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INHIBITION OF METABOLISM-MEDIATED CYTOTOXICITY BY 1,1-DISUBSTITUTED HYDRAZINES IN MOUSE MASTOCYTOMA (LINE P815) CELLS*

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Abstract—Cyclophosphamide (CPA), a compound requiring metabolic activation to form toxic metabolites, has been used as a model compound to validate the use of a hepatocyte/mouse mastocytoma (line P815) cell culture system capable of measuring metabolism-mediated cytotoxicity *in vitro*. A number of hydrazines were tested in this system and were found to be relatively non-toxic. However, an attempt has been made to correlate complex formation between hydrazine metabolites and cytochrome P-450 in rat hepatocytes and the inhibition of the metabolism-mediated cytotoxicity of CPA. *N*-Aminopiperidine (NAP) was the most potent hydrazine tested in this system, since it significantly reduced the metabolism-mediated toxicity of CPA. The concentration dependency of this phenomenon permitted the calculation of the amount of NAP that reduced CPA cytotoxicity by 50%. This value (approximately 1 mM) correlated well with the apparent Michaelis constant (1.2 mM) for the formation of the inhibitory metabolite complex with cytochrome P-450 in microsomal suspensions. There was good correlation between the abilities of a number of 1,1-disubstituted hydrazines to reduce the metabolism-mediated toxicity of CPA and the extent to which they form the inhibitory metabolite complex in microsomes. 1,2-Dimethylhydrazine did not form an observable inhibitory complex in microsomal suspensions and only slightly reduced the metabolism-mediated toxicity of CPA in the mouse mastocytoma cell culture system. However, some other hydrazine derivatives were shown to alter significantly cytochrome P-450 function in isolated hepatocytes. This *in vitro* system may be of use in the evaluation of the biological effects of xenobiotics, particularly those requiring metabolic activation by cytochrome P-450.

Cell culture systems have been widely used to assess the cytotoxicity of a large number of different chemicals [1–3]. It is now well recognized that many chemicals require metabolic activation by oxidative reactions in order for their toxic potential to be realized. Since this specialized cellular function, cytochrome P-450-dependent monooxygenation, is not usually retained in culture [4], most cell culture cytotoxicity tests have been restricted to those compounds which are direct-acting and do not require prior metabolic activation. Liver microsomal fractions have been used as a metabolizing component in some cell culture systems in order to overcome this deficiency [5–7], but this almost certainly leads to an abnormal production of toxic metabolites compared to those produced *in vivo* [8]. We have, therefore, made use of a modification of the mixed cell culture approach described previously [9, 10] employing freshly isolated hepatocytes, which possess the full complement of drug-metabolizing enzymes as the metabolizing component, and cultured mouse mastocytoma cells as the target component of the assay.

Although hydrazine derivatives have been shown to be toxic and/or carcinogenic *in vivo* [11, 12], demonstration of similar biological activity using cell culture or other model systems has not been very successful. In an attempt to develop a successful *in vitro* model for the toxicity of hydrazine derivatives, a mouse mast cell tumor line (P815) was employed, since it has been noted that this particular tumor when implanted subcutaneously in CD_2F_1 mice is susceptible to both oral or parental administration of procarbazine, a methylhydrazine derivative [13]. The *in vivo* transfer of the intact *N*-methyl group of procarbazine, the moiety thought most likely to be involved in its biological effects, into cytoplasmic RNA of P815 mouse ascites cells has also been observed [14]. With this justification, we embarked on a study to assess the *in vitro* metabolism-mediated cytotoxicity of a number of hydrazine derivatives to the P815 cells.

It also has been reported [15] that the addition of 1,1-disubstituted hydrazines to microsomal suspensions in the presence of NADPH and oxygen results in the formation of a metabolite complex with cytochrome P-450, which inhibits the monooxygenase activity of this enzyme system. To study such drug–drug interactions in isolated cell systems, we also have focused our attention, using this hepatocyte/mastocytoma system, on the ability of *N*-aminopiperidine (NAP) and a number of other hydrazines to inhibit the cytotoxicity of an antitumor

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agent, cyclophosphamide (CPA), known to require metabolic activation before its toxicity is expressed.

MATERIALS AND METHODS

Male Sprague–Dawley CD rats (100–125 g body weight) were used throughout this study. The animals were allowed free access to food and water. Treated rats were given a daily intraperitoneal dose of either sodium phenobarbital in 0.9% saline, 80 mg/kg body weight for 4 days, or 5,6-benzoflavone in corn oil, 50 mg/kg body weight for 4 days. Following the last injection, the animals were starved for 18 hr prior to being killed. Tissue culture medium, serum, and cell culture reagents were obtained from Gibco Biocult, Grand Island, NY. Cyclophosphamide (CPA) was a gift from Mead Johnson & Co., Evansville, IN. *N*-Aminopiperidine (NAP), 1,1-dimethylhydrazine (1,1-DMH), monomethylhydrazine (MMH), 1-methyl-1-phenylhydrazine, bis-(2-chloroethyl)-amine hydrochloride (nor-nitrogen mustard) and 4-(*p*-nitrobenzyl)-pyridine (4-NBP) were obtained from the Aldrich Chemical Co., Milwaukee, WI. 1,2-Dimethylhydrazine (1,2-DMH) was purchased from the Sigma Chemical Co., St. Louis, MO. Procarbazine (PCZ) was a gift from Hoffmann-LaRoche, Inc., Nutley, NJ, and 2-diethylaminoethyl-2,2-diphenyl-valerate HCl (SKF-525A) was a gift from Smith Kline & French, Philadelphia, PA. "Falcon" tissue culture flasks were purchased from American Scientific Products, McGaw Park, IL. Cells of mouse mastocytoma (line P815), a gift from Dr. W. T. Garrard, Department of Biochemistry, University of Texas Health Science Center at Dallas, were grown in suspension culture in Leibovitz L-15 medium containing 10% (v/v) fetal calf serum and 10% (v/v) tryptose phosphate broth. All experiments were performed using exponentially growing P815 cells, whose generation time was approximately 10 hr.

Hepatocytes were isolated from rat liver by collagenase digestion as described by Fry *et al.* [16] using sterile techniques throughout and, after assessment of viability by trypan blue dye exclusion [17] (routinely 85–95%), were diluted to 2×10^6 viable hepatocytes per ml of culture medium which was comprised of 5% (v/v) fetal calf serum and 10% (v/v) tryptose phosphate broth in Leibovitz L-15 medium. Samples (2 ml) of this cell suspension were pipetted into 10 ml conical flasks and incubated with various concentrations of the chemical under test at 37° in a shaking water bath (approximately 100 oscillations/min). All of the hydrazines, CPA, and SKF-525A were dissolved in phosphate-buffered saline "A" (PBSA) [18] at ten times the desired final concentration and sterilized by membrane filtration prior to use. The concentrations of the stock sol-

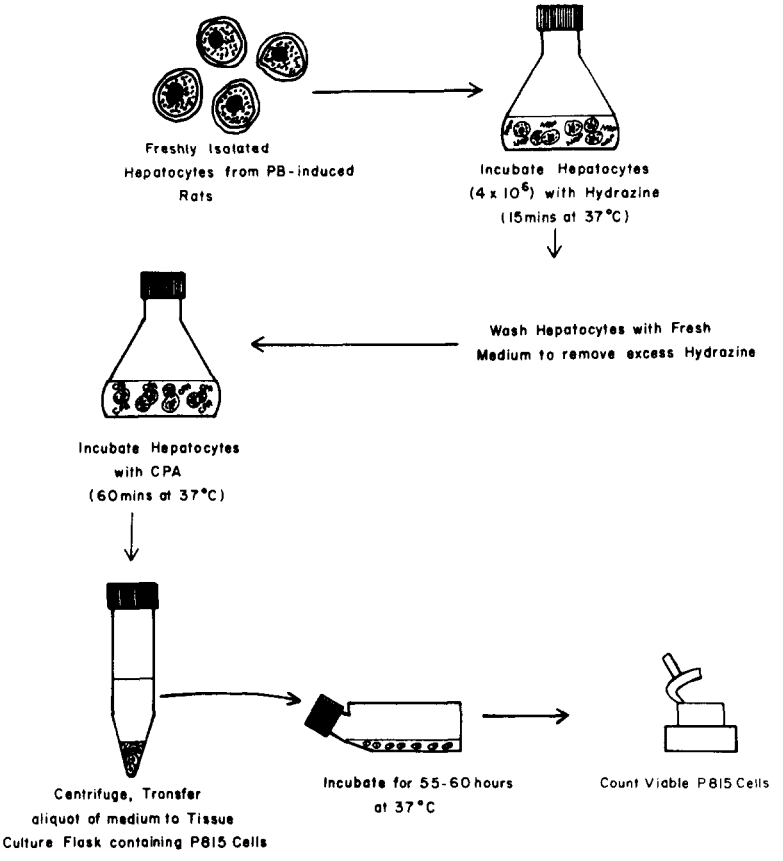

Fig. 1. Protocol of cell toxicity system.

utions of hydrazines and the hydrazines in the media were determined as described previously [15].

The protocol used for most of the studies reported here is shown in Fig. 1. After hepatocytes had been incubated with either CPA alone, hydrazine alone, or hydrazine followed by CPA, the cell suspension was centrifuged at 50 *g* for 1 min to separate the cells from the medium. Aliquots (0.1 ml) of the medium were introduced into 25 cm^2 plastic tissue culture flasks containing 1×10^5 mouse mastocytoma (P815) cells in 5 ml of medium, and the cells were cultured at 37°. After 55 hr in culture, the number of viable mouse mastocytoma (P815) cells was determined by their ability to exclude trypan blue. Production of total alkylating activity was assessed as follows. Freshly isolated viable hepatocytes (4×10^6 in 2 ml of medium) were incubated with CPA for 1 hr at 37°, in the absence or presence of SKF-525A (final concn 8.3 μM). The amount of alkylating agent formed during this time was measured by its reaction with 4-NBP according to the method of Epstein *et al.* [19] as modified by Friedman and Boger [20].

The abilities of certain hydrazines to affect the production of alkylating agent from CPA and the metabolism of 7-ethoxycoumarin in isolated hepatocytes were also studied. Using essentially the same protocol outlined in Fig. 1, hepatocytes (2×10^6 cells/ml) were incubated with the hydrazine (1 mM) for 15 min at 37°, washed with fresh medium to remove the hydrazine, and then incubated for 1 hr at 37° with 1.0 mM CPA or for 15 min at 37° with 7-ethoxycoumarin (70 μM). The amount of alkylating agent formed was measured as described before [19, 20] and the free and conjugated 7-hydroxycoumarin produced was measured fluorimetrically as described by Fry *et al.* [21].

Studies were also performed using liver microsomal fractions from phenobarbital-induced rats prepared as described by Remmer *et al.* [22]. Protein concentration was determined using the method of Lowry *et al.* [23]. Experiments utilizing microsomal suspensions were performed at 37° with a reaction mixture consisting of microsomal protein (2 mg/ml), 3 mM sodium DL-isocitrate, isocitrate dehydrogenase (0.8 I.U./ml), 0.5 mM $NADP^+$, 5 mM $MgCl_2$, and 0.05 M potassium phosphate:0.05 M *N*-tris-(hydroxymethyl)methylglycine–HCl buffer, pH 7.6. NAP and CPA were used at a final concentration of 5 and 1.1 mM respectively. Sterile techniques were employed throughout these studies; all constituents of the reaction mixture were filter sterilized prior to use. Following a 5-min incubation period, the reaction was initiated by the addition of microsomes and NAP. After 5 min of incubation at 37°, CPA was added and the reaction was allowed to continue for 10 min. At the end of this period, 0.1-ml aliquots of the reaction mixture were introduced into 25 cm^2 plastic tissue culture flasks containing 1×10^5 mouse mastocytoma cells in 5 ml of medium. The cells were cultured and counted as described above.

Statistical analysis was performed using an analysis of variance between two sample populations [24]. The data presented in Table 1 describe a single experiment. In this instance, the study was performed at least four times with similar results in each experiment.

RESULTS

When CPA (1 mM) was incubated with freshly isolated hepatocytes from phenobarbital-treated rats prior to addition of aliquots of the CPA mixture to cultures of mouse mastocytoma (P815) cells, there was a marked decrease in the number of viable P815 cells after 55 hr in culture. The effect of varying the CPA concentration on the inhibition of P815 cell growth was studied; the results are shown in Fig. 2. At a CPA concentration of 1 mM (270 μg/ml), the viable P815 cell number after culture was only 6% of that present in the control cultures. When hepatocytes were omitted from the incubation mixture, no toxicity to the P815 cells could be detected even at the highest concentration of CPA used. CPA, at all concentrations, was non-toxic to the hepatocytes; no decrease in viability was seen as judged by dye exclusion over the incubation period employed. The assumption that CPA at the concentrations used in this study is non-toxic *per se*, but is metabolized to extremely toxic entities when incubated in the presence of hepatocytes, was supported by the following findings.

Coincubation of freshly isolated hepatocytes with CPA and SKF-525A, a known inhibitor of microsomal drug metabolism in intact hepatocytes [25], prior to adding a portion of the incubation mixture to P815 cells in culture, resulted in a reduction of cytotoxicity which was paralleled by a decrease in the production of total alkylating activity by the hepatocytes from untreated rats (Table 1). SKF-525A at this concentration was not toxic to the hepatocytes, as judged by dye exclusion, or to the P815 cells, as indicated by a lack of growth inhibition. When hepatocytes were isolated from rats that had been pretreated *in vivo* with phenobarbital, a known

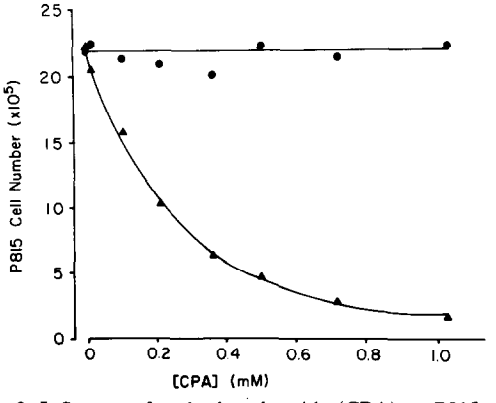

Fig. 2. Influence of cyclophosphamide (CPA) on P815 cell growth in the presence (▲) and absence (●) of hepatocytes from phenobarbital-treated rats. Incubations were carried out for 1 hr at 37° with various concentrations of CPA. Samples (0.1 ml) of the incubation medium were introduced into flasks containing 1×10^5 P815 cells in 5 ml and the flasks were incubated at 37° for 55 hr, after which time viable-cell counts were performed. The concentrations of CPA shown were those present in the incubation mixture; the relative concentrations in cell culture were 1/50 of these values. Three flasks were used for each CPA concentration in the experiment shown.

Table 1. Effect of SKF-525A on the cytotoxicity and alkylating equivalents produced during cyclophosphamide metabolism by isolated rat hepatocytes

Incubation conditions	Hepatocytes from untreated rats		Hepatocytes from phenobarbital rats	
	Alkylating equivalents produced*	% Growth inhibition	Alkylating equivalents produced	% Growth inhibition
CPA†	7.6	45.2	41.5	69.6
CPA + SKF-525A‡	2.1	7.1	3.5	30.4

* Expressed as nmoles nornitrogen mustard equivalents produced in 1 hr per 4×10^6 cells. Each value is the average of three experiments.

† The CPA concentration was 0.3 mM (83.3 μg/ml) and the concentration of CPA in cell culture was 1/50 of this value.

‡ The SKF-525A concentration was 8.3 μM.

inducer of microsomal drug metabolism in cells [26], incubation with CPA produced measurable, but not parallel, increases in the amount of total alkylating activity and P815 growth inhibition, when compared to hepatocytes from untreated rats. From the results of several experiments, it was possible to construct a concentration–response curve for the toxicity of activated CPA (Fig. 3) from which an IC_{50} value (defined as that concentration of CPA which inhibits cell growth by 50%) was calculated. When hepatocytes isolated from untreated rats were used as the metabolizing component, the IC_{50} was 0.3 mM (84 ± 4 μg/ml, N = 4). This value was reduced significantly ($P < 0.001$) to 0.2 mM (51 ± 1 μg/ml, N = 3) when hepatocytes from phenobarbital-treated rats were employed.

Table 2 summarizes the effects of several substituted hydrazines on the growth of P815 mouse mastocytoma cells following incubation in the presence or absence of rat hepatocytes. All of the hydrazines studied appeared to exhibit very little overt toxicity to P815 cells even at a 4.5 mM concentration in the incubation mixture, the highest level of the hydrazine derivatives that could be studied due to cytotoxicity to the hepatocytes at higher levels. It should be noted that the hydrazine concentrations after dilution (1/50) in the culture media were always less than 90 μM.

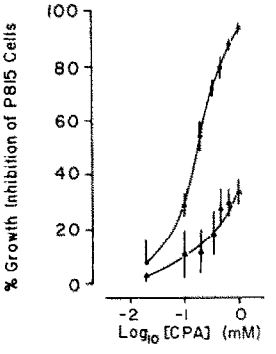

Fig. 3. Effect of *N*-aminopiperidine (NAP) on the dose–response of the metabolism-mediated cytotoxicity of cyclophosphamide (CPA) in mouse mastocytoma cells (line P815). Hepatocytes from phenobarbital-treated rats were incubated for 1 hr at 37° with various concentrations of either CPA alone (●) or with CPA following prior incubation of the liver cells with 4.2 mM NAP (▲). Samples (0.1 ml) of the incubation medium were introduced into flasks containing 1×10^5 P815 cells in 5 ml, and the flasks were incubated at 37° for 55 hr, after which time viable cells counts were performed. The concentrations of CPA shown were those present in the incubation mixture; the relative concentrations in cell culture were 1/50 of these values. From these counts (using three flasks at each CPA concentration), the percent inhibition of cell growth was calculated. Each point is the mean of three determinations with the S.D. as the bar (N = 3).

Table 2. Toxicity of some hydrazine derivatives to mouse mastocytoma cells (line P815) incubated in the presence or absence of rat hepatocytes

Hydrazine	Percent growth inhibition of mouse mastocytoma cells*	
	(+ Hepatocytes)	(− Hepatocytes)
Procarbazine	—†	18 ± 6
1,2-Dimethylhydrazine	—	25 ± 9
1,1-Dimethylhydrazine	17 ± 7	—
Monomethylhydrazine	22 ± 11	—
N-Aminopiperidine	—	—

* Percent growth inhibition ± S.D. (N = 4). The highest concentration of hydrazine derivatives that could be used in the hepatocyte incubation was 4.5 mM since higher concentrations were toxic to the hepatocytes isolated from untreated rats during the preincubation period. The concentration of hydrazine in cell culture was 1/50 of this value.

† Denotes inhibition of growth <10%.

Table 3. Comparison of the abilities of certain hydrazine derivatives to reduce metabolism-mediated cytotoxicity and to form the inhibitory metabolite complex

Hydrazine derivative*	% Reduction of CPA toxicity	Ability to form complex†
	Phenobarbital-pretreated animals‡	
1,1-Disubstituted		
N-Aminopiperidine	52 (100)§	100
1,1-Dimethylhydrazine	37 (69)	23
1-Methyl-1-phenylhydrazine	46 (86)	18
1,2-Disubstituted		
1,2-Dimethylhydrazine	10 (18)	0
Procarbazine	30 (55)	0
Monosubstituted		
Monomethylhydrazine	21 (38)	0
	5,6-Benzoflavone-pretreated animals‡	
N-Aminopiperidine	24 (44)	11
	Untreated animals‡	
N-Aminopiperidine	29 (53)	18

* The hydrazine concentrations used were 1 mM and the cyclophosphamide concentration used was 1.0 mM (270 μg/ml). The concentration of CPA in cell culture was 1/50 of this value.

† Data from Ref. 15.

‡ The hepatocytes used for the metabolic activation system were isolated from rats pretreated as indicated.

§ Numbers in parentheses represent the abilities of the various hydrazines to inhibit the metabolism-mediated toxicity of cyclophosphamide and are expressed relative to the data obtained with *N*-aminopiperidine (N = 4).

The incubation of hepatocytes from phenobarbital-treated rats with NAP (4.2 mM), followed by incubation with different concentrations of CPA prior to adding an aliquot of the mixture to the P815 cell cultures, resulted in a significant change in the concentration–response curve for the toxicity of activated CPA to P815 cells (Fig. 3). At all concentrations of CPA used, prior incubation of hepatocytes with 4.2 mM NAP reduced the inhibition of P815 cell growth by approximately 60%. It should be noted that 4.2 mM NAP had no cytotoxic effect on the hepatocytes as judged by dye exclusion and that 84 μM NAP had no cytotoxic effect on P815 cell growth during the time course of these studies.

Similar results were obtained using liver microsomal fractions from phenobarbital-treated rats. When microsomes were incubated with CPA in the presence of an NADPH-regenerating system, P815 cell growth in the presence of aliquots of the incubation mixture was inhibited by 88 ± 8% (N = 3). Incubation of microsomes with NAP in the presence of a regenerating system followed by incubation with CPA resulted in a significant reversal ($P < 0.001$) in the inhibition of the growth of P815 cells to 26 ± 3% (N = 3). It should be pointed out that growth inhibition of P815 cells occurred only following addition of aliquots of the incubation mixture containing microsomes with CPA alone or to a lesser extent with NAP and CPA together, all in the presence of an NADPH-regenerating system.

The effect of varying the NAP concentration on the reduction of activated CPA toxicity to P815 cells was also studied (Fig. 4). From this concentration–response curve, we have calculated that the concentration of NAP which reduced the cytotoxicity of CPA by 50% was approximately 1 mM.

The results shown in Table 3 indicate that in the case of 1,1-disubstituted hydrazines there is reasonable correlation between the extent of complex formation with cytochrome P-450 observed in micro-

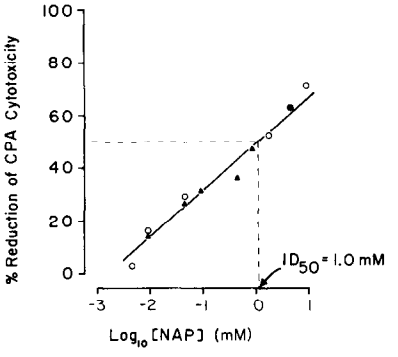

Fig. 4. Concentration–response of the ability of *N*-aminopiperidine (NAP) to inhibit the metabolism-mediated cytotoxicity of cyclophosphamide (CPA) in mouse mastocytoma cells (line P815). Hepatocytes from phenobarbital-treated rats were incubated for 15 min at 37° with various concentrations of NAP followed by centrifugation to remove the hydrazine and incubation of the liver cells for 1 hr at 37° with 1.0 mM (270 μg/ml) CPA. Samples (0.1 ml) of the incubation medium were introduced into flasks containing 1×10^5 P815 cells in 5 ml. The flasks were incubated at 37° for 55 hr, and the viable cell counts were determined. The concentrations of NAP shown were those present in the incubation mixture; the relative concentrations in cell culture were 1/50 of these values. From these counts (using three flasks at each NAP concentration), the percent reduction of CPA cytotoxicity was calculated. Data points from two separate experiments are shown (○) and (▲).

somal suspensions and the degree of inhibition of the metabolism-mediated cytotoxicity of CPA. 1,2-Disubstituted and monosubstituted hydrazines are unable to form a spectrophotometrically observable inhibitory complex in microsomal suspensions, and 1,2-dimethylhydrazine caused only a small inhibition of the CPA metabolite toxicity in the hepatocyte/P815 cell culture system. However, methylhydrazine, a monosubstituted hydrazine, and procarbazine, a 1-methyl-2-benzyl-substituted hydrazine, did produce significant inhibition of CPA toxicity to P815 cells (Table 3). In addition, the ability of NAP to inhibit the formation of the toxic CPA metabolite and thereby to protect the P815 cells from the resultant toxicity was reduced significantly when hepatocytes from untreated or 5,6-benzoflavone-treated rats were used as the "metabolizing component" in this system (Table 3). A similar reduction in the ability of NAP to form the inhibitory metabolite complex with cytochrome P-450 has been noted using liver microsomes from control or 5,6-benzoflavone-treated rats [15].

Table 4 summarizes the effects of three hydrazines, *N*-aminopiperidine (NAP), procarbazine (PCZ) and monomethylhydrazine (MMH), on the formation of nornitrogen mustard equivalents from CPA and the *O*-deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin and its sulfate and glucuronide conjugates by isolated hepatocytes from phenobarbital-treated rats. NAP was the most effective inhibitor of CPA activation (52%). PCZ and MMH also inhibited production of nornitrogen mustard equivalents from CPA in rat hepatocytes by 20 and 6% respectively. In addition, NAP was the most potent inhibitor of 7-ethoxycoumarin metabolism, reducing the total amount of 7-hydroxycoumarin (free and conjugated) produced by 36% when compared to the total amount produced by the untreated hepatocytes. PCZ and MMH were far less effective producing only a 16 and 14% inhibition, respectively, in the overall amount of 7-ethoxycoumarin metabolized by these cells.

DISCUSSION

A number of *in vitro* mixed-cell culture systems have been developed in recent years for the detection of cytotoxicity, mutagenesis and carcinogenicity of xenobiotics, particularly those requiring metabolic activation before their biological effects can be expressed. Isolated hepatocytes, which retain the capacity for metabolizing a wide range of xenobiotics in a manner similar to that found *in vivo* [8, 27], have been used as a metabolizing component in those systems as freshly isolated cell suspensions [9, 10, 28] and in short-term primary culture [29–33]. Using the mixed hepatocyte/mouse mastocytoma cell culture system described here, we have confirmed the studies of Fry and Bridges [9], who reported that CPA toxicity to the fibroblasts was only fully expressed in the presence of isolated rat hepatocytes.

If one compares the relative IC_{50} for CPA using the fibroblast suppression system (20 μg CPA/ml from Ref. 9) and the P815 culture system (29 μg/ml or 0.11 mM), the two methods show very similar responses to the hepatocyte-mediated cytotoxicity of cyclophosphamide. This calculation requires one to take into account the actual concentrations of CPA and its metabolites exposed to the fibroblasts and the P815 cells during the culture period. With this validation of the system, several hydrazine derivatives were tested and found to be relatively non-toxic to the mouse mastocytoma cells, as reflected by their poor inhibition of P815 cell growth after an aliquot of an incubation mixture containing the hydrazines (4.5 mM) in the presence and absence of hepatocytes was added to the P815 cell culture (Table 2). Since it was observed that incubation of freshly isolated rat hepatocyte suspensions with *N*-aminopiperidine (NAP), a 1,1-disubstituted hydrazine which forms an abortive complex with cytochrome P-450 [15], inhibited the metabolism-mediated cytotoxicity of CPA to mouse mastocytoma cells in a concentration-dependent manner (Fig. 3), a more comprehensive study was initiated. The effects of animal pretreatment, concentration dependence, and substrate specificity on the formation of the inhibitory metabolite complex *in vitro* [15] and on the inhibition of CPA toxicity in culture were very similar (Table 3). Therefore, one is led to conclude that a cytochrome P-450–metabolite complex was formed in isolated hepatocytes upon incubation with 1,1-disubstituted hydrazines and that

Table 4. Effects of some hydrazine derivatives on the production of alkylating equivalents during cyclophosphamide metabolism and the metabolism of 7-ethoxycoumarin by isolated hepatocytes from phenobarbital-treated rats

Hydrazine*	Alkylating equivalents produced†	% Inhibition	Total 7-hydroxycoumarin produced‡	% Inhibition
None	23 ± 5		4.2 ± 0.3	
N-Aminopiperidine	11 ± 1§	52 (100)‖	2.7 ± 0.4§	36 (100)‖
Procarbazine	18 ± 3	20 (38)	3.5 ± 0.4§	16 (44)
Monomethylhydrazine	22 ± 4	6 (11)	3.6 ± 0.2§	14 (40)

* The hydrazine concentrations used were 1 mM.

† Data are expressed as nmoles nornitrogen mustard equivalents produced in 1 hr per 10^6 cells. Each value is the average of four separate determinations. The CPA concentration was 270 μg/ml (1.0 mM).

‡ Values ± S.D. are expressed as nmoles 7-hydroxycoumarin formed per 15 min per 10^6 cells (N = 4).

§ Significantly different from controls ($P < 0.05$).

‖ Numbers in parentheses represent the ability of the various hydrazines to inhibit the production of alkylating equivalents from cyclophosphamide metabolism and 7-ethoxycoumarin metabolism in isolated hepatocytes relative to the inhibition obtained with *N*-aminopiperidine.

this complex prevented the metabolism of CPA to its toxic products. It should be noted that the phenomenon must be due to the formation of a complex and not competition of the hydrazine with the CPA as substrates for cytochrome P-450 since the hepatocytes were washed prior to incubation with CPA (Fig. 1). This procedure removed greater than 95% of the hydrazine from the hepatocyte suspension (P. Wiebkin and R. A. Prough, unpublished results). The results obtained when liver microsomes from phenobarbital-treated rats were employed instead of hepatocytes would seem to lend further weight to this argument. It is well established that the activation of CPA occurs primarily in the liver and is mediated by the cytochrome P-450-dependent monooxygenase system [34, 35].

The reasons for the significant decrease in CPA toxicity to P815 cells seen upon prior incubation of hepatocytes with procarbazine (PCZ) or monomethylhydrazine (MMH) (Table 3) were initially unclear, since these hydrazines did not form complexes with cytochrome P-450 which had absorbance maxima near 438 nm [15]. However, recent studies in this laboratory (S. J. Moloney and R. A. Prough, unpublished data) and the results shown in Table 4 have suggested a possible explanation for this phenomenon. We have noted that MMH and PCZ (2.5 mM) in the presence of NADPH and oxygen appeared to destroy approximately 15–20% of the cytochrome P-450 present in liver microsomes from phenobarbital-treated rats as measured by a loss of total heme and CO-reactive cytochrome P-450. NAP at this level does not cause any heme destruction, but does result in a 40% loss of CO-reactive cytochrome P-450, presumably due to the formation of the inhibitory metabolite complex [15]. This explanation is supported by the observation that these hydrazines inhibited the formation of nornitrogen mustard equivalents from CPA and 7-ethoxycoumarin metabolism by isolated hepatocytes from phenobarbital-treated rats (Table 4). Similar reductions in cytochrome P-450-dependent monooxygenase activity and content of CO-reactive cytochrome P-450 following procarbazine administration both *in vivo* and *in vitro* have been noted by a number of other workers [36–39]. Such a loss of cytochrome P-450 within the hepatocytes on preincubation with MMH and PCZ could explain the significant reduction in toxicity to the P815 cells when these hepatocytes are subsequently incubated with CPA.

In conclusion, this study highlights the versatility that such *in vitro* mixed-cell culture systems possess. They can be utilized not only for the evaluation of biological effects of xenobiotics, particularly those requiring activation by microsomal monooxygenases such as cytochrome P-450, but also to elucidate drug–drug interactions that involve enzymes common for metabolism.

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REFERENCES

1. P. C. Rofe, *Fd Cosmet. Toxic.* **9**, 683 (1971).
2. M. Dawson, *Cellular Pharmacology*. Charles C Thomas, Springfield, IL (1972).
3. A. N. Worden, in *Modern Trends in Toxicology* (Eds. E. Boyland and R. Goulding), Vol. 2, p. 216. Butterworth, London (1974).
4. I. S. Owens and D. W. Nebert, *Molec. Pharmac.* **11**, 94 (1974).
5. B. J. Phillips, *Biochem. Pharmac.* **23**, 131 (1974).
6. E. Dolfini, A. Martini, M. G. Donelli, L. Morasca and S. Garattini, *Eur. J. Cancer* **9**, 375 (1973).
7. P. Daudel, D. Papadopoulo, P. Marcovits, M. Hubert-Habart and L. Oielat, *C.r. Seanc. Soc. Biol.* **169**, 507 (1975).
8. J. R. Fry and J. W. Bridges, in *Progress in Drug Metabolism* (Eds. J. W. Bridges and L. F. Chasseaud), Vol. 2, p. 71. John Wiley, Chichester (1977).
9. J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **26**, 969 (1977).
10. P. Wiebkin, J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **27**, 1849 (1978).
11. S. M. Sieber, P. Correa, D. W. Dalgard and R. M. Adamson, *Cancer Res.* **38**, 2125 (1978).
12. B. Toth, *Cancer Res.* **35**, 3693 (1975).
13. V. T. Oliverio, C. Denham, V. T. DeVita and M. G. Kelly, *Cancer Chemother. Rep.* **42**, 1 (1964).
14. W. Kreis, *Cancer Res.* **30**, 82 (1970).
15. R. N. Hines and R. A. Prough, *J. Pharmac. exp. Ther.* **214**, 80 (1980).
16. J. R. Fry, C. A. Jones, P. Wiebkin, P. Bellemann and J. W. Bridges, *Analyt. Biochem.* **71**, 341 (1976).
17. H. Cummings, *Virology-Tissue Culture*, 1st Edn, p. 26. Butterworth, London (1970).
18. J. Paul, *Cell and Tissue Culture*, 4th Edn, p. 91. Churchill Livingstone, London (1973).
19. J. Epstein, R. W. Rosenthal and R. J. Ess, *Analyt. Chem.* **27**, 1435 (1955).
20. O. M. Friedman and E. Boger, *Analyt. Chem.* **33**, 906 (1961).
21. J. R. Fry, P. Wiebkin, J. Kao, C. A. Jones, J. Gwynn and J. W. Bridges, *Xenobiotica* **8**, 113 (1978).
22. H. Remmer, M. Greim, J. B. Schenkman and R. W. Estabrook, *Meth. Enzym.* **10**, 703 (1967).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. J. R. Zar, *Biostatistical Analysis*, pp. 101–15. Prentice Hall, Englewood Cliffs, NJ (1974).
25. P. Wiebkin, J. R. Fry, C. A. Jones, R. Lowing and J. W. Bridges, *Xenobiotica* **6**, 725 (1976).
26. P. Weibkin, J. R. Fry, C. A. Jones, R. K. Lowing and J. W. Bridges, *Biochem. Pharmac.* **27**, 1899 (1978).
27. J. R. Fry and J. W. Bridges, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), Vol. 1, p. 201. Elsevier/North-Holland, New York (1978).
28. M. H. L. Green, B. A. Bridges, A. M. Rogers, G. Horspool, W. J. Muriel, J. W. Bridges and J. R. Fry, *Mutation Res.* **48**, 287 (1977).
29. R. H. C. San and G. M. Williams, *Proc. Soc. exp. Biol. Med.* **156**, 534 (1977).
30. R. Langenbach, H. J. Freed and E. Huberman, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2864 (1978).
31. G. Michalopoulos, G. L. Sattler, L. O'Connor and H. C. Pitot, *Cancer Res.* **38**, 1866 (1978).
32. C. A. Jones and E. Huberman, *Cancer Res.* **40**, 406 (1980).

33. G. Michalopoulos, S. C. Strom, A. D. Kligerman, G. P. Irons and D. L. Novicki, *Cancer Res.* **41**, 1873 (1981).
34. B. F. Hales and R. Jain, *Biochem. Pharmac.* **29**, 256 (1980).
35. N. E. Sladek, *Cancer Res.* **31**, 901 (1971).
36. N. R. Eade, S. M. MacLeod and K. W. Renton, *Can. J. Physiol. Pharmac.* **50**, 721 (1972).
37. I. P. Lee and G. W. Lucier, *J. Pharmac. exp. Ther.* **196**, 586 (1976).
38. D. J. Reed, *Biochem. Pharmac.* **25**, 153 (1976).
39. K. Onoda and I. K. Ho, *Drug chem. Toxic.* **3**, 185 (1980).