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SHORT COMMUNICATIONS

Investigation of the mechanism by which cyclophosphamide alters cytochrome P450 in male rats

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Abstract—The effects of administration of the cytotoxic agent cyclophosphamide on cytochrome P450 have been examined in the liver microsomes of male rats. Microsomes were prepared after cyclophosphamide administration 1, 4 or 7 days prior to killing. The coadministration of cyclophosphamide with *N*-acetylcysteine has also been investigated. The microsomes were assayed for NADPH cytochrome P450 reductase, aminopyrine demethylase, erythromycin demethylase and androstenedione hydroxylase activities. Activities were generally unchanged 1 and 4 days after cyclophosphamide administration and were significantly decreased at 7 days. *N*-Acetylcysteine did not alter the effects of cyclophosphamide at 7 days. The effect of cyclophosphamide *in vitro* has also been examined. Microsomes from untreated animals were subjected to the above assays following *in vitro* metabolic activation of cyclophosphamide in a reconstituted system in the presence and absence of *N*-acetylcysteine. All enzyme activities were significantly reduced by the cyclophosphamide metabolites. The presence of *N*-acetylcysteine prevented this inactivation. The results of these investigations suggest that cyclophosphamide inactivates hepatic cytochrome P450 *in vitro* and *in vivo* via different mechanisms.

Cyclophosphamide (CP*), an oxazophosphorine derivative of the classical alkylating agent nitrogen mustard, is commonly used in cancer chemotherapy and as an immunosuppressant [1]. CP requires metabolic activation by cytochrome P450 to exhibit alkylating activity. The proposed mechanism of CP activation is by an initial hydroxylation at C-4 [2]. Cytochromes 2C11 and 2C6 have been shown to be the major catalysts responsible for the bioactivation of CP in uninduced rat liver microsomes [3]. The resulting 4-hydroxy-CP exists in equilibrium with its ring-opened tautomer, aldophosphamide, which may subsequently undergo β elimination to produce acrolein and phosphoramide mustard. Phosphoramide mustard appears to be the therapeutically significant metabolite. Acrolein has been implicated as the metabolite responsible for CP-induced deactivation of cytochromes P450 *in vivo* [4] and *in vitro* [5]. The underlying mechanism has been suggested by Gurtoo and co-workers [4, 5] to involve covalent interaction between acrolein and cytochrome P450 critical thiols. Chemicals containing free sulphydryl groups have been shown to protect against acrolein-induced denaturation of cytochrome P450 *in vivo* [4] and *in vitro* [5]. Le Blanc and Waxman [6] have examined the effect of CP on specific cytochrome P450 isoenzymes in male rats *in vivo* and reported that the activities of the testosterone-dependent isoenzymes 2A2, 3A2 and 2C11 are suppressed while the activities of the testosterone-independent isoenzyme 2A1 and the P450-independent enzyme steroid 5α-reductase are elevated following CP administration. They conclude that the suppression cannot be attributed to direct inactivation by acrolein. Although the effects of CP on cytochrome P450 have been studied extensively, there are apparent discrepancies in the literature. The present study examines the effects of CP treatment on rat hepatic cytochrome P450 both *in vivo* and *in vitro* and the effects of CP in the presence of NAC, an agent that provides free sulphydryl groups. We propose that CP itself or a metabolite of CP other than acrolein is altering drug metabolizing capacity via an indirect mechanism.

* Abbreviations: CP, cyclophosphamide; NAC, *N*-acetylcysteine; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxy-androst-4-ene-3-one.

Materials and Methods

Materials. Aminopyrine and cytochrome *c* were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). CP was purchased from Farmitalia Carlo Erba (Hawthorn, Vic). NAC was purchased from Glaxo (Boronia, Vic). Sources of other materials were as previously described [7].

Animals. Male Hooded Wistar rats (180–200 g) were obtained from the Animal Resource Centre (Gilles Plains, South Australia). Animals were housed in metal cages and allowed free access to food and water. The animals were randomly assigned to six treatment groups (N = 6). One group was administered NaCl (0.9%) solution as a single i.p. dose and killed 7 days after administration. Three groups were administered CP (200 mg/kg) freshly prepared in NaCl (0.9%) as a single i.p. dose and killed on days 1, 4 and 7. Another group was coadministered CP and NAC where the NAC was given 0.5 hr before (200 mg/kg) and 0.5 hr after (200 mg/kg) CP (200 mg/kg), such that the total NAC dose was 400 mg/kg, and killed 7 days later. The final group received NAC (400 mg/kg) but no CP and were killed after 7 days. The same dose schedule of NAC was used where NAC was given before and after saline.

Microsomes were prepared from animals in a manner described previously [8].

Assays. Protein concentration, P450 specific content and microsomal androstenedione hydroxylase activity were assayed as previously described [7]. The measurement of NADPH cytochrome P450 reductase activity was performed essentially according to the method of Williams and Kamin [9]. The assay was conducted at room temperature where 1.0 mL reaction mixture contained 0.3 M phosphate buffer, pH 7.7, 0.1 mM NADPH, 1 mM KCN and 40 μM cytochrome *c* using an extinction coefficient of 21 $cm^2/mmol$ at 550 nm. Aminopyrine and erythromycin demethylase activities were measured using substrate concentrations of 25 and 0.4 mM, respectively, where the formaldehyde formed was assayed by the method of Nash [10].

Effect of CP metabolites in the presence and absence of NAC on cytochrome P450 in vitro. The incubation mixture in a volume of 3.0 mL in phosphate buffer (0.1 M, pH 7.4) contained the following: CP 5 mM, microsomal protein 2.5 mg, glucose-6-phosphate (4 mM), glucose-6-phosphate dehydrogenase (3 IU), $MgCl_2$ (8 mM) and NADP (1 mM).

Table 1. Influence of CP treatment on hepatic microsomal P450 content, NADPH P450 reductase activity, aminopyrine demethylase activity and erythromycin demethylase activity

Treatment	P450 content (nmol/mg)	NADPH P450 reductase activity (nmol/mg/min)	Aminopyrine demethylase activity (nmol/mg/min)	Erythromycin demethylase activity (nmol/mg/min)
Saline control 7 day	0.60 ± 0.07	118.70 ± 14.91	6.15 ± 0.89	1.52 ± 0.30
CP 1 day	0.62 ± 0.11 (103)	138.90 ± 21.51* (117)	7.01 ± 0.98 (114)	1.47 ± 0.21 (97)
CP 4 day	0.63 ± 0.04 (105)	102.67 ± 14.24 (87)	9.51 ± 0.66* (155)	1.60 ± 0.24 (105)
CP 7 day	0.30 ± 0.09* (50)	60.90 ± 8.22* (51)	4.68 ± 0.68 (76)	0.89 ± 0.30* (59)
NAC 7 day	0.58 ± 0.04 (97)	114.47 ± 7.89 (96)	7.75 ± 0.48* (126)	1.37 ± 0.26 (90)
CP + NAC 7 day	0.30 ± 0.02* (50)	62.92 ± 4.38* (53)	4.14 ± 0.34* (67)	0.49 ± 0.13* (32)

Activity values represent the mean ± SD for N = 6 individual rat liver microsome preparations.
Numbers in parentheses indicate percentage activity relative to saline 7 day control values.
* Significantly different relative to saline 7 day controls at $P < 0.05$.

Table 2. Influence of CP treatment on hepatic microsomal androstenedione 7α-hydroxylase, 6β-hydroxylase, 16β-hydroxylase and 16α-hydroxylase activities

Treatment	Androstenedione 7α-hydroxylase activity (nmol/mg/min)	Androstenedione 6β-hydroxylase activity (nmol/mg/min)	Androstenedione 16β-hydroxylase activity (nmol/mg/min)	Androstenedione 16α-hydroxylase activity (nmol/mg/min)
Saline control 7 day	0.33 ± 0.10	1.51 ± 0.18	0.23 ± 0.08	1.68 ± 0.54
CP 1 day	0.35 ± 0.07 (106)	1.53 ± 0.29 (101)	0.41 ± 0.11* (178)	1.62 ± 0.18 (96)
CP 4 day	0.42 ± 0.05 (127)	1.77 ± 0.22 (117)	0.30 ± 0.05 (130)	2.16 ± 0.57 (129)
CP 7 day	0.13 ± 0.04* (39)	0.47 ± 0.30* (31)	0.09 ± 0.02* (39)	0.58 ± 0.06* (35)
NAC 7 day	0.45 ± 0.05* (136)	2.08 ± 0.23* (138)	0.32 ± 0.05 (139)	2.42 ± 0.24* (144)
CP + NAC 7 day	0.19 ± 0.06* (58)	0.64 ± 0.26* (42)	0.12 ± 0.06* (52)	0.62 ± 0.19* (37)

Activity values represent the mean ± SD for N = 6 individual rat liver microsome preparations.
Numbers in parentheses indicate percentage activity relative to saline 7 day control values.
* Significantly different relative to saline 7 day controls at $P < 0.05$.

The reaction was initiated by the addition of NADP and the incubation continued for 45 min at 37°. Some incubations also contained 5 mM NAC. Control incubations did not contain NADP. At the end of the incubation period, the microsomes were subjected to various assays.

Statistical analysis. Differences between parameters for the different regimens were compared using analysis of variance and subsequently the Dunnett's multiple range test at $P = 0.05$.

Results and Discussion

Results presented in Table 1 demonstrate that 1 and 4 days after CP (200 mg/kg) was administered, there was no significant alteration in cytochrome P450 content and erythromycin demethylase activity. Aminopyrine demethylase activity was not significantly altered on day 1 but was significantly elevated on day 4. In contrast, NADPH cytochrome P450 reductase activity was significantly increased after 1 day with no change at 4 days. However, 7 days after CP administration, there was a significant decrease in all these parameters. Androstenedione undergoes regio- and stereospecific metabolism by distinct cytochrome P450 isoenzymes. The activity of the isoenzymes 2A1/2, 3A2, 2B1 and 2C11 can be determined by measuring the conversion of androstenedione to 7α-, 6β-, 16β- and 16α-hydroxyandrostenedione, respectively [11]. It appears that 1 and 4 days after CP administration there was no significant change in the activity of the isoenzymes 2A1/2, 3A2 and 2C11 in Hooded Wistar rats (Table 2). The activity of 2B1 was significantly increased on day 1 but returns to control values on day 4 (Table 2). In parallel with decreases observed in cytochrome P450 content, NADPH cytochrome P450 reductase, and erythromycin and aminopyrine demethylase activities, 7 days after CP administration there was a significant depression in the activity of the isoenzymes 2A1/2, 3A2, 2B1 and 2C11 (Table 2). As the isoenzyme

Table 3. Influence of CP metabolites on cytochrome P450 *in vitro* in the presence and absence of NAC

	CP *in vitro* (−NADP)	CP *in vitro* (+NADP)	CP + NAC *in vitro* (−NADP)	CP + NAC *in vitro* (+NADP)†
P450 content (nmol/mg)	1.04 ± 0.13	0.54 ± 0.09* (52)	0.95 ± 0.15	1.05 ± 0.13 (111)
NADPH P450 reductase activity (nmol/mg/min)	156.01 ± 16.91	111.6 ± 14.20* (74)	96.73 ± 15.30*	118.30 ± 15.40 (121)
Aminopyrine demethylase activity (nmol/mg/min)	6.02 ± 0.43	4.46 ± 0.49* (74)	6.29 ± 0.64*	6.77 ± 0.52 (108)
Erythromycin demethylase activity (nmol/mg/min)	1.23 ± 0.25	0.61 ± 0.26* (50)		
Androstenedione 7α-hydroxylase activity (nmol/mg/min)	0.20 ± 0.04	0.08 ± 0.05* (40)	0.16 ± 0.06	0.24 ± 0.06 (150)
Androstenedione 6β-hydroxylase activity (nmol/mg/min)	1.40 ± 0.27	0.33 ± 0.13* (24)	1.60 ± 0.13	1.77 ± 0.27 (111)
Androstenedione 16β-hydroxylase activity (nmol/mg/min)	0.23 ± 0.04	0.07 ± 0.03* (30)	0.32 ± 0.09	0.38 ± 0.09 (119)
Androstenedione 16α-hydroxylase activity (nmol/mg/min)	1.79 ± 0.42	0.33 ± 0.15* (18)	2.91 ± 0.53*	3.32 ± 0.59 (114)

Activity values represent the mean ± SD for N = 6 individual rat liver microsome preparations.
Numbers in parentheses indicate percentage activity relative to control values (i.e. − NADP).
* Significantly different relative to CP controls (−NADP) at $P < 0.05$.
† No parameters measured are significantly different relative to CP + NAC controls (−NADP) at $P < 0.05$.

2C11 has been shown to be involved in cyclophosphamide bioactivation in rats [3], it is possible that cyclophosphamide has the ability to alter its own metabolism. Le Blanc and Waxman [6] observed that cyclophosphamide selectively suppressed male-specific rat hepatic cytochrome P450 but the suppression observed in the experiments described in this paper appears non-selective. There was no significant difference in parameters measured when CP and NAC were coadministered compared with CP administered alone. NAC-treated rats appeared to have significantly elevated aminopyrine demethylase, 2A1/2, 3A2 and 2C11 activities when compared with those of saline-treated controls (Tables 1 and 2). This phenomena requires further investigation.

In vitro experiments indicate that acrolein or another metabolite of CP, which is able to react with NAC, significantly depressed apparent cytochrome P450 content (measured as the absorbance of the carbon monoxide-dithionite reduced complex) and NADPH cytochrome P450 reductase and aminopyrine demethylase, erythromycin demethylase, 2A1/2, 3A2, 2B1 and 2C11 activities (Table 3). This suggests that the metabolites of CP directly inactivate some cytochrome P450 isoenzymes and NADPH cytochrome P450 reductase *in vitro*. Although all parameters remain slightly depressed, statistically there was no significant difference between NAC in combination with CP and NAC control incubations (i.e. which did not contain NADP) in all parameters measured (Table 3). This implies that NAC prevents CP-mediated depression in cytochrome P450 activity *in vitro*. Erythromycin demethylase activity was not able to be measured in the presence of NAC. NAC alone *in vitro* appeared to significantly stimulate various parameters, i.e. the activities of the isoenzymes 3A2 and 2C11 (Table 3). In contrast NADPH cytochrome P450 reductase activity was significantly reduced (Table 3).

In male rats, the apparent plasma half-life values of CP, 4-hydroxycyclophosphamide/aldophosphamide and phosphoramide mustard are 29, 34 and 55 min, respectively, following radiolabelled CP administration [12]. The half-lives of cytochrome P450 proteins 2A1/2, 3A2, 2B1 and 2C11 are 14, 12, 19 and 20 hr respectively; the half-life of NADPH cytochrome P450 reductase is 29 hr [13]. Due to the short half-lives of CP and its metabolites and the rapid turnover of the isoenzymes studied, we have speculated that decreases in cytochrome P450 activity *in vivo* due to direct covalent binding by acrolein should be seen 1 day after CP treatment. We also conclude that alterations in cytochrome P450 activity observed 7 days after CP administration cannot be attributed to direct alkylation but reflect the indirect effects of CP and its metabolites i.e. changes in transcription and/or translation. The effects

seen 4 days after treatment should characterize intermediate effects.

The observation that there is generally no change in the parameters measured 1 and 4 days following administration and a significant decrease after 7 days, suggests that direct alkylation by acrolein is unlikely to be the mechanism by which CP decreases mixed function oxidation *in vivo*. The suppression of cytochrome P450 was not altered when *N*-acetylcysteine was coadministered with CP providing further evidence that acrolein is not directly inactivating cytochrome P450. The decrease *in vivo* is likely to be mediated by CP itself or a CP metabolite via changes in transcription and/or translation.

It appears that CP inactivates cytochrome P450 *in vivo* and *in vitro* via different mechanisms. The differences between *in vivo* and *in vitro* results may be attributed to the pharmacokinetics of CP, CP metabolites and NAC, and protein turnover which are operative *in vivo* but absent *in vitro*. There may also be differences in the accessibility of CP, CP metabolites and NAC to cytochrome P450 and the regulatory sites of cytochrome P450 expression. Clearly, the mechanism by which CP alters cytochrome P450 in rats requires further investigation. It also remains to be determined whether such perturbations occur in humans and if so whether the outcome is of clinical significance.

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REFERENCES

1. Kawabata TT, Chapman MY, Kim D-H, Stevens WD and Holsapple MP, Mechanisms of *in vitro* immunosuppression by hepatocyte-generated cyclophosphamide metabolites and 4-hydroxycyclophosphamide. *Biochem Pharmacol* **40**: 927–935, 1990.
2. Colvin M and Hilton J, Pharmacology of cyclophosphamide and metabolites. *Cancer Treat Rep* **65**: 89–95, 1981.
3. Clarke L and Waxman DJ, Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxygenase catalysts of drug activation. *Cancer Res* **49**: 2344–2350, 1989.
4. Berrigan MJ, Marinello AJ, Pavelic Z, Williams CJ, Struck RF and Gurtoo HL, Protective role of thiols in cyclophosphamide-induced urotoxicity and depression of hepatic drug metabolism. *Cancer Res* **42**: 3688–3695, 1982.
5. Marinello AJ, Bansal SK, Paul B, Koser PL, Love J, Struck RF and Gurtoo HL, Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450. *Cancer Res* **44**: 4615–4621, 1984.
6. Le Blanc GA and Waxman DJ, Mechanisms of cyclophosphamide action on hepatic P-450 expression. *Cancer Res* **50**: 5720–5726, 1990.
7. Stupans I and Sansom LN, The inhibition of drug oxidation by anhydroerythromycin, an acid degradation product of erythromycin. *Biochem Pharmacol* **42**: 2085–2090, 1991.
8. Lear L, Nation RL and Stupans I, Influence of morphine concentration on detergent activation of rat liver morphine-UDP-glucuronosyltransferase. *Biochem Pharmacol* **42**: S55–S60, 1991.
9. Williams CH and Kamin H, Microsomal triphosphopyridine nucleotide-cytochrome *c* reductase of liver. *J Biol Chem* **237**: 587–595, 1962.
10. Nash T, The colorimetric estimation of formaldehyde by means of the Hontzsch reaction. *Biochem J* **55**: 416–421, 1953.
11. Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalysed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
12. Sladek NE, Powers JF and Grage GM, Half-life of oxazophosphorines in biological fluids. *Drug Metab Dispos* **12**: 553–559, 1984.
13. Shiraki H and Guengerich FP, Turnover of membrane proteins: kinetics of induction and degradation of seven forms of rat liver microsomal P-450, NADPH-cytochrome P-450 reductase, and epoxide hydrolase. *Arch Biochem Biophys* **235**: 86–96, 1984.

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The effect of glycerol and 4-nitroquinoline 1-oxide on active oxygen formation in sub-cellular fractions of lung tissue

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Abstract—Glycerol enhances pulmonary tumorigenesis in mice treated with 4-nitroquinoline 1-oxide (4NQO). The present study shows that active oxygen formation by glycerol may be responsible for this enhanced tumorigenesis. Male ddY mice were treated with 4NQO and given a 5% glycerol solution instead of drinking water for up to 4 weeks after 4NQO injection. There was no difference in NADH-dependent active oxygen formation of the mitochondria between the 4NQO- and 4NQO-plus-glycerol-treated groups but the ratios of NADH- and NADPH-dependent active oxygen formation of the microsomes and nuclei of the 4NQO-plus-glycerol-treated group to the 4NQO-treated group increased with increasing time after 4NQO injection. Significant differences in the maximum NADH- and NADPH-dependent active oxygen formation were observed 2 or 4 weeks after 4NQO injection.