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Studies on the mechanism of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) -induced hepatotoxicity

II. Biochemical and morphological characterization of the injury and its prevention by phenobarbital*

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Summary. The present study characterizes the biochemical, morphological, and histological sites of CCNU-induced hepatotoxicity and investigates the effect of modifiers of drug metabolism on this toxicity. A single oral dose (100 mg/kg) of CCNU caused four- and ninefold increases in serum GOT and GPT respectively 48 h after administration in rats. A 25-fold rise in serum bilirubin, a total loss of bile flow, and a decrease in BSP clearance were also observed. Cytochrome P-450 content and EM-N-demethylase activity were significantly decreased to 88% and 66% of control values respectively. A histopathological time course study of CCNU-induced injury showed a progression of acute inflammation, edema, and fibrin deposition in portal areas over 24 h with necrosis and sloughing of bile duct epithelium at 24 and 36 h. Treatment of rats with PB (40 mg/kg/day for 4 days, i.p.) 24 h prior to CCNU administration protected against CCNU-induced hepatotoxicity. Thus, the levels of serum GOT, GPT, and bilirubin were only 2.5 and 4 times higher than in untreated or PB-treated controls. Histopathological examination also showed reduced severity of bile duct lesions in PB-pretreated animals. In rats receiving both PB and CCNU, bile flow was restored and BSP clearance was increased compared to the CCNU-treated rats. The mixed-function oxidase activity in PB + CCNU-treated rats was not significantly different from that in PB-treated controls. It is concluded that pretreatment of rats with PB can markedly suppress the hepatotoxic manifestations, including histopathological changes, the rise in serum bilirubin, and the cholestasis observed in CCNU-treated rats.

Introduction

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea is a lipid-soluble antineoplastic agent and a known hepatotoxin. Serum enzymes increases and histologically observable damage have been reported following CCNU administration [5, 6, 9, 10]. Studies in our laboratory and others have revealed hyperbilirubinemia in rats after a single dose of BCNU and CCNU [6, 20]. Prolonged decreases of cytochrome P-450, cytochrome b_5, and heme contents of hepatic microsomes were reported after a single dose of CCNU [6, 13].

The role of metabolism in CCNU-induced hepatotoxic damage is not yet known. The antitumor activity and lethality of nitrosoureas, particularly CCNU, were shown to be decreased by PB pretreatment [11, 12, 16]. This effect was attributed to the PB induction of microsomal enzymes responsible for the detoxification of CCNU. Phenobarbital is also known to induce chloresis in rats [4] as well as to decrease serum bilirubin and bile acid levels in patients with intrahepatic cholestasis [1, 19].

This study is aimed at (a) characterization of the biochemical and histopathological cellular site of CCNU-induced hepatotoxicity and (b) evaluation of the effect of pretreatment with the microsomal enzyme inducer PB on the magnitude of CCNU-induced hyperbilirubinemia and cholestasis. Such investigation will clarify the mechanism by which PB alters the antitumor activity of CCNU and provide some understanding of the mechanism of chemoprevention by PB against nitrosourea toxicity.

Materials and methods

Materials. CCNU was a generous gift from Bristol Laboratories, Syracuse, N. Y. $NADP^+$, glucose-6-phosphate, 4-methylumbelliferone, ATP (equine), UDPGA, L-histidine, imidazole, and sodium deoxycholate were purchased from Sigma Chemical Co., St. Louis, Mo. BSP was purchased from Hynson, Westcott and Dunning, Inc., Baltimore, Md., glucose-6-phosphate dehydrogenase from Boehringer Mannheim Corp., New York, N. Y., and EM hydrochloride from Mallinckrodt Chemical Work, St. Louis, Mo.

Animal treatment. Male Sprague-Dawley rats from Charles River Laboratories, Wilmington, Mass. (weight 200–300 g except for bile flow studies, 300–400 g), were fed Purina Rat Chow ad libitum until killed. Unless otherwise indicated, the number of animals in each group was four. CCNU was given suspended in corn oil in a single oral dose of 12.5, 25, 50, or 100 mg/kg. For the time-course study, animals were sacrificed at 4, 6, 12, and 24 h following CCNU

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Abbreviations: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; PB, phenobarbital; AST, aspartate amine transferase; ALT, alanine amine transferase; AP, alkaline phosphatase; BSP, bromosulfophthalein; UDPGA, uridine diphosphoglucuronic acid; UDPGT, UDP, glucuronyl transferase; GT, glucuronide transferase; ATP, adenosine triphosphate; EM, ethylmorphine; $NADP^+$, nicotinamide adenine dinucleotide phosphate

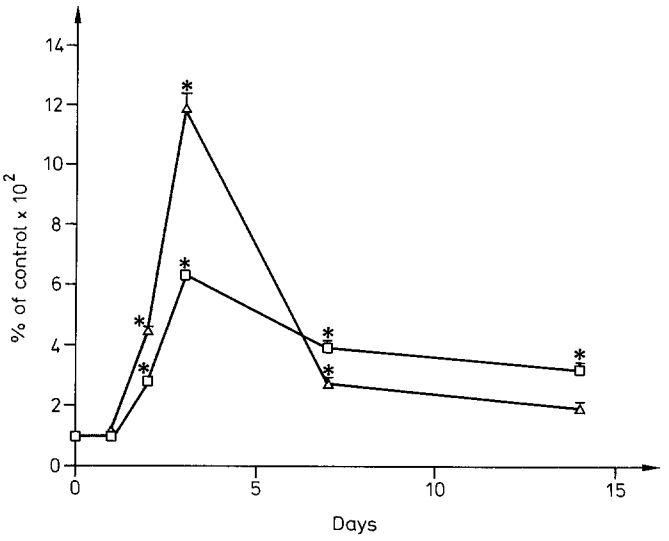

Fig. 1. Effect of CCNU on the serum levels of ALT (■) and AST (▲). Rats were given a single oral dose of CCNU (100 mg/kg) and killed at various time intervals thereafter. GPT and GOT levels were determined as described under *Methods.* *, significantly different from control ($P<0.05$)

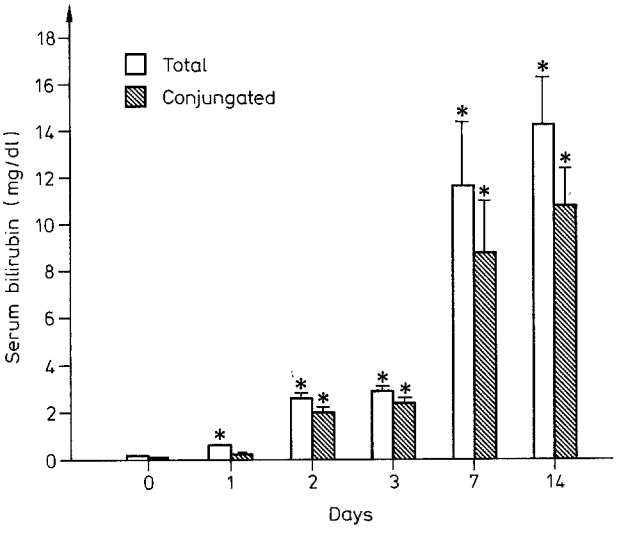

Fig. 2. Effect of CCNU on the serum levels of bilirubin. Rats were given a single oral dose (100 mg/kg) of CCNU, and killed at various times thereafter. Total and conjugated bilirubin were determined as described under *Methods.* $n=4$. *, significantly different from control ($P<0.05$)

administration. Control rats recieved 0.4 ml corn oil (p. o.). PB was injected i.p. in a dose of 40 mg/kg/day for 4 consecutive days. CCNU was given in a single oral dose of 100 mg/kg 48 h prior to killing and 24 h after the last dose of PB.

Sample collection. Animals were killed by decapitation. Blood was collected in conical centrifuge tubes and spun at 2000 rpm for 10 min. Serum was collected for the determination of serum levels of ALT, AST (Worthington Diagnostics, Freehold, N. J.), and total and conjugated bilirubin (Sigma kit 605-C). For metabolic enzyme determination livers were perfused in situ with ice-cold 1.15% KCl, excised, blotted dry, and weighed.

Histological studies. Rats were anesthetized with nembutal, and the portal vein cannulated. Livers were perfused in situ with saline (at 37 °C) and then with fixative (0.33% glutaraldehyde +0.5% paraformaldehyde in 0.05 *M* Pipes buffer at pH 7.35). Liver slices were stored in buffer at 4 °C. Slices were embedded in paraffin and routinely processed for light microscopy; Mallory's trichrome stained sections and PTAH fibrin stains were examined.

Bile flow studies. The main bile duct was cannulated with a PE-10 intramedic polyethylene tubing (Clays Adams, Parsipanny, N. J.) under light ether anesthesia. Bile was collected every half hour over a 6-h period. The body temperature was monitored and controlled by a heat lamp.

BSP clearance studies. Rats were injected i.v. with a 5% solution of BSP in saline, in a dose of 120 mg/kg. Blood (0.1 ml) was collected from a cannulated common iliac vein every 10 min over a 90-min period. An equivalent volume of saline was administered to replace blood. BSP clearance was determined as described by Berk et al. [3].

Enzyme determinations. Microsomes were prepared from liver homogenates as previously described [7]. Microsomal cytochrome P-450 and cytochrome b_5 contents were assayed by the method of Omura and Sato [17], and EM-*N*-demethylase activity was determined as previously described [8]. The method described by Bergmeyer [2] was used for the determinations of microsomal UDPGT activity, using 4-methylumbelliferone as substrate. Protein was determined by the method of Lowry et al. [14].

Statistical analysis. The data were expressed as mean ± SE and analyzed with Student's *t*-test. The 0.05 level of probability was used as the criterion for significance.

Results

Effect of CCNU on serum enzymes and bilirubin

Figure 1 shows the effect of a single oral (100 mg/kg) dose of CCNU on serum enzymes. Increases in ALT and AST to 903% and 415% of control values was first observed after 48 h of CCNU administration. Peak levels of 1190% and 626% of control values for ALT and AST respectively were oserved at 3 days. By day 14 after treatment the enzyme levels were 196% and 315% of controls, repsectively.

Increases in serum bilirubin were first apparent 24 h after CCNU administration: 25- and 20-fold increases of total and conjugated bilirubin over control levels were observed (Fig. 2). However, unlike the serum enzymes, serum bilirubin continued to increase, and by 14 days total and conjugated bilirubin were 140 and 110 times higher than control levels respectively.

Effect of CCNU on serum enzymes and bilirubin in PB-pretreated rats

When rats were pretreated with PB (40 mg/kg/day) for 4 consecutive days the CCNU-induced increases in serum ALT, AST, and bilirubin were sharply reduced (Table 1). When CCNU was given alone, levels of serum GPT and GOT and total bilirubin at 48 h were 9037%, 415%, and 2479% of control values respectively. In animals receiving

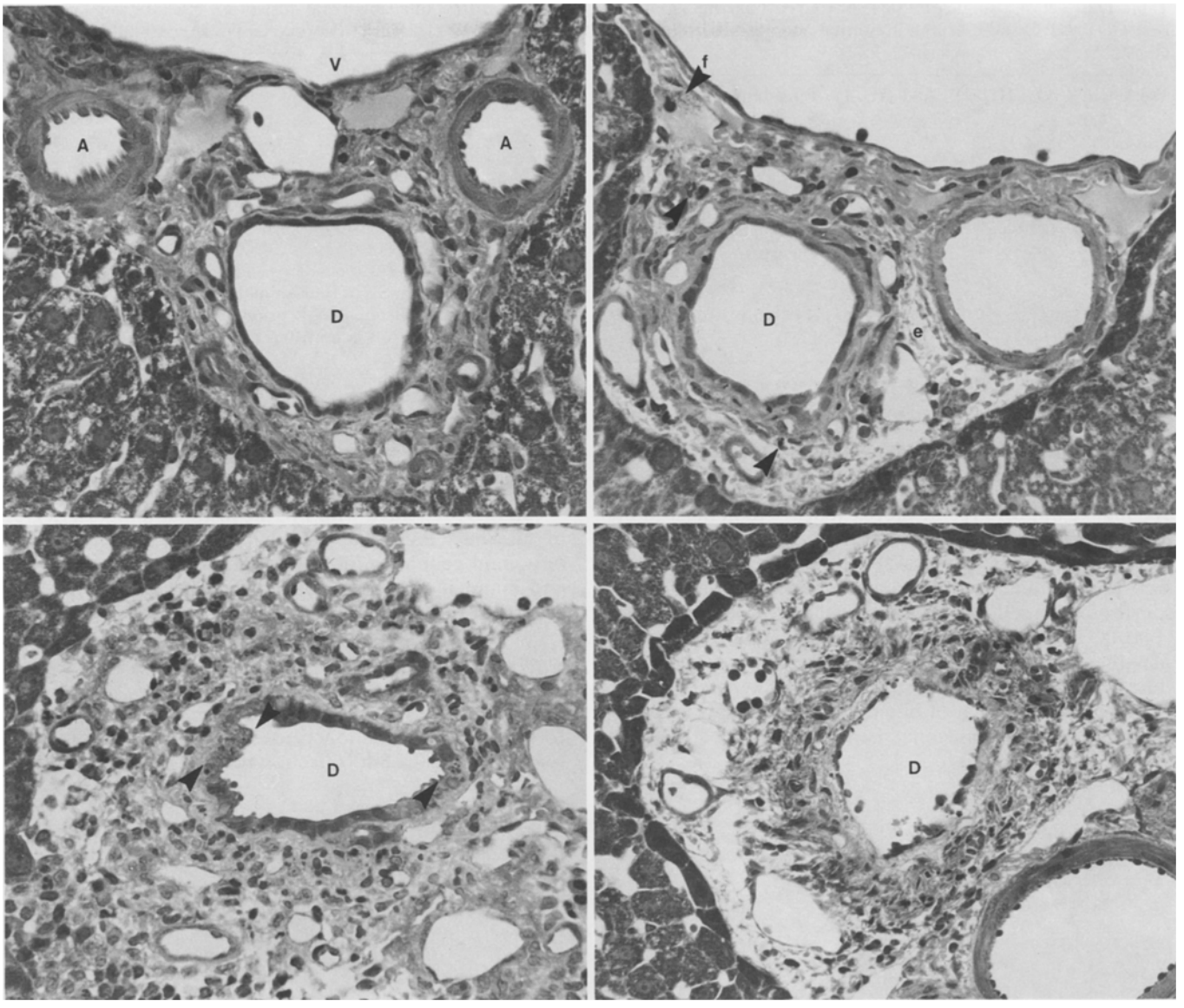

Fig. 3. Histopathological changes following administration of CCNU (50 mg/kg) as a function of time (Mallory's trichrome stain). *Upper left:* Section of portal area of control rat shows bile duct *(D)* lined by normal, low bile duct epithelium. Normal portal area consists of hepatic vein (V) and arteries (A), with a few capillaries and lymphatics also present ×370. *Upper right:* Section of portal area 6 h after CCNU shows a few infiltrating acute inflammatory cells *(arrows)*, interstitial edema *(e)*, and focal fibrin *(f)* which stains positively in special fibrin stains ×380. *Lower left:* Section of portal area 12 h after CCNU shows extensive infiltration by acute inflammatory cells, interstitial edema, and fibrin deposition. There is focal necrosis of bile duct epithelial cells *(arrows)*, although no sloughing of epithelium is seen. ×400. *Lower right:* Section of portal area 24 h after CCNU shows extensive inflammation, edema, and fibrin deposition as seen at 12 h, with complete necrosis of bile duct epithelial cells and sloughing of epithelium. At 36 h (not shown) the epitehlial cells are virtually completely sloughed. ×300

PB prior to CCNU, the corresponding values were significantly lower at 494%, 208%, and 393% of control values. PB treatment alone caused no significant changes in the levels of these enzymes or bilirubin content in serum.

Histopathological changes following CCNU administration

At 6 h following CCNU administration (50 mg/kg), the larger portal areas showed edema and occasional collections of interstitial fibrin and polymorphonuclear leukocytes (Fig. 3). Bile duct epitehlium was intact, and no pathological changes were seen in hepatocytes at this early time. By 12 h, bile duct epithelium showed variable staining of focal cells; increased edema, fibrin exudation, and acute inflammatory cells were present in the periductal interstitium. At 24 h, large areas of bile duct epithelium were necrotic, as evidenced by pyknotic nuclei and clumped cytoplasm, and epithelium was completely sloughed in many portal areas. At 36 and 48 h, the changes were essentially similar to those seen at 24 h but more bile duct epithelial sloughing was evident.

These morphological changes were present at all dose levels examined from 25 mg/kg to 100 mg/kg. Minimal changes were seen at doses less than 25 mg/kg. No clear gradient of changes was discerned over the dose range of 25–50 mg/kg, although the pathological alterations at

Table 1. Effect of a single oral dose of CCNU (100 mg/kg) on serum GOT, GPT, and bilirubin in normal and phenobarbital-treated rats

Treatment	ALT (IU/1)	AST (IU/1)	Bilirubin (mg/dl)	
			Total	Conjugated
Control	12.5± 0.5 (100)	54.4± 1.0 (100)	0.14±0.03 (100)	0.04±0.02 (100)
CCNU	113.8±18.0* (903)	225.6±20.5* (415)	3.47±0.44* (2479)	3.17±0.44* (7925)
PB	13.4± 2.5	58.2± 5.4	0.18±0.02	0.03±0.01
PB+CCNU	61.7±29.7* (494)	113.0±34* (208)	0.55±0.17 (393)	0.57±0.19* (1425)

Values are means ± SE. Values in parentheses are percent of control values
Animals were killed 48 h following CCNU treatment
* Significantly different from controls ($P < 0.05$)

Table 2. Effect of a single oral dose of CCNU (100 mg/kg) on bile flow and BSP clearance in normal and phenobarbital-treated rats

Treatment	Bile flow ($l/min^2/100$ g body wt)	BSP	
		Elimination $t_{1/2}$ (h)	Clearance (ml/min)
Control	2.58±0.50	31.70± 2.8	0.77±0.07
CCNU	0*	143.50±20.6*	0.18±0.03*
PB	7.31±0.59*	17.91± 3.3*	1.93±0.56*
PB+CCNU	1.97±0.52	91.80± 1.5*	0.24±0.01*

Values are means ± SE of four animals
CCNU was administered 48 h prior to bile cannulation or BSP administration. PB was administered as described in *Materials and methods*
* Significantly different from controls ($P < 0.05$)

100 mg/kg appeared to be more extensive than those at the lower doses at comparable times.

Focal hepatocyte necrosis beginning at 12 h after exposure was characterized by vacuolar degeneration of hepatocytes, with cell necrosis and macrophage infiltration (Fig. 4). These changes always affected small clusters of hepatocytes adjacent to the larger portal areas where bile duct injury was occurring, suggesting that hepatocyte injury is secondary to that of bile ducts. No evidence of widespread central or midzonal hepatocellular necrosis was seen.

In rats pretreated with PB, the histopathological degree of bile duct lesions appeared markedly decreased, with only mild cellular infiltration and edema of portal areas, and minimal or absent sloughing of bile duct epithelium observed (Fig. 4).

Effect of CCNU on bile flow and BSP clearance

Administration of CCNU to saline-treated rats 48 h prior to bile duct cannulation caused a complete cessation of bile flow over the 6-h collection period (Table 2). PB treat-

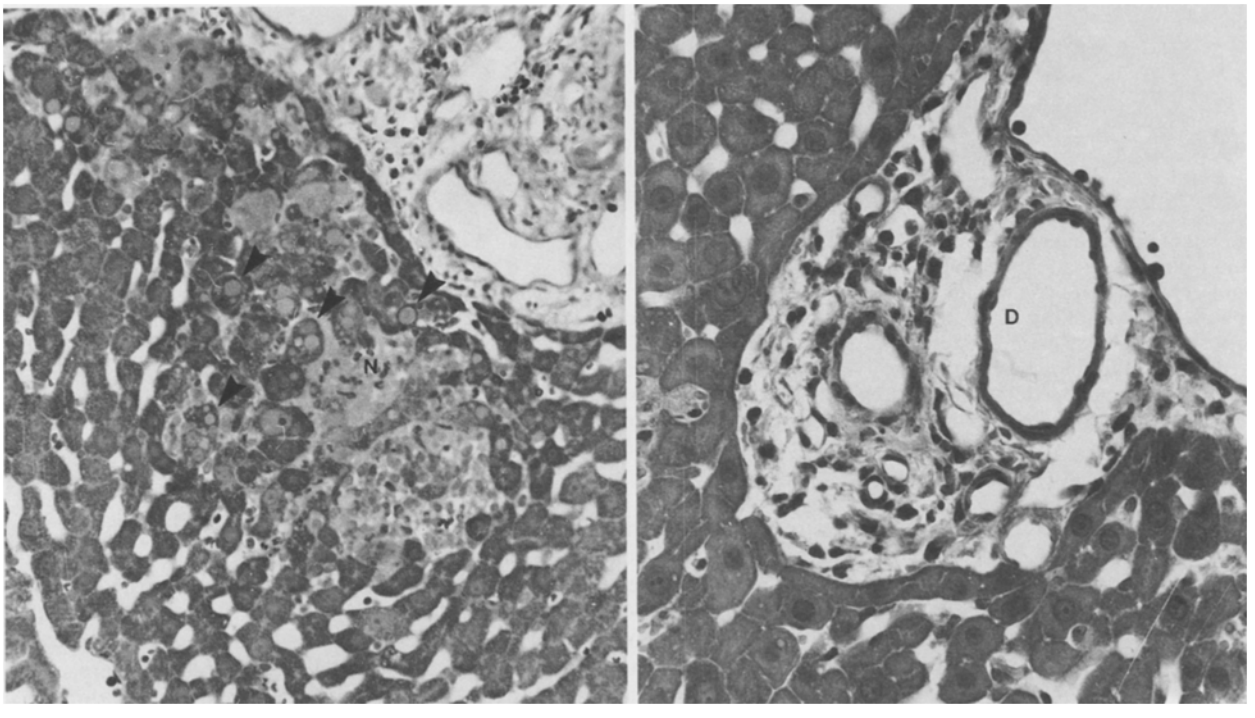

Fig. 4. Hepatocellular damage and protection by PB pretreatment (Mallory's trichrome stain). *Left:* At 12 h after 50 mg/kg CCNU, hepatocytes in areas adjacent to severely involved portal areas such as shown in Fig. 3 display focal necrosis *(N)* and vacuolar degeneration *(arrows)*. Such hepatocellular necrosis was spotty relative to the diffuse portal area changes. ×380. *Right:* Section of portal area from animal pretreated with PB, then given 50 mg/kg CCNU (24 h) shows only mild infiltration by acute inflammatory cells, mild edema, and intact bile duct epithelium *(d)*, in contrast to extensive damage shown in unretreated animals (see Fig. 3, lower right for comparison). ×235

Table 3. Effect of a single oral dose (100 mg/kg) of CCNU on hepatic microsomal hemoproteins and EM-*N*-demethylase activity in normal and phenobarbital-pretreated rats 48 h after CCNU administration

Treatment	Cytochrome b_5 (nmol/mg protein)	Cytochrome P-450 (nmol/mg protein)	EM *N*-Demethylase (nmol HCHO/min/mg protein)
Control	0.49 ± 0.02 (100)	0.48 ± 0.03 (100)	4.48 ± 1.24 (100)
CCNU	0.41 ± 0.02* (85)	0.42 ± 0.05 (88)*	3.09 ± 0.96 (66)*
PB	0.50 ± 0.02 (103)	0.99 ± 0.04* (205)	23.48 ± 2.48* (485)
PB + CCNU	0.49 ± 0.02 (100)	1.23 ± 0.08* (255)	20.28 ± 1.60* (419)

Values are means ± SE of four animals. Values in parentheses are percent of controls values
* Significantly different from controls ($P < 0.05$)

ment alone caused an expected rise in biliary flow to 7.31 µl/min/100 g body wt. When CCNU was given to PB-treated rats, biliary flow was 1.97 µl/min/100 g body wt, which was not significantly different from the saline-treated controls. The data in Table 2 also show the effect of CCNU on BSP elimination rate (t½) and clearance in saline-treated or PB-treated rats. In the saline-treated controls, the elimination t½ and clearance of BSP from plasma were 31.7 h and 0.77 ml/min respectively. Prolongation of elimination t½ to 143.5 h and a decrease in clearance to 0.18 ml/min were observed in CCNU-treated rats. When rats were treated with PB prior to CCNU the t½ (92 h) was intermediate between those of the saline-treated and CCNU-treated animals, and BSP clearance was 0.24 ml/min.

*Effect of CCNU on hepatic microsomal cytochrome P-450, cytochrome b_5, and EM-*N*-demethylase activity*

The data in Table 3 show that a single dose of CCNU caused a decrease in hepatic EM-*N*-demethylase activity to 66% of the control value 48 h after treatment. In PB-treated rats, hepatic cytochrome P-450 and EM-*N*-demethylase activities were 205% and 485% those of saline-treated controls respectively. When CCNU was given to PB-treated rats 48 h prior to killing, cytochrome P-450 and EM-*N*-demthylase activity were at 255% and 419% of control values respectively.

Table 4. Time course of the effect of a single oral dose (100 mg/kg) of CCNU on hepatic microsomal UDPGT activity

Days after treatment	UDPGT (nmol/min/mg protein)
0	23.36 ± 1.50 (100)
2	29.74 ± 2.32* (127)
7	37.60 ± 3.18* (161)
14	33.38 ± 3.74* (143)

Values are means ± SE of four animals. Values in parentheses are percent of controls values
* Significantly different from controls ($P < 0.05$)

Table 5. Effect of CCNU (100 mg/kg) on microsomal UDPGT activity in normal and phenobarbital-treated rats 48 h after CCNU treatment

Treatment	UDPGT (nmol/min/mg protein)
Control	22.34 ± 2.36 (100)
CCNU	32.10 ± 2.04* (144)
PB	44.52 ± 3.22* (199)
PB + CCNU	46.04 ± 1.14* (206)

Values are means ± SE of four animals. Values in parentheses are percent of controls values
* Significantly different from controls ($P < 0.05$)

Effect of CCNU on hepatic microsomal UDP-glucuronyl transferase activity

The data in Table 4 show that CCNU caused a moderate but significant rise in hepatic UDPGT activity as a function of time, reaching 161% of control values at day 7 after administration. In PB-treated rats, UDPGT activity increased to 199% of control values (Table 5). This twofold rise was not significantly altered in rats receiving both PB and CCNU.

Discussion

Our studies, in agreement with previous reports [5, 10], show that CCNU hepatotoxicity is manifested by rises in serum AST, ALT, and bilirubin. In our study, ALT and AST were maximal at day 3 and declining by day 14, whereas serum bilirubin levels continued to increase.

There are several lines of evidence to indicate that the continued elevation of serum bilirubin is due to an accumulation of unexcreted conjugated bilirubin. Serum bilirubin levels were mostly in the conjugated form, which points to the capacity of the hepatocyte to take bilirubin from the circulation and conjugate it, but its inability to excrete it in the bile. In fact, the process of conjugation itself was not affected by CCNU treatment. Indeed, somewhat higher activities of UDPGT were observed in CCNU-treated rats (Table 4).

These findings suggest the involvement of the biliary tree as a primary target in CCNU hepatotoxicity. The total lack of bile flow and the decrease in BSP clearance in rats treated with CCNU further point to a primary lesion in the biliary excretory system. Our histopathological study substantiates this primary biliary lesion. Previous evidence of biliary damage and increases in AP activity, which are markers of bile duct damage, were shown by other authors [9, 10]. Our previous work showed that the increase in serum bilirubin is not due to an increase in the turnover of heme [7]. Therefore, we conclude that the bilirubinemia in CCNU-treated rats is due to the continued accumulation of unexcreted conjugated bilirubin secondary to the primary biliary lesion. Subsequent later hepatocyte injury in liver cells near to the portal areas may, in fact, be secondary to the initial bile duct lesion.

In this study, we show that PB is capable of ameliorating all the hepatotoxic manifestations of CCNU. Thus, the

increases in the serum levels of GOT, GPT, and bilirubin were all significantly reduced in PB-pretreated rats. Biliary flow was restored and BSP clearance was also increased. Thedecline in cytochrome P-450 content and EM-*N*-demethylase activity after CCNU treatment were not observed in PB-pretreated rats. Induction of these enzymes by PB was unaltered in animals receiving both PB and CCNU. Previous results from our laboratory [7] also showed that PB was capable of reversing the depression in heme biosynthesis in the liver, but not spleen, of CCNU-treated rats. PB has also been shown to decrease the antitumor activity and lethality of CCNU in mice [11, 12, 16].

The effects of PB per se on biliary functions are well documented. PB is known to induce hypercholeresis in rats [4]. It has also been shown to decrease the bile acid and bilirubin content of plasma in cases of obstructive intrahepatic cholestasis [1, 19]. It has been suggested [19] that in cholestatic jaundice, bile salt accumulation may lead to a decrease in drug metabolism and cytochrome P-450 content. Induction of cytochrome P-450 as well as GT by PB increases the hydroxylation and conjugation of bile acids, rendering them more polar and excretable.

Although the present study is an example of chemoprevention of the toxicity of a good antitumor agent and a derivative of the environmental carcinogen nitrosamine, no detailed investigations are available on the actual mechanism by which PB prevents or intervenes with nitrosourea hepatotoxicity. Most published investigations using PB pretreatment focus on the protection against animal death or simple measurement of alterations in tumor growth in tumor-bearing animals treated with nitrosourea.

Reversal by PB of the adverse biliary manifestations of nitrosourea may be due to the following mechanisms:

1. PB may alter the metabolism of CCNU, enhancing its detoxification and/or shifting the metabolic profile to a less toxic chemical species.

2. PB may act directly by reversing cholestasis and enhancing biliary excretion and washout of CCNU toxic metabolites and other bile acid derivatives from the liver.

With regard to the first hypothesis, in vitro studies [15, 18] have shown that microsomes from PB-pretreated animals cause a shift in the hydroxylated metabolites of CCNU to more of the cis-4-hydroxy metabolite, compared with microsomes from saline-treated rats. This metabolite, however, is more cytotoxic [21]. So far, the exact chemical species of CCNU metabolites which causes the biliary damage has not been identified.

A question is thus raised regarding the role of PB in reversing the biliary toxicity of CCNU: Does PB reverse the symptoms of cholestasis, or does it reverse the initial damage by CCNU and/or its metabolite by altering the CCNU metabolic pattern? Current work in our laboratory is directed toward answering this question.

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