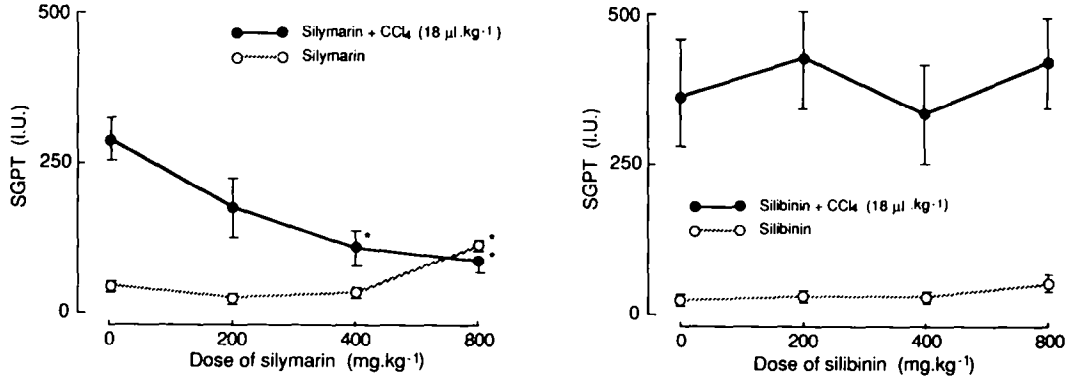

Fig. 2. Effects of various doses of either silibinin or silymarin on SGPT activity 24 hr after the administration of a constant dose of carbon tetrachloride. Silibinin (200, 400 or 800 mg/kg i.p.) or silymarin (200, 400 or 800 mg/kg i.p.) were administered 30 min before the administration of carbon tetrachloride (18 µL/kg i.p.). Results are means ± SE for 15 mice. Asterisks indicate significant differences from values in mice receiving no silymarin, $P < 0.05$.

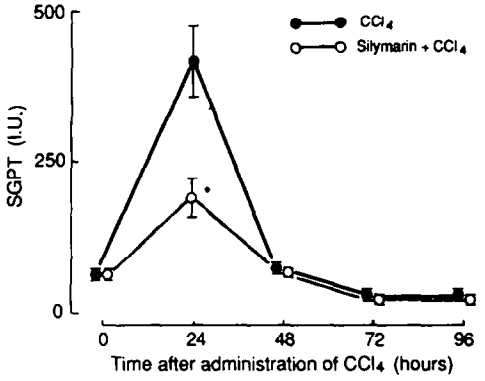

Fig. 3. Time course of SGPT activity after administration of carbon tetrachloride. Mice were pretreated or not with silymarin (800 mg/kg i.p.) 30 min before the administration of carbon tetrachloride (18 μL/kg i.p.). Blood was drawn at various times after the administration of carbon tetrachloride. Results are means ± SE for 10 mice. The asterisk indicates a significant difference from mice receiving CCl_4 alone, $P < 0.01$.

Table 1. *In vivo* effects of silibinin or silymarin administration on the *in vivo* exhalation of ethane after administration of carbon tetrachloride

	Ethane exhalation	
	First hour after CCl_4	Fourth hour after CCl_4
	(pmol/g mice/hr)	
CCl_4 (18 μL/kg)	62 ± 8	20 ± 4
Silibinin (800 mg/kg) + CCl_4	24 ± 7*	31 ± 4
Silymarin (800 mg/kg) + CCl_4	26 ± 7*	11 ± 1†

Mice received silibinin (800 mg/kg i.p.) or silymarin (800 mg/kg i.p.) 30 min before the administration of carbon tetrachloride (18 μL/kg i.p.). Exhalation of ethane was measured either during the first or the fourth hour after the administration of carbon tetrachloride. Results are means ± SE for 5–8 determinations.

* Significantly lower than that in mice receiving CCl_4 alone, $P < 0.05$.

† Significantly lower than that in mice pretreated with silibinin, $P < 0.05$.

(Fig. 2). In contrast, pretreatment with silibinin (800 mg/kg i.p.) had no protective effect (Fig. 2), even when additional but lesser doses of silibinin (400 mg/kg i.p.) were given in addition to the first dose (800 mg/kg i.p.) 3 and 6 hr after the administration of carbon tetrachloride (not shown).

A last series of experiments was performed to determine whether the protective effect of silymarin (800 mg/kg) observed at 24 hr, was followed or not by delayed toxicity. SGPT activity was determined in each mouse 24, 48, 72 and 96 hr after the administration of carbon tetrachloride, 18 μL/kg i.p. (Fig. 3). In mice receiving carbon tetrachloride alone, SGPT activity was maximal at 24 hr and returned to normal values in 72 hr (Fig. 3). No secondary rise in SGPT activity was observed in mice protected by silymarin (Fig. 3).

Liver histology

Liver histology was determined 24 hr after the administration of carbon tetrachloride (18 μL/kg i.p.) given either alone or 30 min after the administration of silibinin or silymarin (both at a dose of 800 mg/kg i.p.). Centrilobular liver cell necrosis was present in 10 of 12 mice (83%) receiving carbon tetrachloride alone, and in each of 9 mice (100%) that had been pretreated with silibinin before the administration of carbon tetrachloride. In contrast, liver cell necrosis was observed in only 5 of 11 mice (45%) pretreated with silymarin before the administration of carbon tetrachloride ($P < 0.02$, as compared to mice receiving carbon tetrachloride only).

In vivo *lipid peroxidation*

The hepatotoxicity of carbon tetrachloride is thought to be due to lipid peroxidation. We therefore determined the exhalation of ethane as an index for *in vivo* lipid peroxidation (Table 1). Pretreatment with either silibinin or silymarin (both at 800 mg/kg i.p.) significantly decreased the exhalation of ethane measured during the first hour after the administration of carbon tetrachloride, 18 μL/kg i.p. (Table 1). When measured during the fourth hour after the administration of carbon tetrachloride, however, the exhalation of ethane tended to be still low in mice pretreated with silymarin, whereas it tended, in contrast, to be high in mice pretreated with silibinin (Table 1). As a consequence the exhalation of ethane was significantly lower in mice pretreated with silymarin than in mice pretreated with silibinin before the administration of carbon tetrachloride (Table 1).

Levels of carbon tetrachloride and metabolites

Reduced *in vivo* lipid peroxidation might have been due to decreased absorption of CCl_4. To rule out such an effect, the total hepatic concentration of [^{14}C]CCl_4 and metabolites was determined 2 hr after the administration of [^{14}C]CCl_4 (18 μL/kg i.p.; 75 μCi/kg). The concentration of [^{14}C]CCl_4 and [^{14}C]metabolites (mean ± SE for 4 mice) was 788 ± 44 nmol/g liver after CCl_4 alone, 789 ± 34 in mice pretreated with silibinin (800 mg/kg i.p.) 30 min before the administration of carbon tetrachloride, and 853 ± 69 in mice pretreated with silymarin (800 mg/kg i.p.).

In vivo *covalent binding*

Lipid peroxidation mediated by carbon tetrachloride is due initially to the formation of the trichloromethyl free radical. This radical (or its peroxyl derivative) may abstract a hydrogen atom from unsaturated lipids and/or may convalently bind to lipids, or proteins [20]. We therefore determined the *in vivo* covalent binding of carbon tetrachloride metabolites to liver lipids and proteins (Table 2).

In mice killed 2 hr after the administration of carbon tetrachloride (18 μL/kg; 75 μCi/kg i.p. in 0.2 mL of corn oil), a ^{14}C-labelled material was covalently bound to hepatic lipids (Table 2). *In vivo* covalent binding to liver lipids was decreased by 38% in mice pretreated with silymarin (800 mg/kg); it was not significantly decreased (by 14%) in mice pretreated with silibinin (Table 2). *In vivo* covalent

Table 2. *In vivo* effects of silibinin or silymarin administration on the *in vivo* covalent binding of carbon tetrachloride metabolites to hepatic lipids and proteins

	Covalent binding of CCl_4 metabolites	
	Lipids (nmol/2 hr/mg lipid or protein)	Proteins
CCl_4 (18 μL/kg)	0.31 ± 0.04	0.13 ± 0.01
Silibinin (800 mg/kg) + CCl_4	0.27 ± 0.03	0.12 ± 0.01
Silymarin (800 mg/kg) + CCl_4	0.19 ± 0.02*	0.10 ± 0.01

Mice received silibinin or silymarin (800 mg/kg i.p.) 30 min before the administration of [^{14}C]CCl_4 (18 μL/kg; 75 μCi/kg). Mice were killed 2 hr after the administration of [^{14}C]CCl_4. Results are means ± SE for 8 mice.

* Significantly different from that in mice receiving CCl_4 alone, $P < 0.05$.

binding to hepatic proteins was much less than that to hepatic lipids (Table 2); it was not significantly decreased by either silibinin or silymarin administration (Table 2).

Monooxygenase activities and in vitro *covalent binding*

The reduced *in vivo* covalent binding of carbon tetrachloride to hepatic lipids suggested that silymarin may inhibit the cytochrome P-450-mediated metabolic activation of carbon tetrachloride. We therefore determined the effects of silibinin and silymarin on monooxygenase activities and on the *in vitro* covalent binding of carbon tetrachloride metabolites to microsomal proteins (Table 3). The rate limiting step in such covalent binding studies is the process of metabolic activation followed by almost immediate covalent binding. Several monooxygenase activities were reduced markedly in the presence of either silibinin or silymarin, 800 μg/mL (Table 3). Aminopyrine *N*-demethylase activity was decreased by 46 and 74% in the presence of silibinin and silymarin, respectively; benzo(*a*)pyrene hydroxylase was decreased by 52 and 46%, respectively, hexobarbital hydroxylase by 40 and 70%, and 7-ethoxycoumarin *O*-deethylase by 82 and 70% (Table 3).

Incubation of [^{14}C]carbon tetrachloride with mouse liver microsomes and a NADPH-generating system resulted in the covalent binding of a ^{14}C-labelled material to proteins (Table 3). This *in vitro* covalent binding was decreased by 20% in the presence of either silibinin or silymarin, 800 μg/mL (Table 3).

Inhibition kinetics were studied with 7-ethoxycoumarin in the presence of silibinin (Fig. 4). Silibinin both decreased the V_{max} for 7-ethoxycoumarin *O*-deethylase activity and increased the apparent K_m for 7-ethoxycoumarin on a double reciprocal plot (Fig. 4). There was marked upward curvature of the plot of the reciprocal of the rate versus the concentration of silibinin (Fig. 4).

Table 3. *In vitro* effects of silibinin or silymarin on some monooxygenase activities and on the *in vitro* covalent binding of CCl_4 metabolites to microsomal proteins

	Aminopyrine *N*-demethylase	Benzo(*a*)pyrene hydroxylase	Hexobarbital hydroxylase	7-Ethoxycoumarin *O*-deethylase	*In vitro* covalent binding of CCl_4 metabolites to microsomal proteins
	(nmol/min/mg microsomal protein)				
Control	1.8 ± 0.1	0.052 ± 0.005	0.62 ± 0.03	5.4 ± 0.2	0.052 ± 0.004
Silibinin (800 μg/mL)	1.0 ± 0.1*	0.025 ± 0.002*	0.37 ± 0.05*	0.9 ± 0.1*	0.041 ± 0.002*
Silymarin (800 μg/mL)	0.5 ± 0.1*	0.030 ± 0.002*	0.21 ± 0.05*	2.0 ± 0.1*	0.041 ± 0.003*

Monooxygenase activities were determined after incubation of various substrates (0.25 mM) with mouse liver microsomes and a NADPH-generating system at 37° for 10 min. *In vitro* covalent binding of CCl_4 metabolites to microsomal proteins was measured after incubation of CCl_4 (18 nL/mL; 0.5 μCi/mL) with mouse liver microsomes (2 mg protein/mL) and a NADPH-generating system at 37° for 10 min. Results are means ± SE for 4–5 determinations.

* Significantly different from control incubations, $P < 0.05$.

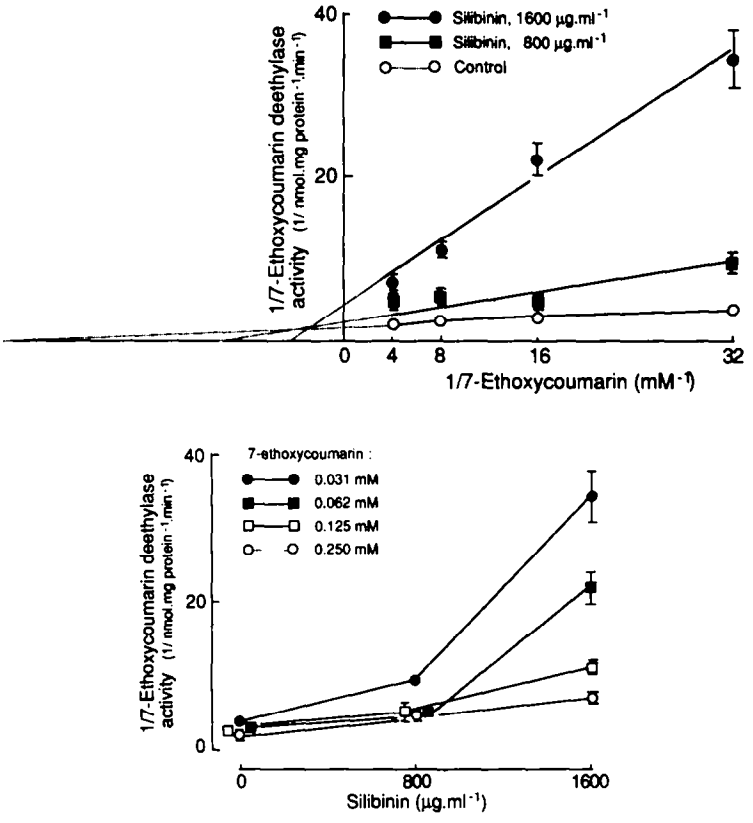

Fig. 4. Inhibition of 7-ethoxycoumarin *O*-deethylase activity by silibinin. 7-Ethoxycoumarin in various concentrations (0.031, 0.062, 0.125 or 0.25 mM) was incubated for 10 min with hepatic microsomes and a NADPH-generating system in the presence of various concentrations of silibinin (0, 800 or 1600 μg/mL). The figures show a double reciprocal plot and a plot of the reciprocal of the rate versus the concentration of silibinin. Results are means ± SE for three experiments.

Table 4. *In vitro* effects of silibinin or silymarin on *in vitro* lipid peroxidation

	TBA-reactants		
	NADPH	NADPH + EDTA	NADPH + EDTA + CCl_4
	(nmol/mg microsomal protein/min)		
Control	0.264 ± 0.028	0.013 ± 0.002	0.124 ± 0.005
Silibinin (800 μg/mL)	0.257 ± 0.012	0.017 ± 0.005	0.085 ± 0.009*
Silymarin (800 μg/mL)	0.035 ± 0.006*	0.016 ± 0.002	0.035 ± 0.005*

Lipid peroxidation was determined as the formation of thiobarbituric acid (TBA)-reactants after incubation of mouse liver microsomes (5 mg protein) with NADPH (0.2 mM), EDTA (0.05 mM), and CCl_4 (18 nL/mL), as indicated. Results are means ± SE for six determinations.
* Significantly different from control incubations, $P < 0.05$.

In vitro *lipid peroxidation*

Lipid peroxidation mediated by the NADPH-induced reduction of endogenous iron was not modified by the addition of silibinin (800 μg/mL), but was decreased by 87% in the presence of a similar amount of silymarin (800 μg/mL) (Table 4). Addition of EDTA to the incubation mixture essentially suppressed this NADPH-triggered, iron-mediated, lipid peroxidation (Table 4). In the third series of experiments, carbon tetrachloride (18 nL/mL) was added together with EDTA and NADPH to determine lipid peroxidation initiated by the trichloromethyl and trichloromethyl peroxyl radicals. Peroxidation mediated by carbon tetrachloride was decreased by only 32% in the presence of silibinin, but by 72% in the presence of silymarin (Table 4).

DISCUSSION

A protective effect of silymarin against the hepatotoxicity of carbon tetrachloride has been reported previously [1–4], but the mechanism for this effect

has not been studied. The present results confirm that silymarin does indeed protect the liver from carbon tetrachloride-induced lipid peroxidation (Table 1) and hepatotoxicity (Figs 1–3). This protection, however, requires very high doses of silymarin (400 or 800 mg/kg i.p.) and is incomplete (Figs 1–3).

The hepatotoxicity of carbon tetrachloride is thought to be due to its reductive dehalogenation by cytochrome P-450 into the trichloromethyl free radical. This radical and the corresponding peroxyl radical create lipid radicals, thereby initiating a process of lipid peroxidation. Our results suggest that the protective effects of silymarin in this model was related to a dual mechanism. A first effect of silymarin was to decrease the metabolic activation of carbon tetrachloride. This is shown by the inhibitory effects of silymarin *in vitro* on several monooxygenase activities, including the *in vitro* covalent binding of carbon tetrachloride metabolites to microsomal proteins (Table 3). Kinetics for the inhibition of 7-ethoxycoumarin *O*-deethylase have been studied with silibinin (Fig. 4), one of the constituents of silymarin. These kinetics proved to be extremely complex, with both a decreased V_{max} and an increased apparent K_m, but also an upward curvature of the plot of the reciprocal of the rate versus the concentration of silibinin (Fig. 4). Further studies will be required to delineate the molecular mechanisms for this inhibition. Silymarin also inhibited the metabolic activation of CCl_4 *in vivo*, as suggested by a decreased covalent binding of carbon tetrachloride metabolites to hepatic lipids *in vivo* (Table 2). Decreased metabolic activation of carbon tetrachloride by cytochrome P-450 will decrease the initial formation of the trichloromethyl free radical and therefore decrease the initiation of lipid peroxidation.

Two pieces of evidence suggest, however, that this is not the only mechanism. *In vitro*, silymarin (800 μg/mL) decreased the covalent binding of carbon tetrachloride metabolites to hepatic proteins by only 21% (Table 3), although it decreased by 72% *in vitro* lipid peroxidation mediated by carbon tetrachloride metabolites (Table 4). *In vivo*, silymarin (800 mg/kg) decreased the covalent binding of carbon tetrachloride metabolites to hepatic lipids by only 39% (Table 2), although it decreased by 60% the exhalation of ethane during the first hour after the administration of carbon tetrachloride (Table 1). These observations suggest that silymarin may decrease lipid peroxidation not only by decreasing the metabolic activation of carbon tetrachloride, but also by another mechanism.

Results reported in Table 4 show that silymarin prevented not only lipid peroxidation mediated by carbon tetrachloride metabolites, but also peroxidation mediated by the addition of NADPH alone (Table 4). In this system, lipid peroxidation is thought to be mediated by the reduction of iron to the ferrous state [20]. Other studies have shown previously that silymarin and/or other flavonoids can prevent lipid peroxidation mediated by the addition of Fe^{2+}-ascorbate, cumene hydroperoxide or *t*-butyl hydroperoxide, suggesting that they can act as chain-breaking antioxidants, presumably as free radical scavengers [7–9, 28]. Taken together, our observations therefore suggest that silymarin prevents carbon tetrachloride-induced lipid peroxidation and hepatotoxicity by two concurrent mechanisms: firstly by decreasing the metabolic activation of carbon tetrachloride into free radicals (Tables 2 and 3), and secondly by scavenging free radicals (Table 4).

Another interesting observation made in the present study was that silibinin, in contrast to silymarin, failed to prevent the increase in SGPT activity and the development of liver cell necrosis in this mouse model (Figs 1 and 2). The reasons for these differences between silibinin and silymarin remain incompletely understood. Silibinin, like silymarin, decreased the exhalation of ethane during the first hour after the administration of carbon tetrachloride (Table 1). During the fourth hour, however, after the administration of CCl_4, the exhalation of ethane was significantly lower in mice pretreated with silymarin than in mice pretreated with silibinin (Table 1). Thus, the protection afforded by silibinin against *in vivo* lipid peroxidation was more short-lived than that afforded by silymarin. This may not result from differences in the half-lives, since the terminal half-life of silibinin in rats is relatively long (96 hr), similar to that of silichristin (109 hr) and much longer than that of silidianin (23 hr) (W. Lang, Dr Madaus and Co., personal communication). Rather, the shorter effects of silibinin may be related, at least in part, to the much lesser effects of silibinin against *in vitro* lipid peroxidation, particularly peroxidation mediated by NADPH alone (Table 4). The latter observation suggests that silibinin is less active as a chain-breaking antioxidant than the combination of silibinin, silidianin and silichristin, at least in this system (Table 4).

In summary, administration of silymarin (800 mg/kg) 30 min before carbon tetrachloride (18 μL/kg) prevents partially both lipid peroxidation and liver cell necrosis in mice. This protective effect is related both to decreased metabolic activation and to a chain-breaking antioxidant effect of silymarin. Silibinin also prevents lipid peroxidation; this effect, however, is less marked than that of silymarin *in vitro*, and is more short-lived *in vivo*. Silibinin (800 mg/kg) failed to prevent liver cell necrosis in this mouse model.

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