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THE DRUG METHOXSALEN, A SUICIDE SUBSTRATE FOR CYTOCHROME P-450, DECREASES THE METABOLIC ACTIVATION, AND PREVENTS THE HEPATOTOXICITY, OF CARBON TETRACHLORIDE IN MICE

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Abstract—Methoxsalen, a potent suicide inhibitor of cytochrome P-450 that can be used in humans, might be of value for the prevention of hepatitis in subjects with carbon tetrachloride poisoning. As a preliminary step, we have determined its effects on the hepatotoxicity of carbon tetrachloride in mice. Several monooxygenase activities, the *in vitro* covalent binding of carbon tetrachloride metabolites to microsomal proteins, and *in vitro* microsomal lipid peroxidation initiated by carbon tetrachloride metabolites were decreased by 60–90% in microsomes from mice killed 2 hr after the administration of methoxsalen (250 μmol·kg^{-1}); microsomal lipid peroxidation mediated by endogenous iron and NADPH was not modified. Administration of methoxsalen (250 μmol·kg^{-1}) 30 min before carbon tetrachloride (0.1 ml·kg^{-1}) decreased both the *in vivo* formation of conjugated dienes in microsomal lipids and the *in vivo* covalent binding of carbon tetrachloride metabolites to lipids and proteins. This pretreatment completely prevented the hepatotoxicity of carbon tetrachloride. Other cytochrome P-450 inhibitors (cimetidine, SKF 525-A or piperonyl butoxide) given at this low molar dose (250 μmol·kg^{-1}) exerted no protective effect. Methoxsalen (500 μmol·kg^{-1}) was also effective, but only partially, when given 30 min after carbon tetrachloride (0.025 ml·kg^{-1}). We conclude that pretreatment with methoxsalen decreases the metabolic activation of carbon tetrachloride, and completely prevents its hepatotoxicity in mice. Post-treatment with methoxsalen must be given early and is only partially effective in mice.

Carbon tetrachloride (CCl_4) is transformed by reduced cytochrome P-450 (P-450 Fe^{2+}) into the trichloromethyl free radical ($^{\cdot}CCl_3$) [1, 2]. This radical may covalently bind to proteins or lipids, may abstract a hydrogen atom from an unsaturated lipid, or may add oxygen to form the highly reactive trichloromethyl peroxy radical ($^{\cdot}OOCCl_3$) [1–4]. Covalent binding of $^{\cdot}CCl_3$ to lipids, or hydrogen atom abstraction by either $^{\cdot}CCl_3$ or $^{\cdot}OOCCl_3$ leads to the formation of lipid radicals (L$^{\cdot}$), which add oxygen to form peroxy radicals (LOO$^{\cdot}$), thereby initiating the process of lipid peroxidation [1–4].

Psoralens are natural furocoumarin derivatives present in many plant species, including some edible ones such as celery, parsley, parsnip or figs [5]. Methoxsalen (8-methoxypsoralen) and several other psoralen derivatives have photosensitizing properties, which are used in the treatment of psoriasis; in so-called "PUVA-therapy", methoxsalen is given orally, followed by exposure to long wavelength ultraviolet light [6]. Following the observation that the clearance of antipyrine was decreased during PUVA-therapy in humans [7], we have studied the effects of methoxsalen on cytochrome P-450 in rats [8]. We found that methoxsalen was a suicide inhibitor of cytochrome P-450 [8]. As a result of both competitive inhibition by methoxsalen itself, and irreversible inactivation of cytochrome P-450 by methoxsalen metabolites, the drug was a potent inhibitor of cytochrome P-450 in rats [8]. Its potency was usually greater than that of SKF 525-A or piperonyl butoxide (two potent experimental inhibitors not used in humans), and much greater than that of the drug cimetidine [8].

Carbon tetrachloride poisoning still occurs in humans and may lead to severe hepatic necrosis [9]. At present, no treatment is known to prevent effectively hepatitis in humans with carbon tetrachloride poisoning [9]. We reasoned that methoxsalen, a potent inhibitor of cytochrome P-450 that can be used in humans, might be perhaps of value in subjects seen shortly after exposure to carbon tetrachloride. As a preliminary step, we determined the effects of methoxsalen on carbon tetrachloride hepatotoxicity in mice.

MATERIALS AND METHODS

Animals and treatments. Male Crl:CD^{R}-1(ICR)BR Swiss mice were purchased from Charles River (Saint-Aubin-les-Elbeuf, France). Animals were fed on a normal standard diet (M_{25} biscuits, Extra Labo, Pietrement, Provins, France) given *ad libitum*.

[^{14}C]carbon tetrachloride (sp. act., 4.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Its radiochemical purity was checked by gas liquid chromatography and found to be higher than

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99%. Methoxsalen was a kind gift from Promedica Laboratories, Levallois-Perret, France.

Methoxsalen (250 or 500 μmol·kg^{-1}; i.e. 55 or 110 mg·kg^{-1}) was administered i.p. in 0.25 or 0.5 ml, respectively of corn oil. Carbon tetrachloride (0.0125–0.1 ml·kg^{-1} i.p.) was given i.p. in 0.2 ml of corn oil. Control mice received similar injections of corn oil.

Hepatic microsomal enzymes and monooxygenase activities. Mice were killed by cervical dislocation; the liver was removed, weighed, minced and homogenized in three volumes of ice-cold 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. The 100,000 *g* supernatant was discarded. The pellet was homogenized in phosphate buffer to make a suspension containing microsomes from 62 mg of liver per ml. Cytochrome P-450 was determined as the CO-difference spectrum of dithionite-reduced microsomes [10]. NADPH-cytochrome *c* reductase activity was measured as reported by Mazel [11]. Benzo(*a*)pyrene hydroxylase activity was measured by the method of Kuntzman *et al.* [12]. Aminopyrine *N*-demethylase activity was measured by the method of Mazel [11]. 7-Ethoxycoumarin *O*-deethylase activity was measured by the method of Greenlee and Poland [13]. Hexobarbital hydroxylase activity was measured as described by Kupfer and Rosenfeld [14]. The concentration of the various substrates in these assays was 0.25 mM.

In vitro *covalent binding.* [^{14}C]Carbon tetrachloride (1 mM, 1 μCi·ml^{-1}) was incubated in 1 ml of 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4, containing microsomes from 125 mg of wet liver and a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system including NADP (1 mM), glucose-6-phosphate (6 mM), and glucose-6-phosphate dehydrogenase (3 enzyme units). An aliquot was taken at zero-time and the mixture was incubated with shaking under air, at 37°, for 15 min. The amount of [^{14}C]metabolites irreversibly bound to microsomal proteins was determined as previously described [15]. Briefly, proteins were precipitated with trichloroacetic acid (TCA), washed 3 times with TCA and repeatedly extracted with various solvents (methanol, twice; *n*-heptane, twice; ethyl ether, once). Radioactivity could not be removed further by introducing additional extraction steps. Proteins were then counted for ^{14}C activity. Radioactivity in the zero-time sample was subtracted from that in the incubated sample.

In vitro *lipid peroxidation.* The peroxidation of microsomal lipids was measured as previously described [15]. In a first system (which measures the peroxidation triggered by the reduction of endogenous iron), microsomes from 125 mg of wet liver were incubated, in a total volume of 3.5 ml of 0.1 M sodium–potassium phosphate buffer, pH 7.4, with 0.2 mM NADPH. In a second system (measuring the peroxidation initiated by carbon tetrachloride metabolites), 0.2 mM NADPH, 1.5 mM ethylenediamine tetraacetate (EDTA) and 1 mM carbon tetrachloride were added. In some flasks, methoxsalen (250 μM) was also added. The mixtures were incubated under air, at 37° for 20 min. The reaction was stopped by adding 2 ml of 20% TCA. After centrifugation, 3 ml of the supernatant was added to 1 ml of a 0.67% solution of thiobarbituric acid and heated at 100° for 10 min. The absorbance was measured at 532 nm. The absorbance of unincubated samples was subtracted, and the amount of thiobarbituric acid-reactants formed *in vitro* was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1}·cm^{-1} [16].

Blood levels and in vivo *covalent binding.* Mice received [^{14}C]carbon tetrachloride i.p. (0.1 ml·kg^{-1}; 75 μCi·kg^{-1}). In some mice, blood was drawn by retroorbital puncture 15, 30, 60, 120, 180 and 240 min later, and counted for ^{14}C activity.

Other mice were killed 2 or 4 hr after the administration of [^{14}C]carbon tetrachloride (0.1 ml·kg^{-1} i.p.; 75 μCi·kg^{-1}). The liver was removed, and a liver fragment was weighed, minced and homogenized in three volumes of 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. An aliquot of the homogenate (50 μl) was counted for ^{14}C activity. The amount of [^{14}C] metabolites irreversibly bound to hepatic proteins was determined as previously described [15]. Proteins were precipitated with TCA, washed 3 times with TCA and repeatedly extracted with various solvents, as described above for the *in vitro* covalent binding studies. [^{14}C]metabolites remaining irreversibly bound to proteins were then counted.

Irreversible binding to hepatic lipids was measured as described by Trudell *et al.* [17], with some modifications. Mice were killed 2 or 4 hr after the administration of [^{14}C]carbon tetrachloride (0.1 ml·kg^{-1} i.p.; 75 μCi·kg^{-1}). The liver was homogenized in 3 vol. of 0.154 M NaCl. To 4 ml of the hepatic homogenate, 5 ml of a chloroform–methanol mixture (2:1; v:v) were added. After shaking and centrifugation, the upper phase was discarded; 2.5 ml of the chloroform phase were added in preweighed tubes and evaporated at 50° under a stream of nitrogen. Lipids were repeatedly dissolved (4 other times) with various solvents (1 ml), which were then evaporated under a stream of nitrogen at various temperatures (chloroform at 50°, carbon tetrachloride at 50°, twice, and ether at room temperature). Radioactivity could not be removed further by introducing additional washings. At the end of these washing procedures, the tubes were weighed again and lipids were taken up in chloroform to give a concentration of 10 mg of lipid per ml; 500 μl were placed at the bottom of scintillation vials and evaporated under nitrogen; 10 ml of scintillation fluid were added, and the vials were counted for ^{14}C activity.

In vivo *lipid peroxidation.* All treatments were given p.o., to prevent the increase in conjugated dienes observed after the intraperitoneal administration of corn oil. Mice were pretreated with phorone (0.5 g·kg^{-1} p.o.) to deplete hepatic glutathione and increase lipid peroxidation; 60 min later, they received either methoxsalen (250 μmol·kg^{-1} p.o. in corn oil) or corn oil alone; 30 min later, mice received carbon tetrachloride (0.1 ml·kg^{-1} p.o.). Mice were killed 30 min after the administration of carbon tetrachloride. The liver was removed and homogenized in 3 vol. of 0.154 M KCl, 0.01 M sodium–potassium

phosphate buffer, pH 7.4, containing 1.5 mM EDTA. Microsomes were prepared as described above. Conjugated dienes present in microsomal lipids were determined according to the method of Recknagel and Ghoshal [18], with modifications [19]. Briefly, microsomal lipids were extracted with chloroform–methanol (2:1) containing 1 $\mu g \cdot ml^{-1}$ of nordihydroguaiaretic acid as an antioxidant. Methanol was discarded after addition of water, and chloroform was evaporated in preweighed tubes, under a stream of nitrogen made free of oxygen by bubbling it in an aqueous phase containing 1% pyrogallic acid. After weighing, the residue was dissolved in cyclohexane to a concentration of 1 mg of lipid per ml. The absorbance was recorded against cyclohexane from 220 to 250 nm. We also recorded the differential absorbance in lipids from rats treated with carbon tetrachloride, against lipids from rats treated with both methoxsalen and carbon tetrachloride.

Liver glutathione and hepatotoxicity. Mice were killed and the livers were removed. A liver fragment was minced and homogenized in 3 vol. of 5% TCA. Total non protein sulfhydryl ("glutathione") in the homogenate was determined by the method of Ellman [20].

Serum glutamic pyruvic transaminase activity (SGPT) was measured by the method of Reitman and Frankel [21], in blood samples drawn by retro-orbital puncture, 24 hr after the administration of various doses of carbon tetrachloride (0.0125–0.1 $ml \cdot kg^{-1}$). In some mice, SGPT activity was determined 24, 48, 72 and 96 hr after the administration of carbon tetrachloride (0.025 or 0.1 $ml \cdot kg^{-1}$).

Some mice were killed 24 hr after the administration of carbon tetrachloride (0.1 $ml \cdot kg^{-1}$ i.p.); liver fragments were placed in Bouin's fluid and embedded in paraffin 24 hr later. Sections were stained with hematoxylin and eosin, and examined for the presence of liver lesions.

RESULTS

In a preliminary experiment, the hexobarbital sleeping time was found to be increased 10-fold 2 hr after the administration of methoxsalen (250 $\mu mol \cdot kg^{-1}$ i.p.), from 33 ± 6 min (mean ± S.E.M. for 5 mice) in control animals, to 318 ± 74 min in methoxsalen-treated mice. This dose (or concentration) of methoxsalen was selected for further studies.

In vitro *effects of methoxsalen*

Incubation of mouse liver microsomes with [^{14}C]carbon tetrachloride (1 mM; 1 mCi/ml) and an NADPH-generating system resulted in the irreversible binding of [^{14}C]metabolites to microsomal proteins (Table 1). This binding was decreased by 90% when 250 μM methoxsalen was added to the incubation mixture (Table 1). Methoxsalen (250 μM) decreased by 60% *in vitro* lipid peroxidation initiated by 1 mM carbon tetrachloride, in the presence of NADPH and EDTA (Table 1), although it had no effect on lipid peroxidation mediated by endogenous

Table 1. Effects of methoxsalen on *in vitro* covalent binding and *in vitro* lipid peroxidation in the presence of carbon tetrachloride

	In vitro covalent binding of CCl_4 metabolites to microsomal proteins (nmol/mg protein/min)	*In vitro* formation of thiobarbituric acid-reactants (nmol/mg protein/20 min)	
		Without EDTA	With EDTA and CCl_4
Effect of methoxsalen *in vitro*			
Microsomes from control mice, incubation without methoxsalen	0.45 ± 0.04	17 ± 3	1.73 ± 0.02
Microsomes from control mice, incubation with 250 μM methoxsalen	0.05 ± 0.01*	16 ± 3	0.75 ± 0.13*
Effect of methoxsalen administration			
Microsomes from mice killed 2 hr after corn oil	0.23 ± 0.02	17 ± 1	2.11 ± 0.18
Microsomes from mice killed 2 hr after methoxsalen (250 $\mu mol \cdot kg^{-1}$ i.p.)	0.06 ± 0.01*	17 ± 1	0.47 ± 0.16*

In vitro covalent binding was measured after incubation of [^{14}C]carbon tetrachloride (1 mM, 1 $\mu Ci \cdot ml^{-1}$) at 37° for 15 min with an NADPH-generating system and mouse liver microsomes. *In vitro* lipid peroxidation was measured with two different systems. In the first system (measuring peroxidation mediated by the reduction of endogenous iron), mouse liver microsomes were incubated with 0.2 mM NADPH. In the second system (measuring peroxidation mediated by CCl_4 metabolites), 1.5 mM EDTA and 1 mM carbon tetrachloride were also added. The flasks were incubated at 37° for 20 min and the amount of thiobarbituric acid-reactants was then determined. These various experiments were first performed with microsomes from control mice, with or without addition of 250 μM methoxsalen in the incubation mixture. In a second series of experiments microsomes from mice killed 2 hr after the administration of corn oil (0.25 ml i.p.) were compared to those from mice killed 2 hr after the administration of methoxsalen (250 $\mu mol \cdot kg^{-1}$ i.p.) given in 0.25 ml of corn oil. Results are means ± SEM for 4–6 determinations.

* Significantly different from that in the corresponding control (Student's *t*-test for independent data), $P < 0.001$.

Table 2. Effects of methoxsalen on monooxygenase activities*

	Cytochrome P-450 (nmol/mg microsomal protein)	Aminopyrine *N*-demethylase	Benzo(*a*)pyrene hydroxylase (nmol/min/mg microsomal protein)	7-Ethoxycoumarin deethylase	Hexobarbital hydroxylase
Control	0.88 ± 0.05	1.09 ± 0.13	0.23 ± 0.02	1.61 ± 0.17	0.82 ± 0.09
Methoxsalen	0.56 ± 0.04*	0.29 ± 0.02*	0.09 ± 0.01*	0.26 ± 0.05*	0.09 ± 0.01*

Mice were killed 2 hr after the administration of methoxsalen (250 μmol·kg^{-1}) given i.p. in 0.25 ml of corn oil; control mice received corn oil. Monooxygenase activities were measured with various substrates (0.25 mM), an NADPH-generating system and hepatic microsomes. Results are means ± SEM for 5 mice.

* Significantly different from that in control mice (Student's *t*-test for independent data), $P < 0.001$.

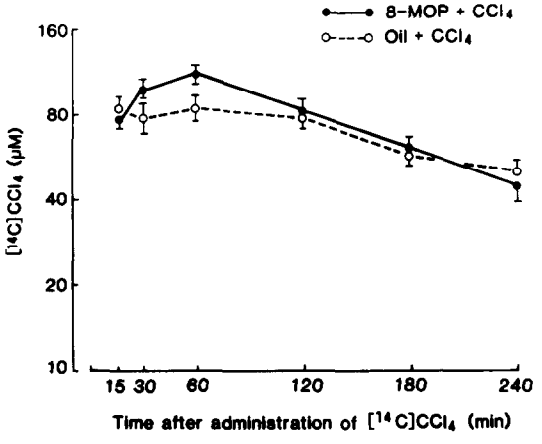

Fig. 1. Effects of pretreatment with methoxsalen on blood levels of ^{14}C after administration of [^{14}C]carbon tetrachloride. Some mice ("8-MOP") received methoxsalen (250 μmol·kg^{-1} i.p.) in 0.25 ml of corn oil, while control mice ("oil") received corn oil only. Thirty minutes later, [^{14}C]carbon tetrachloride (0.1 ml·kg^{-1}; 75 μCi·kg^{-1}) was administered i.p., and blood levels of ^{14}C were determined at different times. Results are means ± SEM for 10 mice.

iron and NADPH, in the absence of EDTA (Table 1).

Effects of methoxsalen given in vivo *on microsomal enzyme activities*

The liver weight, microsomal protein content, and NADPH-cytochrome *c* reductase activity were not modified in mice killed 2 hr after administration of methoxsalen, 250 μmol·kg^{-1} i.p. (not shown); CO-binding cytochrome P-450 was decreased by 40% and monooxygenase activities were decreased by 60–90% (Table 2); this treatment also decreased by 75% the *in vitro* covalent binding of carbon tetrachloride metabolites to microsomal proteins (Table 1) and decreased by 80% the *in vitro* lipid peroxidation initiated by carbon tetrachloride metabolites, although it had no effect on lipid peroxidation mediated by the reduction of endogenous iron (Table 1).

Effects of pretreatment with methoxsalen on carbon tetrachloride metabolism and toxicity

Administration of methoxsalen (250 μmol·kg^{-1}) 30 min before the administration of [^{14}C]carbon

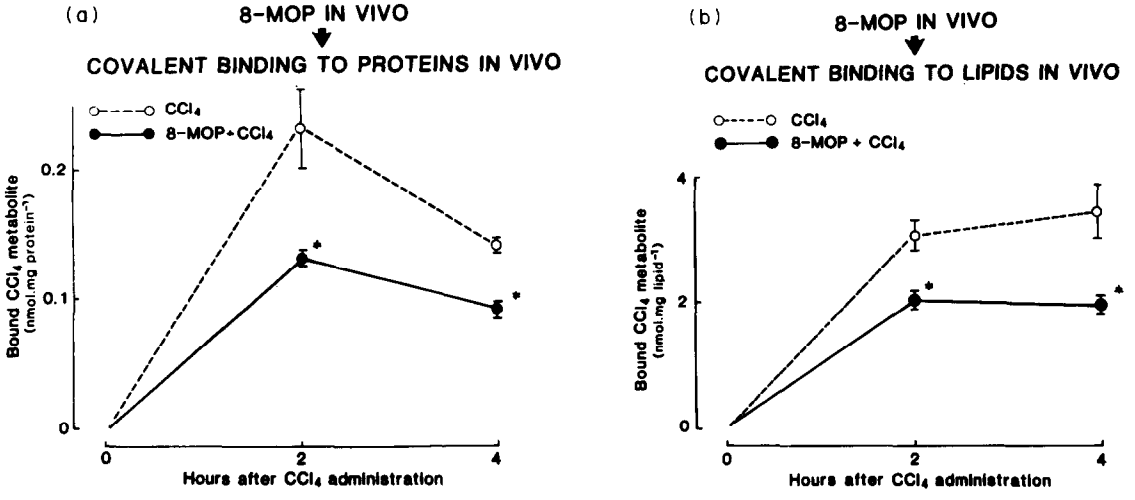

Fig. 2. Effects of pretreatment with methoxsalen on the *in vivo* covalent binding of reactive carbon tetrachloride metabolites to hepatic proteins and lipids. Some mice ("8-MOP") received methoxsalen (250 μmol·kg^{-1}) given i.p. in 0.25 ml of corn oil, while control mice received corn oil only. Thirty minutes later, [^{14}C]carbon tetrachloride (0.1 ml·kg^{-1}; 75 μCi·kg^{-1}) was administered i.p. in 0.2 ml of corn oil. Mice were killed 2 or 4 hr later, and the amount of [^{14}C]metabolites irreversibly bound to hepatic proteins or lipids was determined. Results are means ± SEM for 5–20 mice. The asterisks indicate significant differences from values observed in mice not pretreated with methoxsalen (Student's *t*-test for independent data), $P < 0.01$.

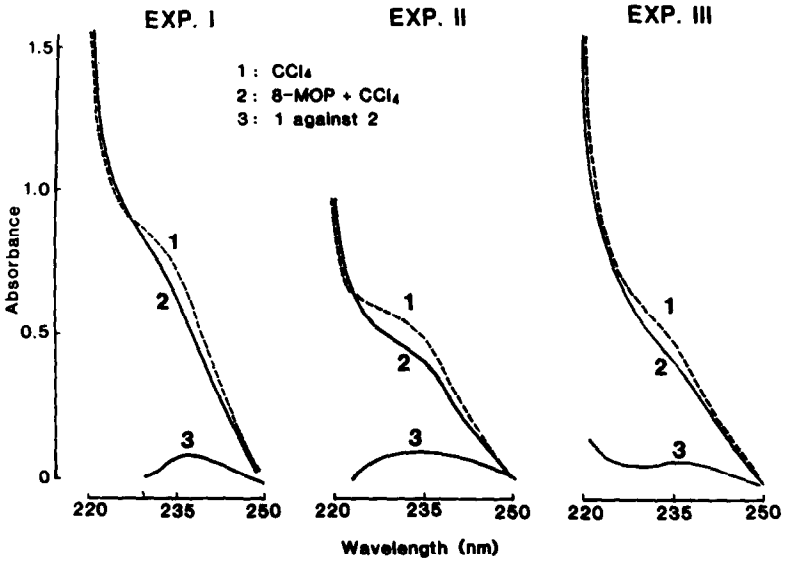

Fig. 3. Effect of pretreatment with methoxsalen on the *in vivo* formation of conjugated dienes in microsomal lipids. All mice were pretreated with phorone (0.5 g·kg^{-1} in 0.2 ml of corn oil p.o.). After 60 min, some mice received methoxsalen ("8-MOP"), 250 μmol·kg^{-1} in 0.25 ml of corn oil p.o., while other mice received corn oil. Thirty minutes later, carbon tetrachloride (0.1 ml·kg^{-1}) was administered in 0.1 ml of corn oil p.o. Mice were killed 30 min later. Hepatic microsomes were prepared. Microsomal lipids were isolated, weighed and dissolved in cyclohexane at a concentration of 1 mg of lipid per ml. Lipids from 3 livers were pooled. The absorption of conjugated dienes around 233 nm was first recorded against cyclohexane (graphs 1 and 2); we then recorded the differential absorption of microsomal lipids from mice intoxicated by carbon tetrachloride against that of lipids from mice receiving first methoxsalen and then CCl_4 (graph 3). The figure shows three successive experiments.

tetrachloride (0.1 ml·kg^{-1}; 75 μCi·kg^{-1}) did not significantly modify blood levels of ^{14}C (Fig. 1) or hepatic levels of ^{14}C, which were 409 ± 61 nmol·g liver^{-1} and 482 ± 56 nmol·g liver^{-1}, respectively in methoxsalen-treated and control mice killed 2 hr after administration of carbon tetrachloride (mean ± SEM for 20 mice) and 158 ± 15 and 179 ± 13 nmol·g liver^{-1} respectively in methoxsalen-treated and control mice killed 4 hr after administration of carbon tetrachloride (mean ± SEM for 10 mice). This pretreatment, however, decreased by 40% the *in vivo* covalent binding of [^{14}C]carbon tetrachloride metabolites to hepatic proteins and lipids (Fig. 2), and decreased the *in vivo* formation of conjugated dienes in microsomal lipids (Fig. 3). Hepatic glutathione content was slightly decreased 2 hr after administration of carbon tetrachloride (0.1 ml·kg^{-1} i.p.) alone, but was not significantly decreased in mice first pretreated with methoxsalen (250 μmol·kg^{-1} i.p.) 30 min before the administration of carbon tetrachloride (Fig. 4).

Pretreatment with methoxsalen (250 μmol·kg^{-1} i.p.), 30 min before the administration of carbon tetrachloride completely prevented the rise in SGPT activity 24–72 hr after the administration of various doses (0.0125–0.1 ml·kg^{-1} i.p.) of carbon tetrachloride (Figs 5 and 6).

Histologic examination showed massive (centrilobular and mediolobular) necrosis in all of 10 mice killed 24 hr after the administration of carbon tetrachloride alone (0.1 ml·kg^{-1} i.p.) but did not show any necrosis in any of 10 mice pretreated with methoxsalen (250 μmol·kg^{-1} i.p.), 30 min before the administration of carbon tetrachloride.

As opposed to the marked effects of methoxsalen, in contrast cimetidine, SKF 525-A, and piperonyl butoxide, given at the same molar dose (250 μmol·kg^{-1} i.p.) 30 min before carbon tetrachloride (0.1 ml·kg^{-1} i.p.) did not decrease SGPT

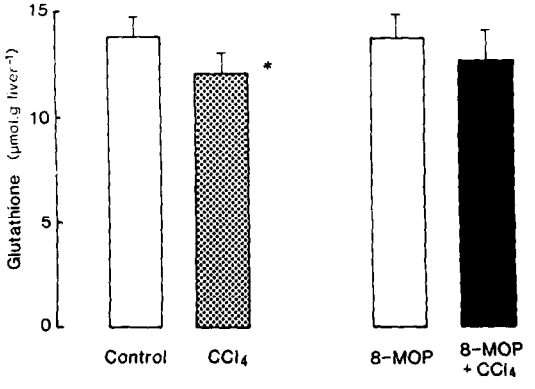

Fig. 4. Effects of pretreatment with methoxsalen on hepatic glutathione concentration after administration of carbon tetrachloride. Some mice received methoxsalen ("8-MOP"), 250 μmol·kg^{-1} i.p. in 0.25 ml of corn oil, while other mice received corn oil only. Thirty minutes later, carbon tetrachloride (0.1 ml·kg^{-1}) was administered i.p. in 0.2 ml of corn oil, while other mice received corn oil only. Total non protein sulfhydryl ("glutathione") content in the liver was determined 2 hr after the administration of carbon tetrachloride and/or corn oil. Values are means ± SEM for 5 mice. The asterisk indicates a significant difference from the value observed in control mice (Student's *t*-test for independent data), $P < 0.01$.

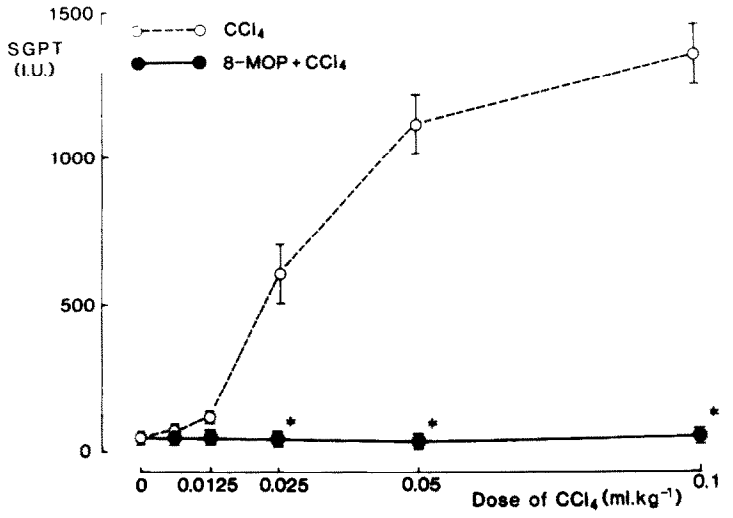

Fig. 5. Effects of pretreatment with methoxsalen on SGPT activity 24 hr after the administration of various doses of carbon tetrachloride. Some mice received methoxsalen ("8-MOP"), 250 μmol·kg^{-1} i.p. in 0.25 ml of corn oil, while other mice received corn oil only. Thirty minutes later, carbon tetrachloride (0.1 ml·kg^{-1}) was administered i.p. in 0.2 ml of corn oil. SGPT activity (mean ± SEM for 5–15 mice) was determined 24 hr later. The asterisks indicate significant differences from values in control mice (Student's t-test for independent data), $P < 0.01$

activity 24 hr after the administration of carbon tetrachloride (Fig. 7).

Post-treatment with methoxsalen

SGPT activity were not significantly decreased when methoxsalen (250 μmol·kg^{-1} i.p.) was administered 30 min after carbon tetrachloride (0.1 ml·kg^{-1} i.p.). To see whether an effect could be obtained under less drastic conditions, we now decreased the dose of carbon tetrachloride to 0.025 ml·kg^{-1} i.p. and increased that of methoxsalen to 500 μmol·kg^{-1} i.p. Under these conditions, methoxsalen was still protective when given 30 min after the administration of carbon tetrachloride (Figs. 8 and 9), whereas other cytochrome P-450 inhibitors, at the same molar dose (500 μmol·kg^{-1}), had no effect (Fig. 10).

DISCUSSION

Our results show that pretreatment with methoxsalen (250 μmol·kg^{-1} i.p.) completely prevented the hepatotoxicity of carbon tetrachloride (950 μmol·kg^{-1} i.p.) in mice (Figs 5 and 6). The initial event in the hepatotoxicity of carbon tetrachloride is its dehalogenation by cytochrome P-

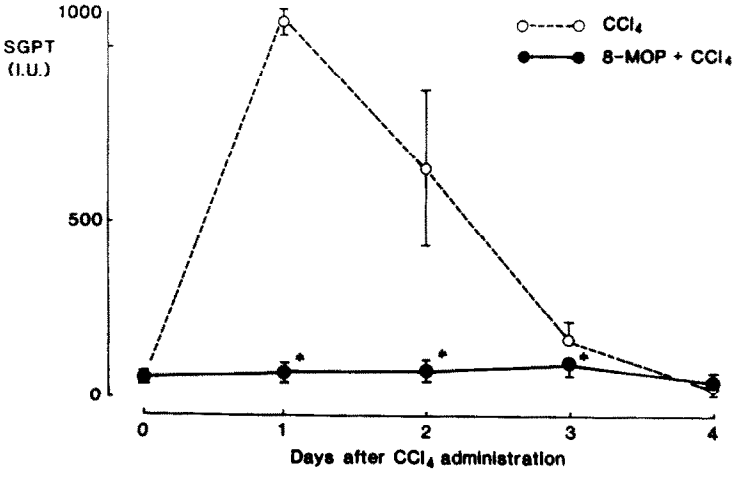

Fig. 6. Effect of pretreatment with methoxsalen on the time course of SGPT activity after administration of carbon tetrachloride. In some mice, methoxsalen ("8-MOP"), 250 μmol·kg^{-1}, was given i.p. in 0.25 ml of corn oil, while other mice received corn oil. Thirty minutes later, carbon tetrachloride (0.1 ml·kg^{-1}) was administered i.p. in 0.2 ml of corn oil. SGPT activity was determined 1, 2, 3 or 4 days later. Results are means ± SEM for 10 mice. The asterisks indicate significant differences from values in control mice (Student's t-test for independent data), $P < 0.01$.

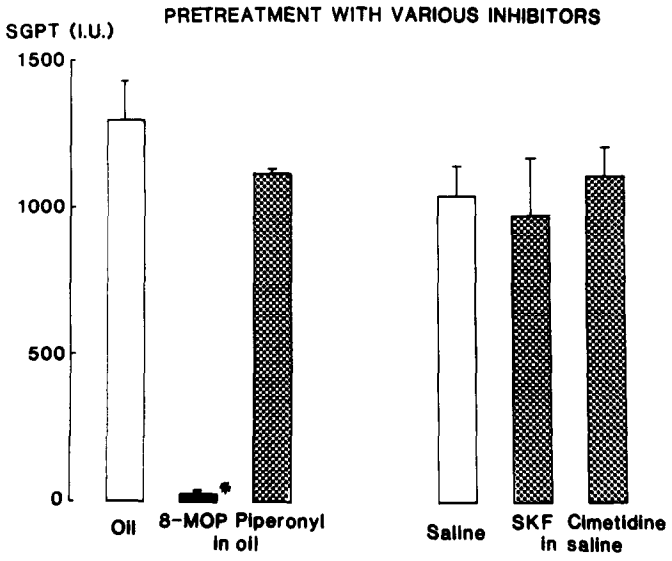

Fig. 7. Comparison of the effects of pretreatment with different cytochrome P-450 inhibitors on SGPT activity 24 hr after carbon tetrachloride. Some mice received 250 μmol·kg^{-1} of either methoxsalen ("8-MOP") or piperonyl butoxide ("Piperonyl") given i.p. in 0.25 ml of corn oil, while other mice received either 250 μmol·kg^{-1} of SKF 525-A ("SKF") or cimetidine in 0.2 ml of saline. Other mice received corn oil or saline alone. Thirty minutes later, mice received carbon tetrachloride (0.1 ml·kg^{-1} i.p.). SGPT activity was determined 24 hr after the administration of carbon tetrachloride. Results are means ± SEM for 5–10 mice. The asterisk indicates a significant difference from the value in mice receiving corn oil before carbon tetrachloride (Student's t-test for independent data), $P < 0.001$.

450Fe(II) to the trichloromethyl free radical [1–4]. Our results indicate that methoxsalen acted at this early activation step. Indeed methoxsalen, a suicide substrate for cytochrome P-450 [8], markedly decreased cytochrome P-450 content and monooxygenase activities (Table 2), and similarly decreased the initiation of lipid peroxidation by carbon tetrachloride metabolites (Table 1, Fig. 3) and their covalent binding to proteins and lipids (Table 1, Fig. 2). Interestingly, lipid peroxidation mediated by reduced endogenous iron was not modified (Table 1), showing that methoxsalen does not act on the development of lipid peroxidation *per se*, but instead prevents its initiation by decreasing the cytochrome

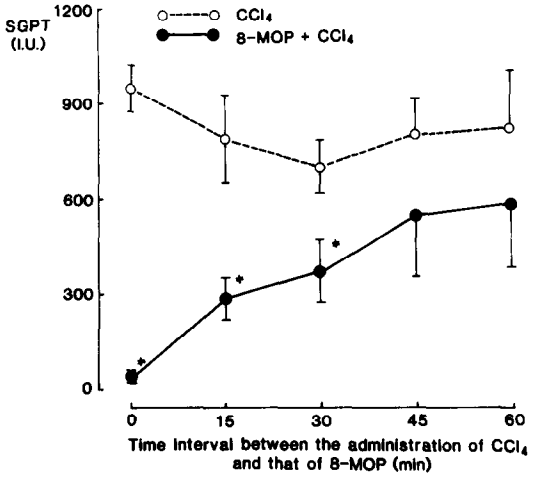

Fig. 8. Influence of the time interval between the administration of carbon tetrachloride and that of methoxsalen on SGPT activity 24 hr after administration of carbon tetrachloride. Mice received carbon tetrachloride (0.025 ml·kg^{-1} i.p.) in 0.2 ml of corn oil. At various times, some mice ("8-MOP") received methoxsalen (500 μmol·kg^{-1}) given i.p. in 0.5 ml of corn oil, while other mice received corn oil only. Results are means ± SEM for 5–20 mice. The asterisks indicate a significant difference from values in control mice (Student's t-test for independent data), $P < 0.05$.

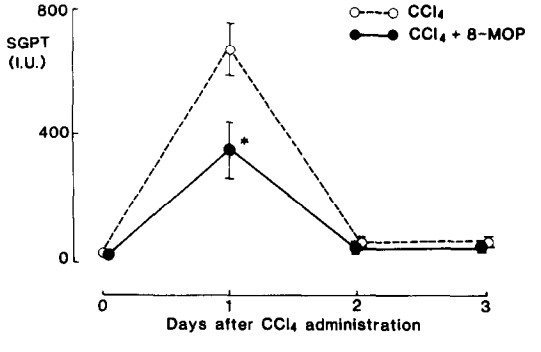

Fig. 9. Effect of post-treatment with methoxsalen on the time-course of SGPT activity after administration of carbon tetrachloride. Mice received carbon tetrachloride (0.025 ml·kg^{-1} i.p. in 0.2 ml of corn oil). Thirty minutes later, mice received either methoxsalen ("8-MOP"), 500 μmol·kg^{-1} i.p. in 0.5 ml of corn oil, or corn oil only. SGPT activity was determined 1, 2 and 3 days after the administration of carbon tetrachloride. Results are means ± SEM for 10 mice. The asterisk indicates a significant difference from value in mice receiving carbon tetrachloride only (Student's t-test for independent data), $P < 0.05$.

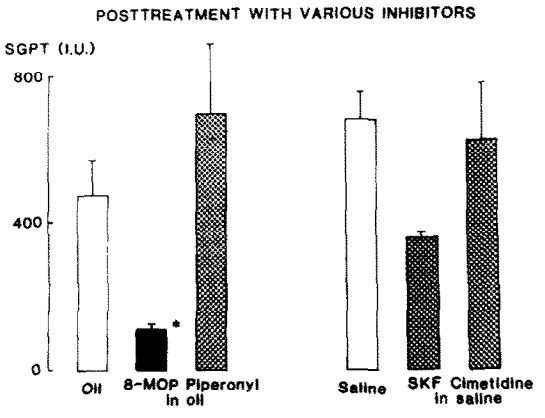

Fig. 10. Comparison of the effects of post-treatment with methoxsalen and other cytochrome P-450 inhibitors on SGPT activity 24 hr after the administration of carbon tetrachloride. Mice received carbon tetrachloride ($0.025\ ml \cdot kg^{-1}$ in 0.2 ml of corn oil i.p.). Thirty minutes later, some mice received either methoxsalen ("8-MOP") or piperonyl butoxide ("Piperonyl"), $500\ \mu mol \cdot kg^{-1}$ i.p. in 0.5 ml of corn oil, while control mice received corn oil. Other mice received either SKF 525-A ("SKF") or cimetidine, $500\ \mu mol \cdot kg^{-1}$ i.p. in 0.2 ml of saline, while other mice received saline. Results are means ± SEM for 5–10 mice. The asterisk indicates a significant difference from the value in mice receiving carbon tetrachloride only (Student's *t*-test for independent data), $P < 0.01$.

P-450 mediated formation of the trichloromethyl free radical and the consequent abstraction of hydrogen atoms from lipids.

In recent experiments [22], we have found that pretreatment with methoxsalen ($125\ \mu mol \cdot kg^{-1}$ p.o. 30 min earlier) also decreased the metabolic activation of acetaminophen ($600\ mg \cdot kg^{-1}$ i.p.) and prevented its hepatotoxicity in mice. Interestingly, cimetidine, SKF 525-A or piperonyl butoxide, given at the same molar doses as methoxsalen (125 or $250\ \mu mol \cdot kg^{-1}$) 30 min before the hepatotoxin, failed to prevent the hepatotoxicity of acetaminophen [22] or carbon tetrachloride (Fig. 7). Indeed, up to now, the inhibitory effects of methoxsalen on monooxygenase activities *in vivo* have always been greater than those of SKF 525-A or piperonyl butoxide [8, 22, this study], suggesting that methoxsalen may be preferred to other inhibitors as a tool to decrease metabolic activation and determine its rôle in the hepatotoxicity of drugs and chemicals.

It is noteworthy that methoxsalen was also effective, albeit only partially, when given 30 min after carbon tetrachloride (Figs 8 and 9). Whether this effect may be useful in subjects seen shortly after exposure to carbon tetrachloride cannot be ascertained at this time. It is noteworthy, however, that in still unpublished experiments, we have shown that methoxsalen is an extremely potent suicide inhibitor of human liver cytochrome P-450 *in vitro*, and that therapeutic doses of methoxsalen decrease the metabolic activation of acetaminophen in human volunteers *in vivo*.

We conclude that pretreatment with the drug methoxsalen decreases the metabolic activation of carbon tetrachloride and completely prevents its hepatotoxicity in mice. Post-treatment with methoxsalen has to be given early and is only partially effective in mice.

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