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Carbon tetrachloride-induced increase in the antitumor activity of cyclophosphamide in mice: A pharmacokinetic study

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Summary. *Carbon tetrachloride is an hepatotoxin that depresses hepatic microsomal cytochrome P-450 and other enzyme activities. Cyclophosphamide is an anticancer drug that is activated by hepatic microsomal cytochrome P-450, while the products of cyclophosphamide metabolism by cytochrome P-450 can be metabolized by other hepatic enzymes. Carbon tetrachloride pretreatment has been found to increase the in vivo antitumor activity of cyclophosphamide against murine leukemia P-388. Carbon tetrachloride did not, however, affect the direct cytotoxicity of cyclophosphamide or 4-hydroxycyclophosphamide to cells in culture. Pharmacokinetic studies in mice revealed a delayed plasma disappearance of cyclophosphamide after carbon-tetrachloride pretreatment with an apparent initial half-time of 20.4 min compared to 9.0 min in non carbon-tetrachloride-pretreated mice. Plasma levels of total alkylating activity and plasma 4-hydroxycyclophosphamide increased more slowly and reached a lower peak, but were maintained for a longer time period in mice pretreated with carbon-tetrachloride than in untreated mice. The half-life for plasma elimination of 4-hydroxycyclophosphamide in untreated mice was 12 min and in carbon-tetrachloride-pretreated mice 27 min. There was, however, no difference in the area under the curve for either plasma total alkylating activity or plasma 4-hydroxycyclophosphamide between the two groups. It is suggested that prolonged exposure of tumor cells to 4-hydroxycyclophosphamide might be responsible for the increased antitumor activity of cyclophosphamide following carbon-tetrachloride pretreatment.*

Introduction

Cyclophosphamide is a pro-drug widely used both as an antitumor and immunosuppressive agent [4]. The first step in the bioactivation of cyclophosphamide is hydroxylation to 4-hydroxycyclophosphamide by the microsomal mixed function oxidase of the liver, and to a lesser extent lung [16, 19]. 4-Hydroxycyclophosphamide is in equilibrium with the open ring aldehyde aldophosphamide. Spontaneous elimination of acrolein from aldophosphamide yields phosphoramide mustard, an active alkylating agent. Oxidation of 4-hydroxycyclophosphamide and aldophosphamide by soluble enzymes, notably aldehyde dehydrogenase, present in liver and kidney produces 4-ketocyclophosphamide and carboxycyclophosphamide, respectively [5, 7]. These compounds lack antiumor activity and their urinary excretion accounts for the majority of an administered dose of cyclophosphamide [9, 40]. 4-Hydroxycyclophosphamide is probably the transport form in plasma of the ultimate cytotoxic species phosphoramide mustard [9, 32]. Levels of cytotoxic metabolites of cyclophosphamide will, therefore, depend on the relative activities of hepatic microsomal mixed function oxidase, aldehyde dehydrogenase, and related enzymes. Hepatic drug metabolizing activity is frequently depressed due to hepatic disease or injury [2, 44]. Despite the fact that many cancer patients exhibit impaired liver function [21, 41], little is known about how this affects the activation and antitumor activity of cyclophosphamide. In the present study the effect, in mice, of carbon tetrachloride induced liver injury on the in vitro and in vivo antitumor activity, and the metabolism of cyclophosphamide has been investigated.

Materials and methods

Male mice (Harlan Sprague Dawley, Madison, Wis.), 6–10 weeks old, weighing 20–30 g, were maintained on a 12-h light-dark cycle and caged on cedar chips in groups of five or six. BDF_1 mice were used for all studies and DBA_2 mice were used to maintain the P-388 leukemia cell line by weekly IP transfer of 10^6 cells/mouse.

Drugs were dissolved in 0.9% sodium chloride and given by IP injection in volumes between 0.1 ml and 0.25 ml/mouse, unless indicated otherwise. BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (Bristol Laboratories, Syracuse, NY), 15 mg/kg, was administered IP dissolved in 10% (v/v) absolute ethanol in 0.9% sodium chloride. 5-Fluorouracil (Sigma Chemical Company, St. Louis Mo.) was given at a dose of 200 mg/kg. Cyclophosphamide (Cytoxan, Mead-Johnson Laboratories, Evansville, Ind.), 45–180 mg/kg, was used for all in vivo antitumor studies. Cyclophosphamide (2H-1,3,2-oxaza-phosphorine monohydrate, Sigma Chemical Company, St. Louis, Mo.), 65 mg/kg, was used for pharmacokinetic and in vitro studies. Carbon tetrachloride (Mallinckrodt, St. Louis, Mo.), 0.32 ml/kg, was administered by IP injection with a Hamilton microliter syringe (Hamilton Co., Reno, New.).

Antitumor activity against murine leukemia P-388 was determined according to National Cancer Institute protocols [18]. Carbon tetrachloride when used, was administered 2 h prior to IP innoculation with 10^6 P-388 leukemia cells. Anticancer drugs were administered 24 h after innoculation with leukemia cells. Mouse liver microsomes were prepared as described by Ernster et al. [11] and hepatic microsomal cytochrome P-450 levels were determined by the method of

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Imai and Sato [25]. Protein was determined the the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.). Serum glutamate pyruvate transaminase (SGPT) activity was determined at room temperature with Beckman Liquid-STAT ALT^{uv} enzyme reagent (Beckman Instruments, Inc, Fullerton, Calif.). Blood was obtained by cardiac puncture from mice anesthetized with ether.

For pharmacokinetic studies, cyclophosphamide (65 mg/kg) was administered IP and blood was collected into heparinized tubes by orbital sinus or cardiac puncture [31] of mice anesthetized with ether. Blood was immediately centrifuged using a Beckman microfuge B (Beckman Instruments, Fullerton, Calif.), and the plasma was assayed for alkylating metabolites and 4-hydroxycyclophosphamide or stored frozen at −70° C until assay for cyclophosphamide. Plasma cyclophosphamide concentrations were measured by a modification of the gas chromatographic procedure of Juma et al. [27] using a 12-m OV-1 capillary column with isophosphamide as an internal standard. Alkylating activity was determined by a colorimetric procedure using 4-(p-nitrobenzyl)-pyridine according to the method of Friedman and Boger [15]. 4-Hydroxycyclophosphamide was measured by the fluorometric procedure of Voelcker and Hohorst [43]. Area under the plasma concentration time curve for alkylating activity and for 4-hydroxycyclophosphamide was determined by cutting out a linear plot of the concentration-time data on graph paper and weighing.

In vitro studies were conducted with Chinese hamster ovary cells. Cells were grown in flasks containing Dulbecco's modified Eagle's medium with 30 mM sodium bicarbonate, 0.3 mM streptomycin sulfate, penicillin 200,000 U/l, and 10% fetal calf serum (growth medium). Cells in log-phase growth were detached with 0.25% trypsin, 0.1% EDTA in phosphate buffered saline, and plated on 60-mm plastic petri dishes to obtain a final colony count, after drug treatment, of between 100 and 200. Petri dishes were placed in a 37° C incubator for 24 h to allow attachment of cells. Growth medium was replaced with prewarmed growth medium containing drug and incubated at 37° C for 1 h. Petri dishes were rinsed five times with prewarmed growth medium to remove drug and then incubated at 37° C in a CO_2 incubator for 10 days. Media was removed and dishes washed with warm 0.9% NaCl. Colonies were stained with 0.2% crystal violet in methanol for 10 min, rinsed with tap water and counted. Quadruplicate petri dishes were employed for each drug concentration. Rat hepatocytes were isolated by the method of Stewart and Inaba [38] and incubated with Chinese hamster ovary cells and drug for 1 h at a hepatocyte to cell ratio of 100:1.

Mouse survival data were analyzed by the Mann-Whitney test with correction for ties, other groups of data were analyzed by Student's *t*-test [35].

Results

Hepatotoxicity of carbon tetrachloride

The effect of carbon tetrachloride pretreatment on hepatic microsomal cytochrome P-450 levels in mice is shown in Fig. 1. Maximum lowering of cytochrome P-450 occurred 24 h after giving carbon tetrachloride and levels of cytochrome P-450 remained below 50% of the control for at least 3 days. Serum glutamate pyruvate transaminase activity increased from a mean control value of 39 IU/l to greater than 11,000 IU/l 24 h after giving carbon tetrachloride, indicating marked hepatic damage.

Antitumor activity

The effect of administering carbon tetrachloride 2 h before injecting P-388 tumor cells and 26 h before giving BCNU, 5-fluorouracil or cyclophosphamide on the antitumor activity of these drugs is shown in Table 1. Carbon tetrachloride pretreatment alone did not alter the survival of tumored mice but did further increase the survival of mice treated with BCNU and cyclophosphamide. The antitumor activity of 5-fluorouracil was not affected by carbon tetrachloride pretreatment. To confirm the increase in the antitumor activity of cyclophosphamide in carbon tetrachloride pretreated mice another study was undertaken, employing a range of cyclophosphamide doses (Fig. 2). The highest dose of cyclophosphamide employed was 90 mg/kg, which is the optimum for antitumor activity against leukemia P-388 and is just below the

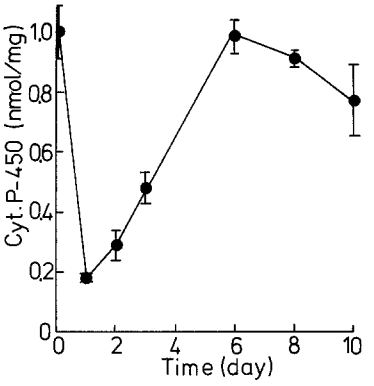

Fig. 1. Effect of carbon-tetrachloride pretreatment on hepatic microsomal cytochrome P-450 in mice. Groups of three mice were administered IP carbon tetrachloride, 0.32 ml/kg. *Bars* are SE of mean

Table 1. Effect of carbon-tetrachloride pretreatment on the antitumor activity of BCNU, 5-fluorouracil, and cyclophosphamide

Treatment	$-CCl_4$		
	n	%ILS	30-Day survivors
Control	8	0	0
BCNU (15 mg/kg)	8	70	1
5-Fluorouracil (200 mg/kg)	6	80	0
Cyclophosphamide (60 mg/kg)	8	70	0
Treatment	$+CCl_4$		
	n	%ILS	30-Day survivors
Control	8	− 10	0
BCNU (15 mg/kg)	8	160	3
5-Fluorouracil (200 mg/kg)	6	95	0
Cyclophosphamide (60 mg/kg)	8	140	3

Groups of six or eight mice were injected IP with 10^6 leukemia P-388 cells; BCNU, 5-fluorouracil or cyclophosphamide was administered IP 24 h later. Carbon tetrachloride (0.32 ml/kg) was administered IP 2 h before giving tumor cells. Antitumor activity is expressed as percent increase in the median life span of drug treated compared to non-drug-treated tumored mice (%ILS). The study was terminated at 30 days. Non-drug-treated tumored mice exhibited a median survival time of 10 days

LD_{10} for cyclophosphamide in BDF_1 mice, in our hands 105 mg/kg. Carbon tetrachloride pretreatment produced an increase in the antitumor activity of cyclophosphamide, which was statistically significant at cyclophosphamide doses of 60 and 90 mg/kg. At a dose of cyclophosphamide of 45 mg/kg the increase in antitumor activity was not statistically significant, but there was one 60-day survivor. Althought not designed as a toxicity study, the results also suggest an increased lethality when mice were given cyclophosphamide after carbon tetrachloride pretreatment. One, two, and two mice died before day 5 in the carbon-tetrachloride-pretreated groups given 45, 60, and 90 mg cyclophosphamide/kg, respectively. These animals were automatically excluded from the analysis of antitumor activity [18]. No mice died when carbon tetrachloride or cyclophosphamide at any of the doses used was given alone.

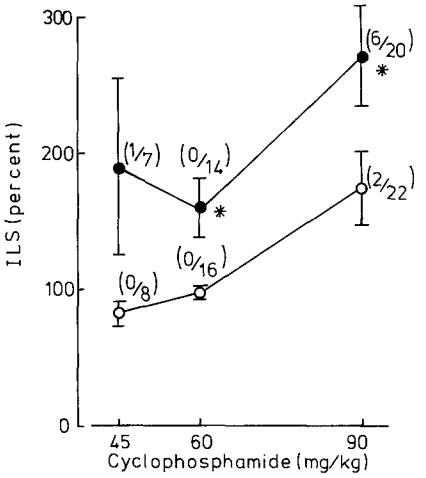

Fig. 2. Effect of carbon-tetrachloride pretreatment on antitumor activity of cyclophosphamide against murine leukemia P-388. Mice were adminstered IP carbon tetrachloride, 0.32 ml/kg, 2 h before IP innoculation with 10^6 leukemia P-388 cells and 24 h before IP injection of cyclophosphamide. Antitumor activity was measured as increase in life span (*ILS*). (○) Control mice; (●) carbon-tetrachloride-pretreated mice. *Bars* are SE of mean. *Significantly different ($P < 0.05$) to the non-carbon-tetrachloride-treated group. *Figures in parentheses* are the number of 60-day survivors/number of animals in each group

Pharmacokinetics

Plasma concentrations of cyclophosphamide in untreated and carbon-tetrachloride-pretreated mice are shown in Fig. 3. The peak cyclophosphamide plasma concentration measured in this study 5 min after IP administration of the drug was 48% higher in carbon-tetrachloride-pretreated mice compared to untreated mice. Cyclophosphamide plasma concentrations remained higher in carbon-tetrachloride-pretreated compared to untreated mice at all time points. Elimination of cyclophosphamide was biphasic, but there was insufficient data to accurately characterize the second phase of disposition. The apparent half-time for the initial phase of cyclophosphamide disappearance, ignoring the contribution of the second phase, was 9.0 min in untreated mice and 20.4 min in carbon-tetrachloride-pretreated mice. Plasma total alkylating activity after cyclophosphamide administration reached a peak later, but was maintained at higher levels for longer in carbon-tetrachloride-pretreated mice compared to untreated mice (Fig. 4). The ratio of the area under the concentration time curve (AUC) for plasma alkylating activity in carbon-tetrachloride-pretreated compared to untreated mice was 1.04. Plasma concentrations of 4-hydroxycyclophosphamide after cyclophosphamide administration reached a peak later, and

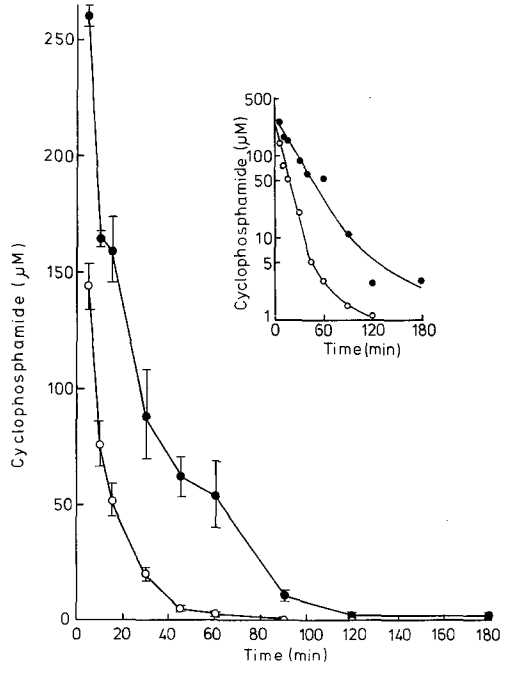

Fig. 3. Effect of carbon tetrachloride pretreatment upon plasma concentrations of cyclophosphamide following an IP dose of 65 mg/kg cyclophosphamide. (○) Mice receiving cyclophosphamide alone; (●) mice treated with IP carbon tetrachloride, 0,32 ml/kg 24 h previous to receiving cyclophosphamide. *Each point* represents mean of six mice, *bars* are SE of mean. *Inset* is a semilogarithmic plot of the same data

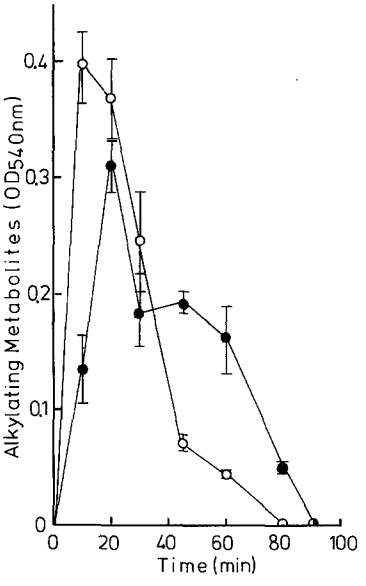

Fig. 4. Effect of carbon-tetrachloride pretreatment on plasma total alkylating activity following an IP dose of 65 mg/kg cyclophosphamide. (○) Control mice, $n = 3$; (●) mice treated IP with 0.32 ml carbon tetrachloride 24 h previously, $n = 5$. *Bars* are SE of mean, n = number of mice

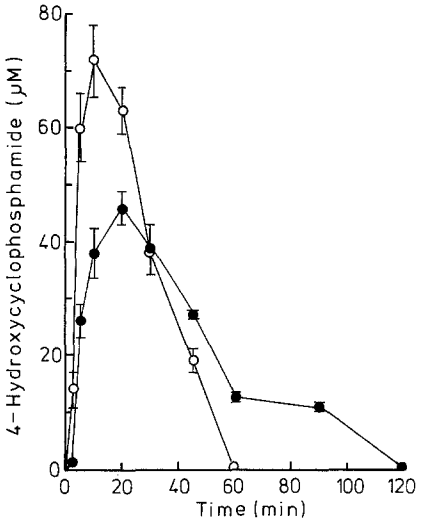

Fig. 5. Effect of carbon-tetrachloride pretreatment on plasma 4-hydroxycyclophosphamide following an IP dose of 65 mg/kg cyclophosphamide. (○) Control mice; (●) mice treated IP with 0.32 ml/kg carbon tetrachloride 24 h previously. *Each point* is the mean of six mice, *bars* are SE of mean

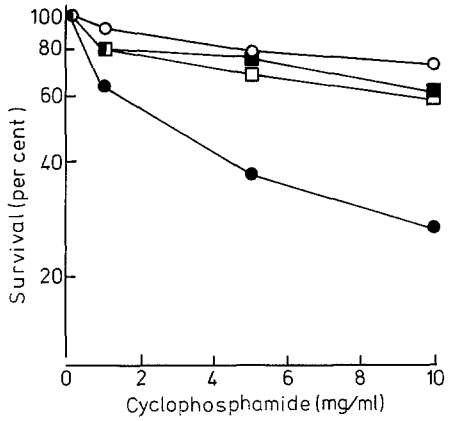

Fig. 6. Effect of carbon tetrachloride on the cytotoxicity of cyclophosphamide to Chinese hamster ovary cells in culture. (○) Cyclophosphamide alone; (●) cyclophosphamide and rat hepatocytes; (□) cyclophosphamide, 1 mM CCl_4 and rat hepatocytes; (■) cyclophosphamide and hepatocytes isolated from rats treated with carbon tetrachloride 24 h previously. Cells were exposed to drugs for 1 h and cell growth was measured by colony formation 10 days later.

over the first 30 min were lower in carbon-tetrachloride pretreated compared to untreated mice (Fig. 5). Between 30 and 90 min plasma 4-hydroxycyclophosphamide concentrations were higher in carbon-tetrachloride-pretreated than in untreated mice. The approximate half-life for plasma elimination of 4-hydroxycyclophosphamide in untreated mice was 12 min and in carbon-tetrachloride-pretreated mice 27 min. The ratio of AUC for plasma 4-hydroxycyclophosphamide in carbon-tetrachloride-pretreated compared to untreated mice was 1.06.

In vitro studies

The direct cytotoxicity of carbon tetrachloride and its effects on the cytoxicity of cyclophosphamide was studied using Chinese hamster ovary cells in culture. Exposure to 1 mM carbon tetrachloride for 1 h was not directly cytotoxic to cells in culture. Cyclophosphamide had little cytotoxicity even at high concentrations unless a rat hepatocyte activating system was included in the incubation. One millimolar carbon-tetrachloride did not affect the minimal cytotoxicity of cyclophosphamide in the absence of an acitvating systems (results not shown), nor did it affect the cytotoxicity of 4-hydroperoxycyclophosphamide. 4-Hydroperoxycyclophosphamide is known to break down rapidly under physiological conditions to 4-hydroxycyclophosphamide [30]. The IC_{50} of 4-hydroperoxycyclophosphamide alone was 6.5 g/ml and in the presence of 1 mM carbon tetrachloride 6.1 g/ml. Addition of 1 mM carbon tetrachloride to the incubation medium, however, blocked the activation of cyclophosphamide by rat hepatocytes, as did pretreating rats with carbon tetrachloride 24 h before preparing hepatocytes (Fig. 6).

Discussion

Although some reports suggest that the lethality of cyclophosphamide can be modified by treatments which alter microsomal mixed function oxidase activity [3, 6, 20], studies designed to improve the therapeutic efficacy of cyclophosphamide by pretreatment of experimental animals with drugs that alter the rate of cyclophosphamide activation by the hepatic microsomal mixed function oxidase have generally yielded inconclusive results [1, 10, 14, 17, 20, 22, 34]. It was surprising to find that pretreatment of mice with carbon tetrachloride, a well-known hepatotoxin and inhibitor of hepatic microsomal mixed function oxidase activity, increased the antitumor activity and toxicity of cyclophosphamide. The effect of carbon tetrachloride on the in vitro cytotoxicity of cyclophosphamide, as well as on the antitumor activity of other anticancer drugs, was as expected. Carbon tetrachloride alone exhibited no antitumor activity in vivo and in vitro exposure to 1 mM carbon tetrachloride in the incubation medium for 1 h was not toxic to Chinese hamster ovary cells. Carbon tetrachloride had no effect on the in vitro cytotoxicity of cyclophosphamide or 4-hydroxycyclophosphamide (formed from 4-hydroperoxycyclophosphamide), suggesting that the permiability of the cell membrane to these agents was not affected by carbon tetrachloride. However, the addition of 1 mM carbon tetrachloride to the incubation medium blocked the cytotoxic activation of cyclophosphamide by isolated hepatocytes, as did carbon tetrachloride pretreatment of rats from which hepatocytes were prepared. This is probably because carbon tetrachloride depresses levels of hepatic microsomal cytochrome P-450 and consequently blocks the bioactivation of cyclophosphamide by the isolated hepatocytes. The antitumor activity of BCNU, a drug which is inactivated by the hepatic microsomal mixed-function oxidase [29], was increased by carbon-tetrachloride pretreatment while the antitumor activity of 5-fluorouracil, a drug which is not metabolized by the hepatic microsomal mixed-function oxidase [37], was not altered by carbon-tetrachloride pretreatment.

In order to understand further the mechanism for the increase in antitumor activity of cyclophosphamide by carbon tetrachloride, the pharmacokinetics of cyclophosphamide and some of its metabolites was studied in untreated and carbon tetrachloride pretreated mice. The plasma elimination of cyclophosphamide in carbon-tetrachloride-pretreated mice was delayed with an apparent plasma half-time for the first phase of disappearance of 20.4 min compared to 9.0 min in untreated mice. Cyclophosphamide is metabolized by the

microsomal mixed-function oxidase [16] and the decrease in hepatic microsomal cytochrome P-450 produced by carbon-tetrachloride pretreatment probably accounts for the delayed elimination of cyclophosphamide. The rate of formation of plasma total alkylating metabolites, measured by reaction with 4-(p-nitrobenzyl) pyridine [15], was delayed and peak plasma alkylating activity was not as high in carbon-tetrachloride-pretreated as to in untreated mice. The AUC for total alkylating acitivy was, however, the same in carbon-tetrachloride-pretreated mice as in untreated mice because of the prolonged elevation of alkylating activity in carbon-tetrachloride-pretreated animals. The results of studies measuring total alkylating activity are difficult to interpret because the 4-(p-nitrobenzyl) pyridine reaction for alkylating activity is non specific and detects non alkylating cyclophosphamide metabolites such as carboxycyclophosphamide, in addition to alkylating metabolites [26].

4-Hydroxycyclophosphamide is thought to be the transport form of activated cyclophosphamide which is taken up by cells and which releases phophoramide mustard the ulimate alkylating species [9]. The rate of formation of 4-hydroxycyclophosphamide was decreased by carbon-tetrachloride pretreatment, but so also was its apparent rate of elimination. Measurable plasma concentrations of 4-hydroxycyclophosphamide were maintained for a longer period of time in carbon-tetrachloride-pretreated compared to untreated mice. It has been suggested that the actual plasma half-life for 4-hydroxycyclophosphamide is substantially shorter than its apparent plasma half-life and that the major determinant of the apparent plasma half-life is the rate of 4-hydroxycyclophosphamide formation from cyclophosphamide rather than its rate of removal [27, 32]. The slower apparent rate of elimination of 4-hydroxycyclophosphamide in carbon-tetrachloride-pretreated mice could, therefore, be a consequence of the delayed elimination of cyclophosphamide. Indeed, there is a good correlation between the rate of plasma elimination of 4-hydroxycyclophosphamide and the initial phase of plasma cyclophosphamide disappearance in both untreated and carbon-tetrachloride-pretreated mice. Another explanation for the delayed elimination of 4-hydroxycyclophosphamide could be that carbon-tetrachloride pretreatment inhibits enzymes involved in the further metabolism and inactivation of 4-hydroxycyclophosphamide. Hepatic aldehyde dehydrogenase, and enzyme thought to metabolize 4-hydroxycyclophosphamide [5, 7], is known to be inhibited by carbon-tetrachloride pretreatment in mice [24]. It is possible that both of the above mechanisms could contribute to the prolonged elevation of measurable levels of total alkylating activity and 4-hydroxycyclophosphamide in plasma, even though there is a decreased rate of formation of these metabolites from cyclophosphamide due to a carbon-tetrachloride-induced lowering of hepatic cytochrome P-450. Whatever the mechanism, the increased antitumor activity of cyclophosphamide in carbon-tetrachloride-pretreated mice is associated with lower, but prolonged measurable plasma concentrations of 4-hydroxycyclophosphamide or alkylating activity. These results suggest that the time of exposure of the tumor to a threshold concentration of 4-hydroxycyclophosphamide or other alkylating metabolite is more important for antitumor activity than either the peak concentration or the AUC of 4-hydroxycyclophosphamide or other alkylating metabolites. Voelcker and Haeglsperger [42], however, have concluded from studies in which cyclophosphamide and its metabolites were administered to nude mice with human breast cancer xenografts that tumor growth is more inhibited by high peak plasma concentrations than by maintained low concentrations of 4-hydroxycyclophosphamide. The reason for the differences in the two studies is not clear.

It might be possible to simulate the effects of the delayed elimination of cyclophosphamide metabolites produced by carbon tetrachloride by the administration of cyclophosphamide over a longer period of time. Evans et al. [2] have reported that the therapeutic index of cyclophosphamide in mice pretreated with cyclophosphamide can be improved by administering the drug in divided doses. Solidoro et al. [36] have administered cyclophosphamide to patients with acute lymphocytic leukemia as a continous 5-day IV infusion and reported preliminary evidence for an improved response rate compared to patients receiving cyclophosphamide as an oral daily single dose or an IV weekly injection [13, 23].

Cancer patients frequently exhibit signs of hepatic damage which can be due to their tumor or to drug treatment [41]. Hepatic malignancy can lead to lowered microsomal mixed-function oxidase activity [21]. Several anticancer drugs lower hepatic microsomal mixed-function oxidase in animals [8, 28, 33] and might do the same in human subjects. It cannot be assumed, however, that a lowered mixed-function oxidase activity is the only effect of liver damage. Changes in cyclophosphamide disposition similar to those produced by carbon tetrachloride in mice might occur in human subjects with hepatic disease. This could depend upon the extent of the decrease in the activity of the different enzyme systems responible for activation and inactivation of cyclophosphamide. The effects that such change might have upon the therapeutic efficacy and toxicity of cyclophosphamide in human subjects are not known.

In summary, pretreating mice with the hepatotoxin carbon tetrachloride results in an increase in the antitumor activity of cyclophosphamide. This is not due to a direct increase in the cytotoxicity of cyclophosphamide or its metabolites. The rate formation of alkylating metabolites and 4-hydroxycyclophosphamide from cyclophosphamide is decreased, presumably due to a marked decrease in hepatic microsomal cytochrome P-450, but measurable plasma levels of total alkylating activity and 4-hydroxycyclophosphamide are maintained for a longer time period in carbon-tetrachloride-pretreated compared to untreated mice. It is suggested that the maintained plasma concentrations of 4-hydroxycyclophosphamide might explain the increase in antitumor activity of cyclophosphamide.

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