

Role of Hepatic Cytochrome P450s in the Pharmacokinetics and Toxicity of Cyclophosphamide: Studies with the Hepatic Cytochrome P450 Reductase Null Mouse

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Abstract

Cyclophosphamide (CPA) is an anticancer prodrug that is dependent on cytochrome P450 (CYP) metabolism for its therapeutic effectiveness. In spite of the use of CPA in the clinic for over 50 years, little is known about the relationship between its toxicokinetics and therapeutic response. We have employed a powerful new model, the Hepatic Cytochrome P450 Reductase Null (HRN) mouse, which has almost no hepatic cytochrome P450 activity, to study the toxicokinetics of CPA and to establish *in vivo* the role of hepatic P450 metabolism in its pharmacokinetics. In HRN mice the *in vitro* metabolism and intrinsic clearance of CPA was over 6-fold lower than in wild-type animals. This change in CPA metabolism was also reflected *in vivo*, with a profound difference in the pharmacokinetics of both CPA and its metabolites. At a CPA dose of 100 mg/kg, the C_{max} , plasma area under the curve (AUC) and half-life were increased by 2.6-, 6.2-, and 3.2-fold, respectively, in the HRN mice. Similar changes were also observed at a dose of 300 mg/kg. These data confirm that hepatic metabolism is the major route of CPA elimination and disposition. The primary metabolites of CPA, 4-hydroxycyclophosphamide (4-OH-CPA) and 3-dechloroethylcyclophosphamide, were still formed, but at altered rates in the HRN mice. At 100 mg/kg the $t_{1/2}$ for 4-OH-CPA was increased 1.8-fold, the C_{max} reduced 1.7-fold, and the AUC remained unchanged. This latter finding shows that P450-mediated oxidative metabolism is essential for the clearance of this compound. Toxicokinetic analysis of CPA-induced myelosuppression and granulocytopenia showed that at high doses (≥ 100 mg/kg) there was no difference in myelotoxicity between the wild-type and HRN mice. However, at lower doses (≤ 70 mg/kg) a significant difference was observed, with little toxicity seen in HRN mice but at least a 45% reduction in the bone marrow granulocyte population in wild-type mice. Meta-analysis of the toxicity experiments showed the myelotoxicity of CPA was found to be closely correlated with the C_{max} of 4-OH-CPA ($r^2 = 0.80$, $P = 0.002$). As the therapeutic effectiveness of CPA has been linked to the AUC for 4-OH-CPA, the finding that 4-OH-CPA C_{max} may determine its level of myelotoxicity indicates that the

therapeutic index could be altered by changing the method of CPA administration. Furthermore, monitoring 4-OH-CPA C_{max} may identify individuals at most risk of CPA side effects. (Cancer Res 2005; 65(10): 4211-7)

Introduction

Cyclophosphamide (CPA) is an anticancer prodrug that is dependent on cytochrome P450 (CYP) metabolism for its therapeutic effectiveness. CPA has been in the clinic since the late 1950s and is still one of the most widely used drugs in cancer chemotherapy (1, 2). However, due to the complex metabolic activation and detoxification pathways of CPA, making predictions about factors that determine its pharmacokinetics, toxicokinetics, and therapeutic effectiveness has been difficult. CPA is metabolized by hepatic CYPs via two major pathways. The first involves 4-hydroxylation to the active metabolite, 4-hydroxycyclophosphamide (4-OH-CPA). In humans, this is carried out predominantly by cytochrome P450 CYP2B6 (3, 4). 4-Hydroxy-CPA exists in equilibrium with aldophosphamide, which breaks down to form the DNA cross-linking agent, phosphoramidate mustard, and the toxic metabolite, acrolein (1, 2, 5). The alternative pathway involves a CYP3A4-mediated *N*-dechloroethylation of CPA to form the inactive metabolite, 3-dechloroethylcyclophosphamide (DECP), and the toxic by-product, chloroacetaldehyde (1, 2, 6, 7). In humans, this pathway is secondary to the activation pathway and accounts for less than 10% of the dose (5).

To date, most research on the involvement of hepatic CYPs in drug metabolism and on potential drug-drug interactions has been carried out *in vitro*, including liver microsomes, fresh and cryopreserved hepatocytes, or recombinant enzymes (8, 9). Although these *in vitro* tools have provided valuable insights into the pathways of disposition mediated by the cytochrome P450 system, a definitive method for correlating *in vivo* effects from these *in vitro* approaches remains elusive. Factors such as the route of administration, absorption, involvement of drug transporters, renal clearance, and the contribution of extrahepatic CYPs are all complicating factors in understanding drug pharmacokinetics, toxicokinetics, and efficacy.

There have been some significant advances in understanding the involvement of CYPs in drug metabolism and disposition *in vivo* with the use of transgenic mice and models where specific enzymes have been deleted (10–12). However, due to the large number of CYP isoforms and the broad and overlapping substrate specificity of these enzymes, their application has been restricted. Our group has recently developed a mouse model where the key electron transfer protein in all CYP-mediated reactions, cytochrome P450 reductase (POR), has been conditionally deleted in the liver

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[Hepatic cytochrome P450 Reductase Null (HRN)]. These mice have a profoundly reduced capacity for hepatic CYP-mediated drug metabolism (13), providing a powerful approach to establish the role of hepatic versus extrahepatic drug metabolism and drug disposition and in toxicokinetic studies. In this report, we have used the HRN mouse to investigate the pathways of CPA disposition and its toxicokinetics.

Materials and Methods

Chemicals

CPA was obtained from the Ninewells Hospital Pharmacy (Tayside University Hospital Trust, Dundee) as a solution (20 mg/mL) in saline. Ifosfamide and methoxyamine were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals and solvents were of analytic reagent grade.

Animals

All experiments were carried out in accordance with the Animal Scientific Procedures Act (1986), and after local ethical review. The generation of HRN knockout mice by this group has been described elsewhere (13). Male $POR^{lox/lox}$ (wild-type) and $POR^{lox/lox} + Cre^{ALB}$ (HRN) mice between 12 and 16 weeks of age were used in all experiments. All mice were maintained under standard animal house conditions with *ad libitum* access to standard rodent diet and water, and 12 hours of light/dark cycle.

In vitro Cyclophosphamide Metabolism

Liver microsomes were prepared from the livers of three wild-type and three HRN mice by differential centrifugation (14) and protein concentration of the microsomal suspensions was estimated as previously described (15). Hepatic POR levels were determined either by immunoblotting using hepatic microsomal fractions (by Western Blotting) or immunostaining of liver sections. Assays were carried out as previously described (12) using an antibody to rat POR (13). For the determination of Michaelis-Menten kinetic variables, microsomal incubations with CPA were carried out in Dulbecco's PBS using a microsomal protein concentration of 1 mg/mL and CPA concentration ranging from 1 to 1,000 $\mu\text{mol/L}$. Incubations were carried out in a total volume of 250 μL in the presence of NADPH (2 mmol/L). The nucleophilic trapping agent, methoxyamine (25 μL , 10 mmol/L in water), was included in the incubation mixture for stabilization of the activation metabolite, 4-OH-CPA (16). Incubations were carried out at 37°C for 30 minutes and these conditions were in the linear range for the activation metabolite, 4-OH-CPA, as determined from preliminary experiments (data not shown). After incubation, internal standard was added (ifosfamide, 10 μL of 10 $\mu\text{g/mL}$ solution in water) followed by 1 mL of acetonitrile to precipitate proteins. The samples were then vortex mixed and centrifuged at $3,000 \times g$ for 10 minutes. The supernatant was transferred to a clean tube and the sample volume was evaporated to $\sim 150 \mu\text{L}$ under a stream of N_2 at 50°C. The concentrated sample was then placed in a high-performance liquid chromatography (HPLC) vial for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

Cyclophosphamide Pharmacokinetics

CPA was administered as a single i.p. dose at either 100 mg/kg (stock of 10 mg/mL, using 10 mL/kg) or 300 mg/kg (stock of 20 mg/mL, using 15 mL/kg) body weight. For both dosing experiments, two wild-type mice and two HRN mice were used per time point. At both doses, blood samples were collected at 15, 30 minutes, 1, 2, 4, 6, and 12 hours after administration of CPA. Animals were sacrificed by a rising concentration of CO_2 and blood was obtained by cardiac puncture and placed in heparinized vials on ice for later centrifugation to separate the plasma fraction. The plasma sample was then snap frozen in liquid N_2 and stored at -70°C .

Three additional mice from each group were given a single i.p. dose at 100 mg/kg body weight and placed in individual metabolic cages (Tecniplast, Buguggiate, Italy). Urine was collected at room temperature for a 24-hour period, after which urine volume was recorded, samples transferred into clean tubes and frozen at -20°C . A small aliquot of urine ($\sim 25 \mu\text{L}$) was also taken at the time of collection to test for protein,

bilirubin, and blood using urinalysis reagent strips (N-Multistix SG, Bayer, plc, Berkshire, United Kingdom). Mice were then sacrificed and blood obtained by cardiac puncture. Plasma was collected as described above and frozen at -70°C for subsequent analysis for creatinine and alanine aminotransferase (ALT) using commercially available kits (ThermoTrace, Noble Park, Australia) on a Cobas Fara II centrifugal analyser (Roche Molecular Biochemicals, Mannheim, Germany).

Myelosuppression Experiments

Initial experiments were carried out to establish a dose-response curve for CPA myelosuppressive effects. CPA was administered i.p. at 100 mg/kg body weight (stock of 10 mg/mL, using 10 mL/kg) and bone marrow and spleen cells harvested 12 hours, 1, 2, 4, and 7 days later. Two wild-type and two HRN mice were used for each time point. Bone marrow cells were obtained from the two femurs by flushing with a known volume of cold PBS containing 1% FCS. Spleen cells were obtained by pushing the spleen through a fine mesh using a syringe plunger in a known volume of cold PBS. Both bone marrow and spleen cells were passed through a 23-G needle several times to ensure a single cell suspension. The cells obtained from both femurs were pooled and bone marrow and spleen cell numbers were determined using a haemocytometer. Forward and side scatter plots of cells were also obtained by flow cytometry for further characterization of cell lineage distributions.

The above experiments established that the toxic effect of CPA on both bone marrow and spleen cells was greatest 2 days after dosing. Therefore, using this data, further experiments in both mouse groups were then carried out to correlate the myelosuppressive effect of CPA with the CPA dose, the circulating level of CPA itself, and the activation product, 4-OH-CPA.

To establish the pharmacokinetics of CPA and metabolites over a dose range, CPA was administered i.p. at a dose of 35, 50, 70, or 100 mg/kg from stock solutions adjusted to yield a final volume of 10 mL/kg for each treatment. Three HRN and three wild-type mice were used at each dose and serial blood samples (10 μL) were taken from the lateral tail vein at the following time points: 10, 20, 30, 45, 60, 120, 240, and 360 minutes. Terminal blood samples were collected by cardiac puncture at 420 minutes. For serial sampling, blood was collected using a pipette directly from the tail vein and placed immediately into an Eppendorf tube containing 10 μL of methoxyamine (40 mmol/L) for the stabilization of 4-OH-CPA (16). Tubes were capped and incubated at 50°C for 20 minutes and then snap frozen in liquid N_2 and stored at -70°C . A 10- μL aliquot of the terminal blood was treated in the same way. For the myelosuppression experiment, CPA was given to mice ($n = 3$ for each group) at the same doses outlined above. Two days after CPA administration, mice were sacrificed and bone marrow harvested as detailed above.

Overall bone marrow cellularity was determined by haemocytometer and the degree of neutropenia, as measured by quantitation of the granulocytic cell population, was determined using flow cytometry. Briefly, bone marrow cells (1 million in 500 μL) were incubated with 1 μg of primary antibody (PE-conjugated RB6-8C5 or PE-conjugated A95-1 as control for nonspecific binding; Pharmingen, Oxford, United Kingdom) for 30 minutes at 4°C in the dark. After incubation, cells were washed once with PBS containing 1% FCS (3 mL) and then the pellet was resuspended and fixed in 1% formaldehyde in PBS (300 μL). The fixed cells were stored in the fridge and, the following day, the cells were analyzed by flow cytometry. A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) was used and data obtained were analyzed using the BD Cell Quest software. Forward and side scatter plots were prepared and granulocytes (Gr-1) were counted relative to 10,000 cells acquired from the fluorescence-activated cell sorting instrument.

Plasma, Urine, and Blood Analysis

Sample preparation. For the pharmacokinetic studies, 50 μL of plasma were transferred into a tube containing 450 μL of water. Internal standard was then added (ifosfamide, 100 ng in 10 μL of water) and this mixture was extracted into ethyl acetate (1.5 mL). The sample was centrifuged and the ethyl acetate extract removed and placed into a clean tube. This process was repeated a further two times with 600 μL ethyl acetate. The extracts were pooled and evaporated to dryness under a stream of N_2 at 40°C.

The pellet was then reconstituted in 100 μ L of 50% MeCN/20 mmol/L ammonium acetate buffer (pH 5.0) and placed into a HPLC vial for analysis.

For blood and urine samples, 10 μ L of internal standard (ifosfamide, 100 ng) were added to each tube and protein precipitation was carried out using four times the sample volume of acetonitrile. Samples were vortex mixed and centrifuged. The supernatant was removed and, for the urine samples, evaporated to dryness under a stream of N_2 at 50°C. The pellet was then reconstituted in HPLC mobile phase as for the plasma samples. For the blood samples, the supernatant was transferred directly into HPLC vials for analysis.

Standard curves for CPA were constructed for quantifying CPA plasma and blood levels by spiking blank plasma or blood samples with known amounts of CPA, followed by extraction or protein precipitation as outlined above for the test samples.

Liquid chromatography tandem mass spectrometry conditions.

Plasma, blood, urine, and microsomal extracts were analyzed for CPA and its metabolites by LC-MS/MS. LC-MS/MS was carried out using a Waters 2795 HPLC coupled to a Quattro Micro mass spectrometry system (MicroMass, Manchester, United Kingdom) in the positive electrospray ionization mode. The capillary temperature and voltage were 350°C and 3.2 kV, respectively. Multiple reaction monitoring data were acquired with the following variables: m/z 260.9 > 140.1 for CPA, m/z 260.9 > 154.1 for ifosfamide (internal standard), m/z 305.7 > 220.9 for the derivatized product of 4-OH-CPA, and m/z 198.9 > 170.7 for DECP. A cone voltage of 35 V was used for all analyses, with the exception of 4-OH-CPA, where the cone voltage was 20 V. A collision energy of 20 V was used for each analyte.

Chromatography was done using a Kromasil C_{18} (5 μ m, 150 \times 3.2 mm) column (Phenomenex, Torrance, CA). An isocratic mobile phase consisted of 35% acetonitrile/ammonium acetate buffer (20 mmol/L, pH 5.0) and a flow rate of 0.4 mL/min was used. The total run time was 6.5 minutes with analytes eluting in the following order: DECP, CPA, ifosfamide, and 4-OH-CPA. The injection volume was 10 μ L. MS-MS data were processed using the quantify option of MassLynx software version 4.0 from Micromass.

Data Analysis

Pharmacokinetic variables were calculated using the WinNonLin software, version 3.1. A simple noncompartmental model was used to calculate plasma area under the curve (AUC_{plasma}), $t_{1/2}$, C_{max} , and clearance (CL). Differences between wild-type and HRN mice in the level of myelosuppression were analyzed by unpaired t test and considered significant with a $P < 0.05$. The relationship between granulocytes and the pharmacokinetic variables for CPA and 4-OH-CPA was determined by regression analysis. Statistical analysis was carried out using the Statview program for Macintosh, version 4.5 (Abacus Concepts, Berkeley, CA).

Results

In vitro cyclophosphamide kinetics. Analysis of hepatic POR expression in the HRN mice used in these studies showed a profound reduction in POR levels and activity related to wild-type animals in that almost all hepatocytes were devoid of detectable POR expression (Fig. 1A). As the deletion of the POR was carried out genetically, the negative hepatocytes could not contain any active reductase. By immunostaining, the reductase deletion in hepatocytes was estimated to be >99%. Associated with this change, there was a marked reduction in the microsomal metabolism of CPA (Fig. 1B). Due to the lack of availability of metabolite standards, it was not possible to quantify the amount of 4-OH-CPA formed. However, the relative V_{max} for 4-OH-CPX formation was more than 6-fold higher in wild-type versus HRN microsomal fractions. As the K_m values for 4-OH-CPA formation were the same, being 340 ± 46 and 401 ± 32 μ mol/L for wild-type and HRN mice, respectively ($P = 0.786$), this resulted in a 6.2-fold higher intrinsic clearance (relative V_{max}/K_m) for this pathway in wild-type versus HRN mice.

Pharmacokinetic analyses. To evaluate the role of hepatic metabolism in the *in vivo* CPA pharmacokinetics, in initial

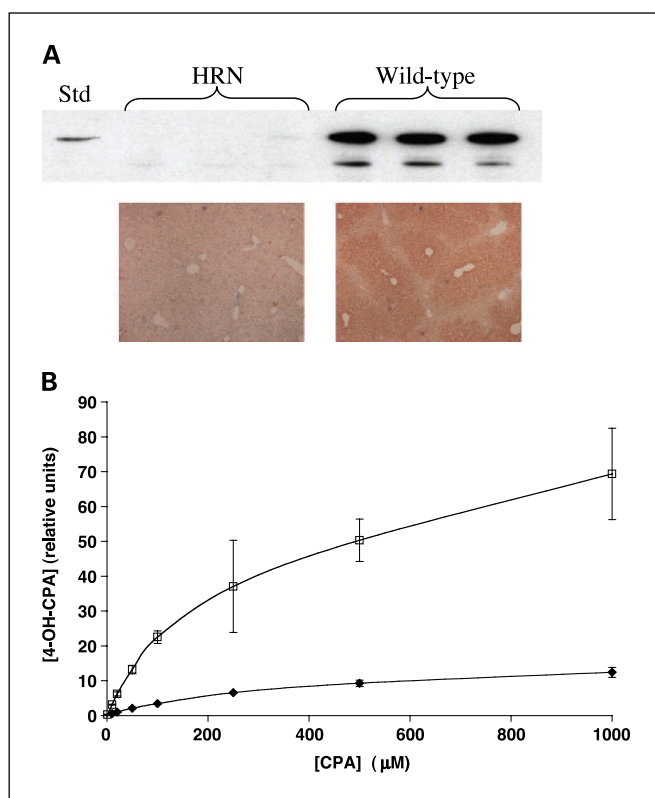


Figure 1. Hepatic expression of cytochrome P450 reductase and microsomal metabolism of cyclophosphamide in HRN and wild-type mice. 4-Hydroxy-CPA formation was determined in microsomal incubations containing CPA (1-1,000 μ mol/L) by LC-MS/MS as described in Materials and Methods. ◆ and □, HRN and wild-type mice, respectively. Points, mean of three determinations; bars, SD. A, immunoblot of POR protein levels in liver microsomes (top) and immunohistochemical staining of liver sections obtained from HRN and wild-type mice (bottom), where brown zonal staining represents POR positive cells (see Materials and Methods for details). B, Michaelis-Menten plot. 4-Hydroxy-CPA concentrations are presented as relative units as no 4-OH-CPA standard was available (see Materials and Methods).

experiments CPA pharmacokinetic variables were determined in HRN and wild-type mice at two doses (Table 1). Plasma concentration profiles over time are shown in Fig. 2 and indicate a marked reduction in CPA elimination in the HRN mice. At 100 mg/kg CPA there was a 6.2-fold increase in the plasma AUC and a 3.2-fold increase in the half-life in the HRN mice, whereas at 300 mg/kg CPA, the plasma AUC was increased 4.2-fold and the half-life by 3.1-fold.

Measurement of CPA metabolites in blood samples from the dose response study showed that in spite of the reduction in hepatic P450 activity, considerable metabolism occurred in the HRN mice and the same oxidized metabolites were formed as in the wild-type animals. Wild-type mice produced significantly higher blood concentrations of 4-OH-CPA, however, the peak concentration of DECP was found to be lower than that reached in the HRN mice. A marked difference between the blood concentration profiles with time for the two metabolites was also observed (Fig. 3). In both mouse lines, the 4-hydroxylated metabolite had a similar half-life to the parent drug, indicating its pharmacokinetics was formation rate limited. Although the shape of the blood concentration time profile for this metabolite differed between wild-type and HRN mice, there was no difference in the AUC. In contrast to the DECP metabolite, the

Table 1. Pharmacokinetics variables of CPA and its metabolites in wild-type and HRN mice

Parameter	Cyclophosphamide				4-OH-CPA		DECP	
	100 mg/kg i.p.		300 mg/kg i.p.		100 mg/kg i.p.		100 mg/kg i.p.	
	HRN	Wild-type	HRN	Wild-type	HRN	Wild-type	HRN	Wild-type
C_{max} ($\mu\text{g/mL}$)	97	38	255	181	12*	20*	19*	16*
$t_{1/2}$ (h)	0.9	0.3	1.1	0.4	0.7	0.4	1.6	1.3
$CL_{(0.25-\infty)}$ (mL/h)	18.9	118.3	15.4	65	—	—	—	—
$AUC_{0-\infty}$ (h $\cdot\mu\text{g/mL}$)	162	26	571	135	27*	22*	82*	46*

NOTE: Wild-type or HRN mice were dosed with either 100 or 300 mg/kg CPA i.p. and kinetic variables were determined as described in Materials and Methods.

*Units for C_{max} and AUC of metabolites are expressed as relative to internal standard area ratios from mass chromatograms.

kinetic profile indicated that its elimination was rate limiting (i.e., displaying a much longer terminal half-life than the parent compound). For this metabolite, there was an increase in the AUC and C_{max} for the HRN relative to the wild-type, probably

reflecting a decrease in the metabolic clearance of DECP as it undergoes further metabolism, which in turn is consistent with the increased half-life.

Urine analysis showed no difference between wild-type and HRN mice in the level of CPA excreted unchanged and, for both groups, this was only ~1% of the total CPA dose. Similarly, no detectable differences were observed in protein, bilirubin, or blood in urine samples or in plasma creatinine and ALT levels in both groups of mice (data not shown). All of these variables were not significantly different for untreated mice, indicating that in spite of the increased blood concentrations, the CPA did not become more toxic to the liver or other tissues. This shows the importance of hepatic metabolism in the toxicity of CPA to tissues such as the kidney.

Myelosuppression. To investigate the relationship between CPA pharmacokinetics and toxicity, we studied whether the pharmacokinetic changes were reflected in the level of myelosuppression. Initial experiments were carried out at a CPA dose of 100 mg/kg. Bone marrow and spleen hematopoietic cellularity were determined over a 7-day period and, interestingly, no difference in response between the HRN and wild-type mice at any of the time points was observed. In both groups, an initial decrease in bone marrow cells (by ~75%) was measured over the first 2 days, followed by an overshoot of control levels by ~50% at day 7 (not shown).

The myelosuppression was accompanied by a large decrease in the mature granulocyte population, with a nadir between 2 and 4 days (Fig. 4). After these time points, an increase in the mature granulocyte cell population was then observed which, after 7 days, represented 60% and 68% of the total bone marrow cell population for wild-type and HRN mice, respectively.

In the spleen, overall cellularity in both groups fluctuated initially and then decreased to around 30% of control levels on day 2 (data not shown). This was followed by a slight recovery to around 40% (wild-type) and 60% (HRN) of control values. The rate of recovery seemed to be slightly faster in the HRN mice; however, this was not significant.

In the light of the surprising lack of difference between the HRN and wild-type mouse lines in the level of myelosuppression at high CPA doses, in spite of the change in the rates of metabolite formation, we carried out a toxicokinetic study where CPA dose was altered and drug pharmacokinetics was compared with the

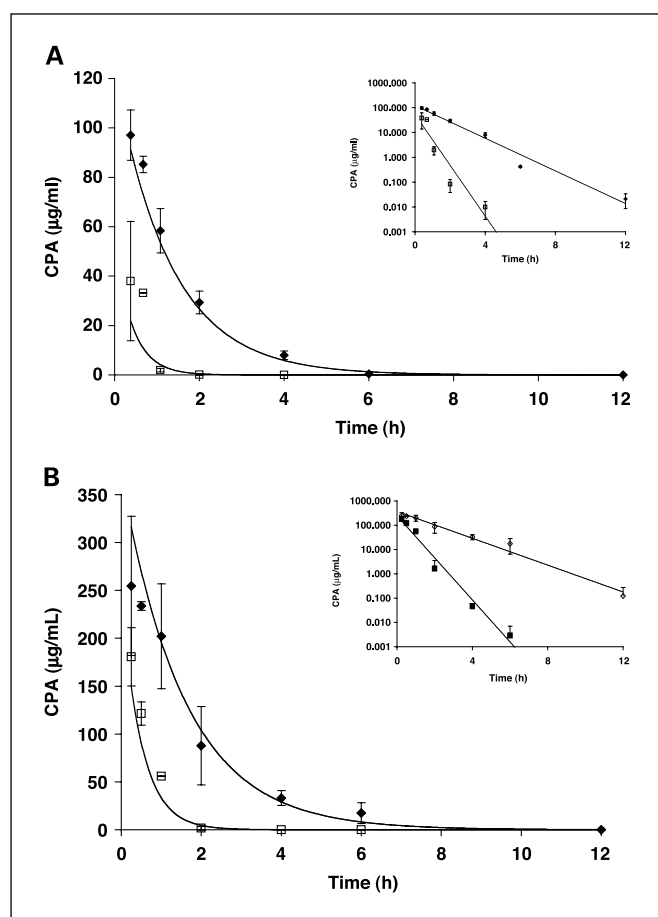


Figure 2. *In vivo* pharmacokinetics of cyclophosphamide in HRN and wild-type mice. CPA was administered i.p. to mice at doses of (A) 100 mg/kg and (B) 300 mg/kg. \blacklozenge and \square , HRN and wild-type mice, respectively (*inset*, logarithmic plot of the data). Plasma CPA levels were determined at the times shown by LC-MS/MS as described in Materials and Methods. *Points*, mean of two determinations; *bars*, range.

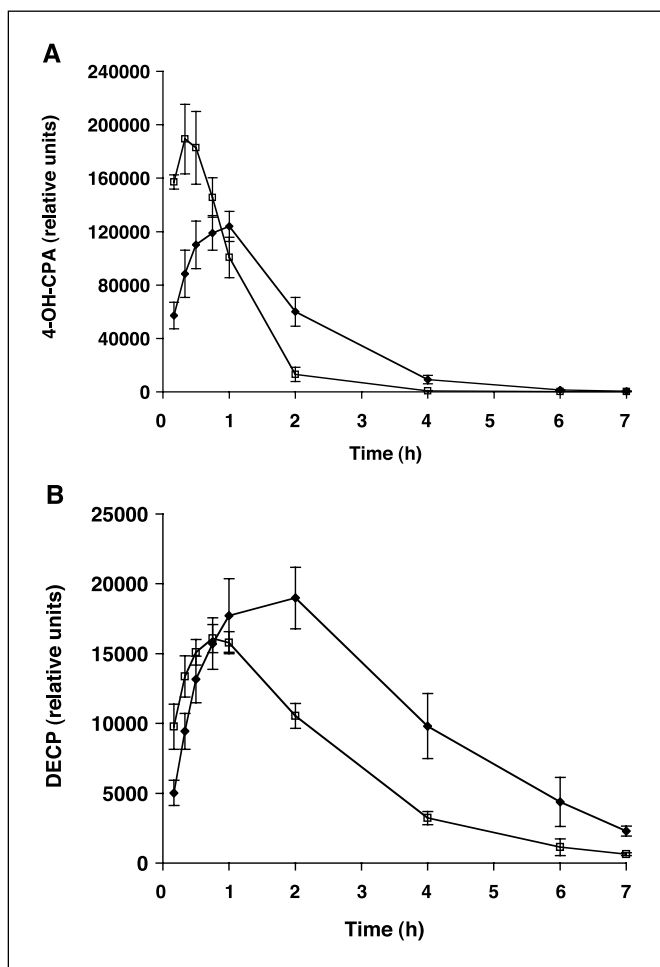


Figure 3. Blood concentration profile of cyclophosphamide metabolites in HRN and wild-type mice. Values are expressed as chromatogram area ratios relative to internal standard (ifosfamide); A, 4-OH-CPA; B, DECP. Animals were treated with 100 mg/kg CPA i.p. ◆ and □, HRN and wild-type mice, respectively. Blood metabolite levels were determined at the times shown by LC-MS/MS as described in Materials and Methods. Points, mean of three determinations; bars, SD.

level of myelotoxicity (Fig. 5). In wild-type mice, a dose-dependent increase in granulocyte toxicity was observed (Fig. 5A-C). This reduction was directly proportional to all three pharmacokinetic variables measured [i.e., CPA exposure (AUC_{CPA}), 4-OH-CPA exposure ($AUC_{4-OH-CPA}$), and acute 4-OH-CPA exposure (C_{max}), respectively; Fig. 5A-C]. However, in the HRN mice, the 4-OH-CPA C_{max} correlated much closer to the myelotoxicity than the CPA dose or the AUC. No significant myelotoxicity was observed in HRN mice at all doses except at 100 mg/kg. At this dose, myelotoxicity was similar to wild-type mice. At all doses, there was no significant difference in the overall exposure to 4-OH-CPA between groups, whereas the 4-OH-CPA C_{max} was around 2-fold lower in the HRN mice at all but the highest dose and this mirrored the reduction in granulocytes. A meta-analysis using all the pharmacokinetic data obtained in these experiments showed that again the C_{max} for 4-OH-CPA correlated extremely closely with the loss in granulocyte cell number ($r^2 = 0.80$, $P = 0.002$; Fig. 5D), compared with r^2 values of 0.01 ($P = 826$) and 0.20 ($P = 0.256$) for the AUC_{CPA} and $AUC_{4-OH-CPA}$, respectively (not shown and Fig. 5E, respectively).

Discussion

The metabolism and pharmacokinetics of the anticancer prodrug, CPA, have been extensively studied in several species over the last three decades (1, 17, 18) and its mechanism of action and toxicity has been associated with hepatic activation by the cytochrome P450 system. However, these conclusions have been predominantly based on the use of *in vivo* cytochrome P450 inducers or inhibitors. As inhibitors can inhibit the P450 system in any tissue and experiments with inducers only provide evidence for the induced state, the role of hepatic P450s in CPA disposition remains unproven. In this study we provide unequivocal evidence on the importance of the hepatic enzymes in the disposition of this compound. This has been achieved by employing a genetic approach where hepatic cytochrome P450 activity has been essentially inactivated by the conditional deletion of hepatic POR. Western blot and immunohistochemical analysis on the liver samples used in these studies showed an almost complete loss of POR (Fig. 1), yet a low level of cytochrome C activity as well as NADPH-dependent CPA metabolism was observed *in vitro*. Based on these the data, this level of residual activity is difficult to rationalize, but could be explained if cells other than hepatocytes have some metabolic capacity or, alternatively, if there is cross-electron transfer in microsomal fractions from membrane vesicles that still contain POR activity to those which do not. *In vivo*, significant levels of CPA metabolism, as well as formation of oxidized metabolites, were still detected. The *in vitro* kinetic studies showed that the rate of 4-OH-CPA formation was 6-fold lower in HRN mice. Similar differences were observed in the *in vivo* kinetic variables. It therefore remains to be shown whether the residual CPA metabolism is due to hepatic or extrahepatic metabolism. In any event, it is clear that hepatic metabolism is a major determinant in CPA pharmacokinetics, and metabolism is a prerequisite for CPA elimination.

There are limited reports on the effect of impaired liver function on the metabolism and pharmacokinetics of CPA in humans. In a study on one patient with cirrhosis, Bagley et al. (19) found that the half-life of CPA was significantly longer compared with patients with normal liver function. In a more comprehensive report, Juma (20) found that the plasma CPA half-life after an i.v. dose was significantly longer in patients with severe liver failure, and the

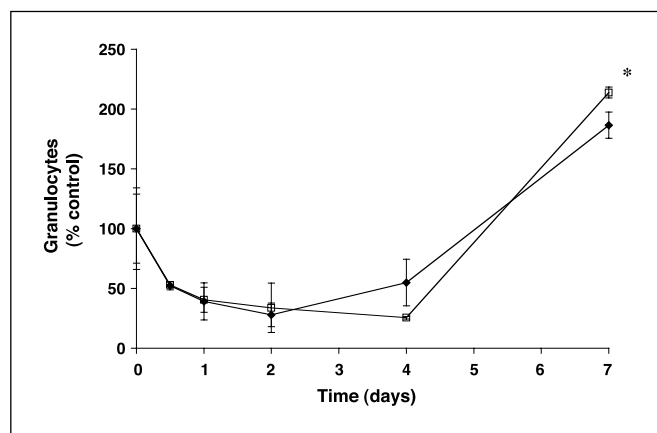
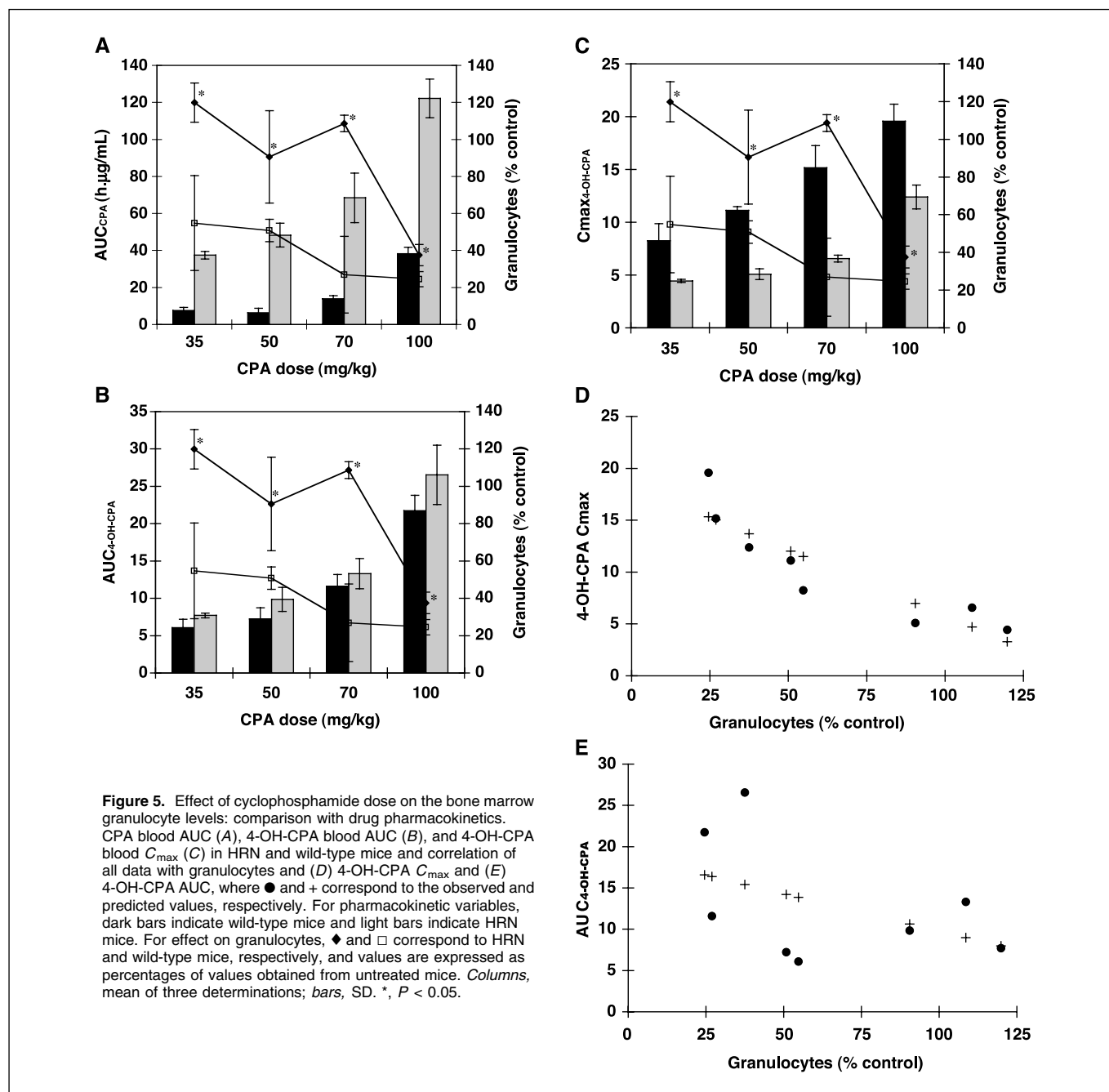


Figure 4. Effect of cyclophosphamide on bone marrow granulocyte cellularity. Mice were dosed with CPA (100 mg/kg) and cellularity determined as described in Materials and Methods; ◆ and □, HRN and wild-type mice, respectively. Values are expressed as percentages of values obtained from untreated mice. Points, mean of two determinations; bars, range. *, $P < 0.05$.



total body clearance was reduced, indicating a reduced capacity for elimination of this compound. These workers also observed a decrease in the adverse effects of CPA, such as haemorrhagic cystitis and anaemia, side effects that are known to be a result of the CPA metabolites, acrolein and phosphoramidate mustard. This lack of toxicity with liver disease supports the many reports associating oxidative metabolism of CPA with hepatic CYPs.

At high doses of (100 and 300 mg/kg), we observed little difference in associated myelosuppression in the HRN mice compared with wild-type mice. In both experiments a similar profound decrease in bone marrow and spleen cellularity after CPA administration was observed. The number of mature granulocytic cells reached a nadir between 2 and 4 days for both HRN and wild-type mice, which was comparable with other studies carried out

on mice looking at the effect of this compound on hematopoiesis (21–23). Urine analysis for markers of liver, kidney, and bladder toxicity also showed no difference between the two groups. There was some evidence of CPA-induced nephrotoxicity with all urine samples showing a positive result for protein. However, as this effect was not increased in the HRN mice, in spite of the increased circulating CPA levels, this would indicate that the nephrotoxicity is due to hepatic rather than local metabolic activation. Similarly, plasma ALT and creatinine levels indicated no CPA affect within groups, indicating that the overall toxicity of CPA at high doses is not changed. The lack of difference between HRN and wild-type mice at high doses of CPA may be due to the finding that although exposure to CPA itself is around 5-fold greater in the absence of hepatic POR, exposure to the activated metabolite, 4-OH-CPA, was

almost identical in both groups. Alternatively, the acute exposure to this metabolite may have reached a saturable level such that even in the HRN mice, the same level of toxicity is observed at higher doses (i.e., the toxic pathway was saturated). Toxicokinetic analysis indicated that the latter is probably the most likely explanation (see below).

Although the use of CPA in cancer therapy dates back to the late 1950s, until now, a systematic study investigating the relationship between CPA dose, exposure to parent drug and active metabolite, and the myelosuppressive toxic effect of this compound has not been carried out. Such studies are essential for the optimal use of this and similar cytotoxic antitumor agents. In the wild-type mice, we found a relatively linear relationship between the CPA dose and the C_{max} and AUC of CPA and 4-OH-CPA, respectively. Similarly, the degree of neutropenia was inversely proportional to all of these variables. As a result, this data alone does not allow an evaluation of whether or not neutropenia is caused by the overall exposure to 4-OH-CPA (AUC) or by the acute exposure to this metabolite. By combining these data with data obtained using the HRN model, we have found that the myelotoxicity correlated to the maximum blood concentration of 4-OH-CPA compared with the overall exposure to this metabolite. Two lines of evidence suggest this. First, at CPA doses of 35, 50, and 70 mg/kg, the level of myelosuppression did not correlate with the AUC for either CPA or 4-OH-CPA, but did parallel the 4-OH-CPA C_{max} . In addition, meta-analysis of all data points gave a much higher correlation with the C_{max} than the combined AUC values. These data are intriguing as the literature suggests that the antitumor effects of this DNA cross-

linking agent are more closely related to the overall exposure or AUC. On this basis, it would be predicted that the optimal method for the administration of CPA would be as a continuous infusion over an extended period. This would result in a reduced C_{max} of 4-OH-CPA, without compromising therapeutic efficacy. Voelcker et al. (24) measured the pharmacokinetics of 4-OH-CPA in both cancer patients and mice and correlated this with tumor growth inhibition. These workers reported that CPA was more therapeutically efficacious if 4-OH-CPA blood concentrations were maintained at lower levels over longer time periods. This was achieved by giving lower doses over set time periods, compared with a bolus injection of the same dose which resulted in a higher acute exposure to 4-OH-CPA. At present, CPA is normally given as a single dose over a short time period of up to 1 hour.

In conclusion, the use of the HRN mouse provides a means of carrying out detailed toxicokinetic analysis of currently used anticancer drugs or anticancer drugs in development. Such information is of vital importance for the appropriate design of clinical trials and subsequent clinical use and suggests that the mode of administration of many currently used antitumor agents may need to be revisited.

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