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Prolonged depression of hepatic microsomal drug metabolism and hemoprotein levels following a single dose of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)

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CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] is an alkylating and carbamoylating drug with antineoplastic activity against numerous human and animal tumors. Animal toxicity of high doses of CCNU has been noted toward bone marrow, lymphoid tissue, gastrointestinal tract, liver, and kidney [1]. A delayed and serious hepatotoxicity has been observed to occur at low doses as well [1–3]. Increases in serum alkaline phosphatase, along with increases in lactic dehydrogenase (LDH) isoenzyme No. 5 have been reported to occur in dogs as long as 60–90 days after a single oral dose of the drug [3], and various histologic changes in liver first occur 3–4 weeks after treatment and persist for 12 weeks after a single dose [3]. In patients, hepatotoxicity has occurred 2 weeks after treatment with nitrosoureas and persisted for up to 4 months [4]. CCNU has been shown to be metabolized by the hepatic cytochrome P-450-dependent monooxygenase system [5, 6] and to bind to cytochrome(s) P-450 [6]. A single dose of CCNU reduced *in vitro* microsomal drug metabolism for up to at least 2 weeks [7]. To investigate the mechanism of this prolonged effect, the temporal aspects of the response of other hemoproteins, in addition to cytochrome P-450, to CCNU treatment, and the interaction of CCNU with microsomal enzyme induction have been examined, and the results are reported in this communication.

Male Sprague–Dawley rats (200–225 g; Taconic Farms, Pearl River, NY) were treated intraperitoneally (i.p.) with 40 mg/kg of CCNU. This dose was approximately an LD_{10} (30 days). Although body weights were often very low and animals appeared quite debilitated, no more than 25 per cent mortality ever occurred. This dose was selected because preliminary experiments showed that a higher dose (60 mg/kg) produced large numbers of animal deaths and that a lower dose (20 mg/kg) produced similar effects which were quantitatively less significant. The vehicle was absolute ethyl alcohol–Emulphor 620–0.9% NaCl (1:1:10). At various times after a single dose, rats were killed by cervical dislocation. Livers were perfused *in situ* with cold 0.15 M KCl containing 50 mM Tris–HCl (pH 7.4; KCl–Tris) to remove as much residual blood as possible. Livers were then removed and homogenized in 3 vol. of KCl–Tris. The homogenate was centrifuged at 9000 *g* for 20 min, and the supernatant fraction was centrifuged at 105,000 *g* for 60 min. The 105,000 *g* pellet was resuspended in KCl–Tris, and the suspension was centrifuged again at 105,000 *g* for 60 min to remove traces of hemoglobin. The washed microsomes were resuspended in KCl–Tris and the protein content was determined [8]. NADPH–cytochrome *c* reductase, aminopyrine *N*-demethylase, and biphenyl 4-hydroxylase activities were determined by standard methods described in detail previously [9]. The NADH-reduced difference spectrum of CO-exposed cytochrome P-450 was determined by the method of Omura and Sato [10] using extinction coefficients of 185 cm^{-1} mM^{-1} (Δ426–409 nm) and 91 cm^{-1} mM^{-1} (Δ450–490 nm) respectively. Total heme was determined by the pyridine hemochromogen technique as described by Falk [11] using an extinction coefficient of 32.4 cm^{-1} nM^{-1} (Δ556–575 nm). Phenobarbital-Na (PB; 75 mg/kg) was administered i.p. in 0.9% NaCl daily for 4 days except where noted. 3-Methylcholanthrene (3-MC; 25 mg/kg) was administered i.p. in corn oil daily for 2 days. Animals were killed and microsomal drug metabolism was analyzed 24 hr after the last dose of the inducing agent. Control animals received equivalent volumes of vehicle. Each treatment group contained four animals, except in induction studies where there were three animals per group. Results are presented as the mean ± 1 S.D. Differences between control and treatment means were evaluated for significance using a two-tailed Student's *t*-test at $P < 0.05$.

Figure 1 shows the onset of the effect of a single dose of CCNU on microsomal hemoprotein content and *in vitro* drug metabolism. No effect was observed at 24 hr after treatment, but by 36 hr aminopyrine demethylase and cytochrome P-450 were reduced significantly. By 48 hr all variables except total heme were reduced, and all four variables were reduced by 72 hr after treatment. Figure 2 shows the persistence of the effect. Cytochrome P-450 levels remained at approximately 60–70 per cent of control for 5 weeks after treatment. Cytochrome b_5 was decreased significantly only 3, 14, and 21 days after treatment. The activity of aminopyrine demethylase was the most severely reduced of all variables studied, with values that were approximately 50 per cent of normal persisting for 6 weeks after treatment. The nadir of the effect of CCNU on indicators of microsomal drug metabolism and hemoprotein levels was between 2 and 5 weeks after treatment for all affected variables; significant recovery began after 5–6 weeks. Neither NADPH–cytochrome *c* reductase activity nor microsomal protein contents were consistently affected by CCNU treatment at any of the times assayed.

The ability of PB to induce microsomal enzymes concomitant with CCNU treatment is demonstrated by the

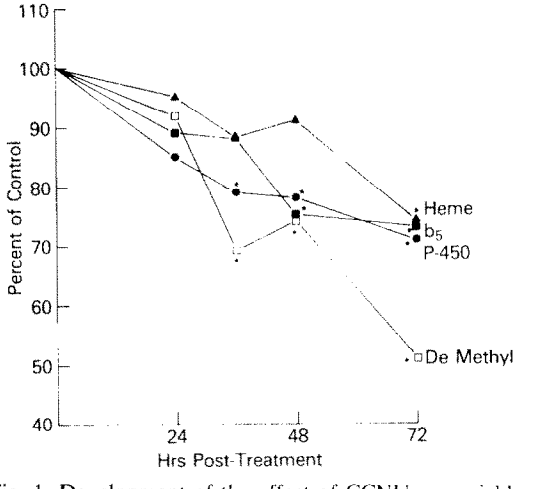

Fig. 1. Development of the effect of CCNU on variables of hepatic microsomal drug metabolism during the first 72 hr after a single i.p. dose. Each point is the mean of four animals; an asterisk (*) indicates a significant difference from the control value at $P < 0.05$. Control values for the four variables are: heme, 1.41 ± 0.18 nmoles/mg; P-450, 1.11 ± 0.16 nmoles/mg; b_5, 0.54 ± 0.09 nmole/mg; and demethylation, 9.51 ± 1.34 nmoles · mg^{-1} · min^{-1}.

Table 1. Interaction between CCNU effects on microsomal drug metabolism and microsomal enzyme induction following phenobarbital treatment*

Experiment†	Treatment	Cytochromes P-450	Cytochromes b_5	Total Heme	Aminopyrine demethylation
1	PB	158‡	81	159‡	163‡
	CCNU	65‡	85	82	55‡
	PB + CCNU	200‡	94	153‡	226‡
2	PB	178‡	108	202‡	234‡
	CCNU	79‡	81‡	89	87
	PB + CCNU	193‡	134‡	214‡	263‡
3	PB	186‡	96	153‡	159‡
	CCNU	73‡	84	82	50‡
	CCNU + PB	216‡	107	157‡	248‡

* Data are presented as percent of control. Control values (N = 5) were: P-450, 1.17 ± 0.12 nmole/mg; b_5, 0.43 ± 0.09 nmole/mg; total heme, 1.35 ± 0.13 nmole/mg; and demethylation, 8.16 ± 0.57 nmoles·mg^{-1}·min^{-1}.

† Experimental protocols were as follows: Exp. 1: CCNU administration on day 1; PB administration days 14–17; analysis on day 18. Exp. 2: PB administration Monday through Thursday; CCNU administration on Wednesday; analysis on Friday. Exp 3: CCNU administered on Sunday; PB treatment on Monday through Thursday; analysis on Friday.

‡ Significantly different from control value ($P < 0.05$, N = 3).

data presented in Table 1. Using several different treatment sequences, PB was in all cases able to produce a normal increase in enzyme activity or hemoprotein level. In addition, PB induction was normal 4 weeks after CCNU treatment and also when PB was administered 1 hr after CCNU treatment and assayed 24 hr later (data not presented). In separate experiments using a standard inducing regimen of 3-MC in treatment protocols similar to experiments 1 and 3 in Table 1, 3-MC was similarly shown to produce normal induction of biphenyl hydroxylase activity and cytochrome(s) P-450 following CCNU treatment (data not presented).

Results presented in this paper show that a single nonlethal dose of CCNU reduced microsomal drug metabolism, including levels of cytochrome(s) P-450, by 40–60 per cent for up to 6 weeks. To explain such a prolonged effect, the interaction of CCNU with the genetic mechanism controlling P-450 synthesis and/or degradation is probably required. The interaction of CCNU with nucleic acids is well established [12] and it has been proposed [13] that the long-term effects of nitrosoureas such as CCNU and its structural analog BCNU are due to a selective inhibition of DNA transcription, a mechanism that would be compatible with the prolonged effect of CCNU observed in the present study. In addition, a single dose of BCNU has been shown to reduce *in vivo* thymidine incorporation into tumor DNA by 90 per cent for at least 8 days [14]. This striking decrease in DNA synthesis is similar to the present result, where a 40–60 per cent decrease in drug metabolism was observed for 4–6 weeks. Results similar to those cited [14] have not been published for CCNU or for liver, but it is reasonable to postulate that CCNU might have a similar prolonged effect on P-450 metabolism in liver.

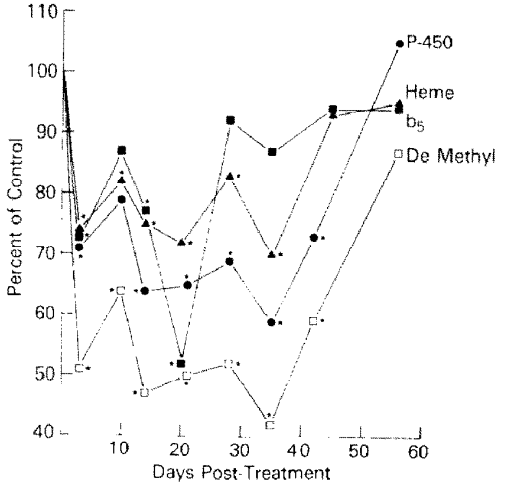

Fig. 2. Persistence of the effect of a single dose of CCNU on variables of hepatic microsomal drug metabolism. Each point is the mean of four animals; an asterisk (*) indicates a significant difference from the control value at $P < 0.05$. Control values are the same as presented in the legend of Fig. 1.

The 36-hr delay between drug treatment and the decrease in P-450 levels is surprising considering the short biological half-life of CCNU. Hilton and Walker [15] showed that only 50 per cent of an i.v. dose of CCNU was recoverable as parent drug 5 min after administration. If, as has been suggested [7], CCNU decomposes with the formation of reactive intermediates, a rapid interaction of CCNU with reactive tissue sites might be expected. The 36-hr delay approximates the 42- to 44-hr half-life of the heme moiety of P-450 [16, 17] and suggests a defect in either synthesis of heme or in assembly of the P-450 molecule. The experiments showing normal response to phenobarbital and 3-MC following CCNU treatment, however, suggest that the mechanisms for syntheses of both heme and protein have not been altered permanently by exposure to CCNU.

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