

## The Effect of Repeated Administration of Cyclophosphamide on Cytochrome P450 2B in Rats

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**Abstract Purpose:** The prodrug cyclophosphamide (CPA) is activated by cytochrome P450 (CYP) enzymes. CPA is one of the corner stones in all cancer treatment. We have studied the effect of repeated doses of CPA given at different time intervals on the mRNA, protein levels, and enzyme activity of CYPs in rats.

**Experimental Design:** Two groups of animals (A-75 and A-150) were treated with four doses of CPA (75 and 150 mg/kg, respectively) at short time intervals (6 h). The third group of animals (B-150) was treated with 150 mg/kg at 24-h intervals. Three animals were killed 30 min after administration, and three animals immediately before the next dose.

**Results:** CYP2B1 and CYP2B2 mRNAs were significantly induced at 6 h after each dose in group A-75 (maximum of 2100-fold and 60-fold after the third dose, respectively), whereas the mRNA levels measured at 6 h postadministration in group A-150 were 1,490-fold and 36-fold after the second dose. In group B-150, no significant induction of mRNA levels was observed. CYP2B1 and CYP2B2 protein levels also increased with increased mRNAs. Plasma levels of 4-hydroxy-CPA measured at 30 min after dose correlated well with the increase in protein levels.

**Conclusion:** Up-regulation of CYP2B mRNA, with a concomitant increase in protein expression and activity, were observed after repeated administration of low doses of CPA compared with that found using higher doses, possibly due to toxicity counteracting induction. These results may help in designing more effective dosing schedules for CPA.

Cyclophosphamide (CPA) is an alkylating agent, mainly used in the treatment of a wide range of malignancies and autoimmune disorders (1). CPA is extensively metabolized in the liver by cytochrome P450 (CYP) enzymes via 4-hydroxylation and *N*-dechloroethylation. About 20% of the given dose are excreted in the urine as unchanged compound (2, 3).

CYP2B6, CYP3A4, CYP2C9, and CYP2C19 have been shown to be the major CYPs responsible for the metabolism of CPA (4–7). Among these enzymes, CYP2B6 was pointed out as the main CYP isozyme for the formation of 4-hydroxy-CPA (4-OH-CPA; ref. 6). 4-OH-CPA penetrates the cell membrane and decomposes to phosphoramid mustard that alkylates DNA

and the urotoxic compound (acrolein) which is believed to be involved in hemorrhagic cystitis (5, 6). *N*-dechloroethylation generates an inactive compound (dechloroethyl-CPA) and chloroacetaldehyde, which is both nephrotoxic and neurotoxic (2).

CPA is used as stem cells mobilizing agent before autologous stem cell transplantation (SCT) in lymphoma patients (8, 9). CPA is also used in high doses as a part of the conditioning regimen before SCT. The doses used in the conditioning vary between 50 and 100 mg/kg/day and are usually given for 2 or 4 consecutive days, primarily to facilitate engraftment of donor cells. Patients treated with high doses of CPA before SCT suffer from side effects, including nausea and vomiting, bone marrow suppression, alopecia, and hemorrhagic cystitis. McDonald et al. have reported liver toxicity and mortality after giving repeated high doses of CPA in SCT patients (10). After the administration of repeated doses, a significant decrease in CPA elimination half-life has been observed without changes in urinary excretion of CPA or increased exposure to activated metabolites (11–13). Schuler et al. have reported that the area under the plasma concentration-time curve of 4-OH-CPA (measured as acrolein) increased by 73% between the first and second days (13, 14). The authors suggested that this might be due to autoinduction of CPA metabolism through enzyme induction (13). Moreover, patients who were treated with high-dose CPA as conditioning before SCT showed shorter elimination half-life and increased clearance, which correlated well with an increase in CYP-dependent CPA metabolism (13–17).

In a culture of primary human hepatocytes, an increase in CYP3A4, CYP2C8, and CYP2C9 levels, but not CYP2B6 levels,

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was observed after exposure to CPA, which surprisingly was correlated with an increased rate of 4-hydroxylation (18). In another *in vitro* study, no detectable change in CYP3A4 protein expression was observed after 48-h exposure to CPA, whereas CYP2B6 was induced at both mRNA and protein levels (19). Lindley et al. (2) have reported that CPA treatment of primary human hepatocyte cultures for 3 days produced a concentration-dependent increase in CYP3A4 and CYP2B6 activities and immunoreactive proteins that peaked at 250 and 125  $\mu\text{mol/L}$  CPA, respectively.

Several studies in rats have illustrated that CPA suppresses a variety of CYP activities via multiple mechanisms. However, these studies were carried out 6 to 14 days after CPA treatment (4, 20–24). Recently, we (25) have shown that induction effect of CPA on CYPs in rats occurred within 5 days after a single administration of CPA. The induction of mRNA, protein, and microsomal activity of CYP2B1, CYP2B2, CYP3A2, and CYP2C11 was observed after both high and low doses of CPA in rats. The maximum levels of mRNA were reached between 4 and 8 h after the administration of a single dose. A decrease in CPA concentration and an increase in 4-OH-CPA levels after repeated administration were observed (25).

The aim of this study was to investigate the effect of repeated doses of CPA given at different time intervals on CYPs in rats by assessing mRNA levels, protein levels, and CPA 4-hydroxylation activity.

## Materials and Methods

### Chemicals

The CPA monohydrate was purchased from Sigma Chemicals (Sweden). Maphosphamide was used to produce 4-OH-CPA by hydroxylation in water. Maphosphamide was kindly provided by Professor Ulf Niemeyer, Baxter Oncology GmbH.

TEMED, 40% acrylamide solution, ammonium persulfate, SDS, trizma base, glycine,  $\beta$ -mercaptoethanol, bromophenol blue, coomassie brilliant blue, glycerol, and Tween 20, used for electrophoresis and Western blotting, were purchased from Bio-Rad Laboratories.

$\beta$ -Nicotinamide adenine dinucleotide phosphate, reduced form ( $\beta$ -NADPH), was from Sigma Chemicals. SYBR Green PCR master mix containing AmpliTaq Golds DNA polymerase and optimized buffer components was purchased from Applied Biosystems. All other chemicals and solvents used were of high performance liquid chromatography (HPLC) or analytical grade and were purchased from Merck.

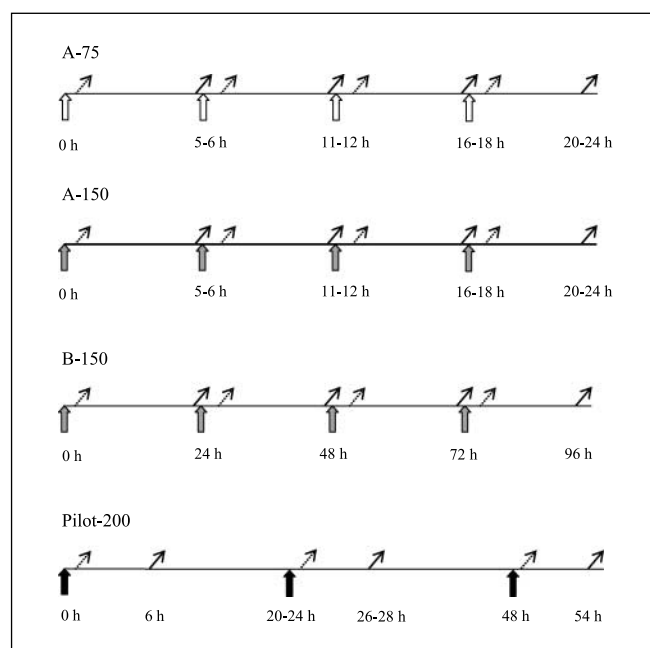
### Animals, treatment and sampling

**Animals.** Inbred male Wistar/Fu rats (B&K Universal) were housed as three animals per cage and received food and water *ad libitum*. Animals were housed in acclimatized room with a 12-h light-dark cycle. The average rat body weight upon arrival was 240 to 270 g. The Animal Ethical Committee at Karolinska Institutet approved all experimental protocols used in this study.

**Treatment.** The CPA was dissolved in physiologic saline and was given *i.v.* through the tail vein.

**Pilot study.** Rats received 200 mg/kg every 24 h for three consecutive doses (pilot-200). Three rats were sacrificed at 0.5 h and another three rats at 6 h after each dose (Fig. 1).

**Main study design.** We used the results obtained from the pilot study to design the main study. Animals were divided into two groups: group A was treated with four repeated doses at short dosing intervals (6 h), and group B was treated with four repeated doses with longer dosing intervals (24 h). Group A was divided into two subgroups: group A-75 was treated with 75 mg/kg of CPA, whereas group A-150 received higher dose at 150 mg/kg. After each administration, three rats



**Fig. 1.** Treatment groups. Wide arrows and numbers under arrows, CPA administration and time. White, 75 mg of CPA/kg body weight; gray, 150 mg of CPA/kg body weight; black, 200 mg of CPA/kg body weight. Arrows above the lines, time points when three rats were sacrificed; dotted arrows, sampling at 0.5 h postadministration; solid line arrows, second sampling after each dose.

were killed 0.5 h after dosing, wherein  $C_{\text{max}}$  of 4-OH-CPA is reached as we have reported in our previous study (26). Another three rats were killed 6 h after dosing (just before the next dose). Animals in group B (B-150) received CPA 150 mg/kg, once daily, for 4 days. In this group, three rats were killed 0.5 h after each administration and another three rats were sacrificed 24 h after each administration (Fig. 1). Untreated rats were used as controls. The dosage regimen used to study the autoinduction was based on the short half-lives of the parent compound CPA and its metabolite 4-OH-CPA (6, 26).

**Sampling.** Blood was collected in prechilled heparinized vacutainer tubes by heart puncture under anesthesia. Plasma was separated from blood cells by centrifugation ( $3,000\times g$ , 5 min,  $4^{\circ}\text{C}$ ). Plasma (500  $\mu\text{L}$ ) was added to 500  $\mu\text{L}$  of acetonitrile for protein precipitation. The mixture was vortexed for 30 s and centrifuged ( $3,000\times g$ , 3 min,  $4^{\circ}\text{C}$ ), the supernatant was separated, and aliquots were stored at  $-70^{\circ}\text{C}$  until determination of 4-OH-CPA. The remaining plasma was stored at  $-70^{\circ}\text{C}$  until assayed for CPA. Livers were removed, snapped frozen in liquid nitrogen, and kept at  $-70^{\circ}\text{C}$  for subsequent total RNA extraction and microsomal preparation (25, 27).

### Pharmacokinetics of CPA and 4-OH-CPA

The concentrations of CPA and 4-OH-CPA in rat plasma were measured by HPLC as described previously (25, 28).

Briefly, for CPA analysis, 1 mL of plasma was added to 25  $\mu\text{L}$  of internal standard (1 mmol/L ifosfamide). The samples were extracted in 5 mL ethyl acetate, vortexed, and then centrifuged ( $3000\times g$ ) for 10 min. The organic layer was evaporated to dryness under nitrogen gas. The residue was dissolved in 100  $\mu\text{L}$  of the mobile phase (acetonitrile–0.05 mol/L  $\text{KH}_2\text{PO}_4$  buffer, pH 4.8, 24:76 v/v). Thirty microliters were injected into the HPLC system, which consisted of an LKB 2150 HPLC pump, a Gilson 234 autoinjector with a 100- $\mu\text{L}$  sample loop, and a UV detector (Milton Roy Spectro Monitor 310) operated at a wavelength of 195 nm. A Zorbax 150  $\times$  4.6 mm and 5  $\mu\text{m}$  microsphere Extend C18 (Agilent) with a C18 guard column was used, and the flow rate was set to 1 mL/min. The standard curve, prepared using blank plasma added to CPA, was linear within the range of 1 to 200  $\mu\text{g/mL}$ . Chromatograms

were recorded, and the determination of the concentrations was carried out using a CSW32 chromatographic station.

4-OH-CPA was stabilized by adding dansylhydrazone (2 mg/mL) and hydrochloric acid (1 mol/L) as described previously (28). The mixture was vortexed and heated at 50°C for 5 min. Thirty microliters of the final sample was injected into the HPLC system as described above. The detector was a Shimadzu RF-10XL fluorescence detector. The detection was carried out with an excitation wavelength of 350 nm and an emission wavelength of 550 nm. The column was the same as used for the CPA analysis. The mobile phase was phosphate buffer (pH 3.5)/acetonitrile (2:1 v/v) and the flow rate was 2 mL/min. The calibration curve was linear between 60 and 2000 ng/mL.

#### RNA extraction and cDNA synthesis

Total RNA was isolated from the rat livers with QuickPreps Total RNA Extraction kit (Amersham Pharmacia Biotech). The concentrations and the purity of the RNA prepared were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Beckman 530 Life Science UV/Vis). RNA samples were stored at -70°C or processed directly by reverse transcription using the TaqMan reverse transcriptase-cDNA kit (Applied Biosystems). One microgram of total RNA was used to produce cDNA in a total volume of 35 µL. The samples were incubated at 37°C for 1 h using oligo (dT) 18 as the primer for reverse transcription, and heat denatured at 95°C for 5 min. cDNA samples were stored at -70°C.

#### Real-time PCR

The primers and PCR conditions used for CYP2B1, CYP2B2, CYP2C11, CYP3A1, and CYP3A2 amplifications were as described earlier (26). Briefly, the specific PCR conditions were optimized to produce PCR product with a minimum background. PCR was done in a GeneAMP PCR system 9700 (Applied Biosystems). For the assessment of the specific mRNAs, real-time quantitative PCR was done in an ABI Prism 7700 system (Applied Biosystems). The quantitative PCR was monitored by measuring the increase in fluorescence by the binding of SYBR Green I dye to the generated double-stranded cDNA. A volume of 2.5 µL of cDNA and primers (0.5 mmol/L) were added to SYBR Green master mix to make a final reaction volume of 25 µL. Cycling condition for reverse transcription-PCR was also used for the real-time PCR (26). Nonspecific amplification was checked by analysis of the PCR amplification product by agarose gel electrophoresis (data not shown). By this method, we were able to reach high specificity for each gene.

Standard curves were prepared from dilution of cDNA that were obtained from a single rat liver. The samples were analyzed using a standard curve, in which 32 cycles gave 10,000 copies per mRNA reaction (total RNA, 33 ng). Endogenous ribosomal 18S was used as an internal standard. Specific CYP mRNAs were presented as a copy number based on the ratio of CYP/18S.

#### Preparation of rat liver microsomes

Microsomes from rat liver specimens were prepared by subcellular fractionation as described elsewhere (25, 27). All procedures were done at 0 to 4°C unless stated otherwise. Pieces of rat liver (~3 g) were weighed and homogenized in three volumes of potassium phosphate buffer (10 mmol/L, pH 7.4) containing 1.15% KCl using a Potter-Elvehjem homogenizer. The microsomal fraction was obtained after successive centrifugations at 10,000×g for 20 min and 100,000×g for 70 min. The pellets containing the microsomes were resuspended in 1 mL of potassium phosphate buffer (50 mmol/L, pH 7.4) and stored at -70°C until use. The protein content of the microsomes was determined using a method modified from Lowry (29) with bovine serum albumin as standard.

#### Western blotting

Western blotting was carried out for rat hepatic CYPs as previously described (25). Briefly, microsomal proteins (2.5 µg) were separated by SDS-polyacrylamide gel electrophoresis (8.7% acrylamide), run for

45 min in a Mini-Protean II at 200 V, and transferred to PolyScreen polyvinylidene difluoride transfer membranes (NEN) by semidry blotting in a Bio-Rad Trans Blot SD semidry transfer cell (Bio-Rad Laboratories) using ~1.5 mA/cm<sup>2</sup> for 50 min by transfer buffer (20 mmol/L Tris base, 154 mmol/L glycine, 20% methanol, and 0.1% SDS). Prestained broad range molecular weight standards (Bio-Rad) were used on all gels as controls for separation and transfer. Blots were blocked with 5% nonfat milk in TBS buffer [200 mmol/L NaCl, 0.05% Tween 20, and 50 mmol/L Tris-HCl (pH 7.4)] and incubated for 2 h with primary antibodies in TBS buffer containing 1% nonfat milk at 37°C. The primary antibodies were rabbit polyclonal primary antibody against rat CYP2B1/2 (Chemicon), goat anti-rat CYP2C11 (Daiichi), rabbit anti-rat CYP3A1 (Chemicon), and rabbit anti-rat CYP3A2 (Chemicon). All of the primary antibodies were used at a dilution of 1:2,000 except the antirat 3A2 that was diluted to 1:1,000. The membranes were washed and incubated with 1:1,500 to 1:5,000 diluted secondary antibodies (alkaline phosphatase-antirabbit/antigoat IgG, Sigma) for 1 h at 37°C. The blots with alkaline phosphatase-conjugated antibodies were developed by the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, and band intensities were quantified by densitometric reading in a Bio-Rad GS-700 Imaging Densitometer with Molecular Analyst (v 1.4) software.

#### Microsomal activity assay *in vitro*

The auto induction was determined by measuring the formation rate of 4-OH-CPA from CPA using liver microsomes isolated from each individual rat in all treatment groups.

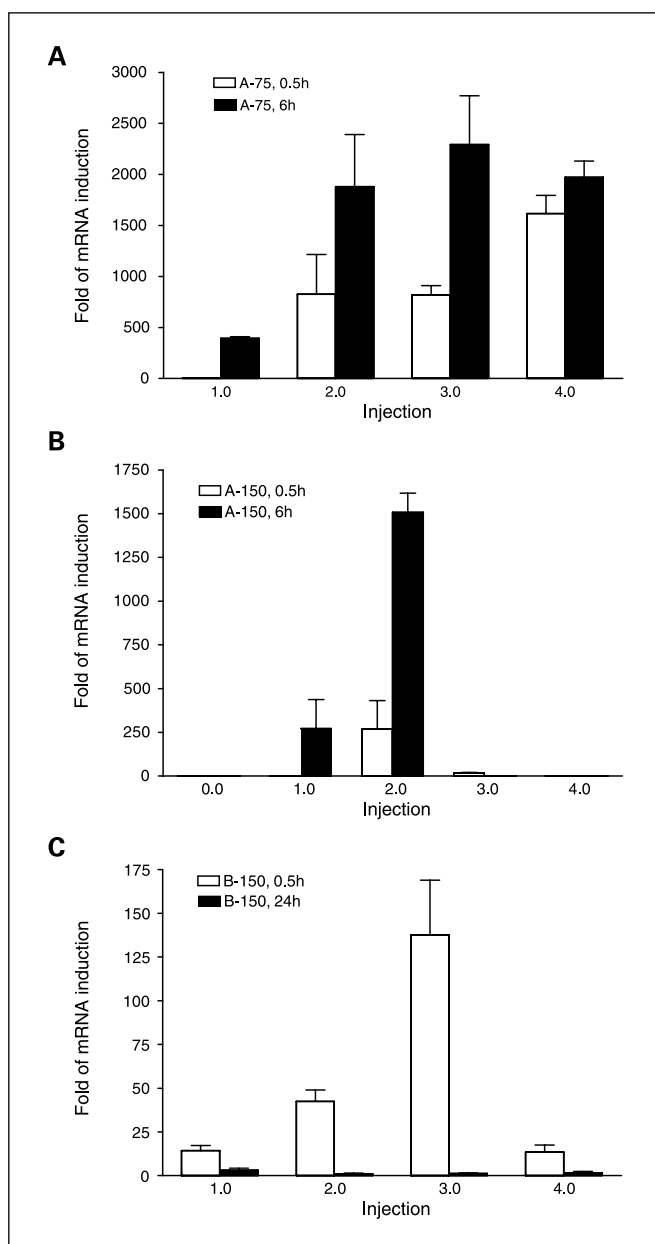
Microsomal assays were conducted with microsomal protein 1 mg/mL and 0.1 mmol/L CPA except in the pilot study, in which 1, 0.5, or 0.1 mmol/L CPA in 50 mmol/L potassium phosphate buffer (pH 7.4) were used. The total incubation volume was 0.5 mL. Reactions were initiated by the addition of β-NADPH (final concentration, 1 mmol/L). Samples were incubated at 37°C for 10 min, and the reaction was stopped with 0.5 mL of ice-cold acetonitrile. The mixtures were vortexed and centrifuged at 3000×g for 5 min to remove proteins. The concentration of 4-OH-CPA was determined as described previously using HPLC. The results were presented as the concentration ratios of 4-OH-CPA of treated/untreated animals.

#### Data analysis

Independent *t* test was done for the comparison of mRNAs at different time points with those from control groups using STATISTICA 6 (StatSoft). An increase or decrease in mRNA, protein, enzyme activity, or drug concentration more than 2-fold was considered as significant.

## Results

**CYP mRNA levels.** Induction of CYP2B1 mRNA expression was observed in group A-75 with a maximum of 2,000-fold compared with the control animals at 6 h after the third administration (Fig. 2A). In group A-150, the maximum induction of CYP2B1 mRNA (1,500-fold) was observed 6 h after the second dose but declined to 9-fold after the third injection. Six hours after the fourth injection, the mRNA level returned to the baseline (Fig. 2A). In group B-150, in which CPA was given at 24-h intervals, no induction was observed at any time point (Fig. 2B). The induction of CYP2B1 mRNA expression in the pilot group is shown in Fig. 3. The induction was increased by 425-fold and ~1,000-fold 6 h after the first and the second administration, respectively. However, it had declined to 340-fold at 6 h after the third injection. CYP2B2 mRNA increased by 7-fold and 100-fold after the first and the second administration, respectively, and also declined (to 33-fold) after the third injection. In all our experiments, we used the endogenous house keeping gene 18S as internal standard because 18S



**Fig. 2.** The effect of repeated administration of 75 and 150 mg/kg CPA into the rats on CYP2B1 mRNA levels. *A* and *B*, A-75 and A-150 groups were treated with repeated doses of CPA at 6-h dosing intervals. White bars, sampling at 0.5 h after administration; black bars, samples at 6 h postadministration. *C*, B-150 group was treated with CPA at 24-h time intervals. White columns, sampling at 0.5 h after administration; black columns, samples at 24 h postadministration. Y axes are the mRNA level (fold of induction), and X axes show the number of doses. Columns, mean of three rats; bars, SD.

expression was stable during all experiments (copy number,  $2.25$  to  $4.0 \times 10^5$ ), whereas the  $\beta$ -actin expression showed high variability between different treated rats ( $3.01 \times 10^0$  to  $4.3 \times 10^3$ ). All mRNA data are presented as ratios of treated animals to controls.

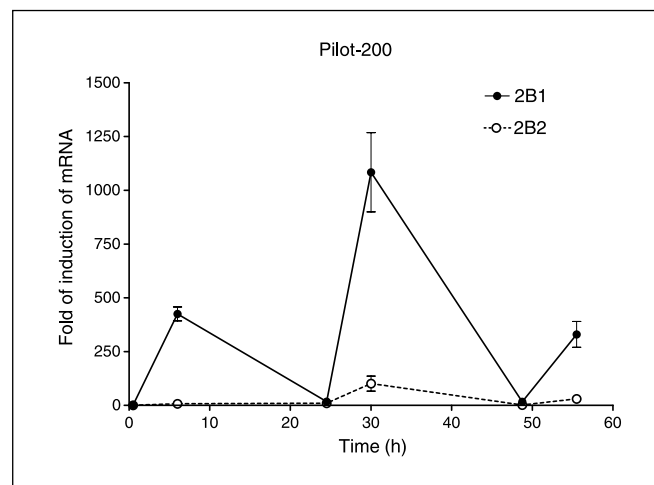
CYP2B2 mRNA induction showed a similar pattern to that of CYP2B1 but with lower magnitudes. In group A-75, the maximum level of CYP2B2 mRNA was reached after the third dose (60-fold), whereas it decreased to 20-fold after the fourth administration. No induction effect on 3A and 2C mRNA was observed in our pilot study.

**Western blotting analysis.** CYP protein levels in rat liver microsomes were detected using antibodies against rat CYP2B1/2, CYP3A1, CYP3A2, and CYP2C11. An increase in the CYP2B1/2 protein levels was observed in the animals treated at 6 h intervals; groups A-75 and A-150 (Fig. 4A). In group A-150, maximum levels were reached after the third dose but decreased at 6 h after the last dose. In two groups treated at 24-h intervals, B-150 and pilot-200, the maximum level of CYP2B protein was reached after the second dose at 48 h (Fig. 4B).

No alterations in the protein levels of CYP3A1/2 and CYP2C11 were observed in any group.

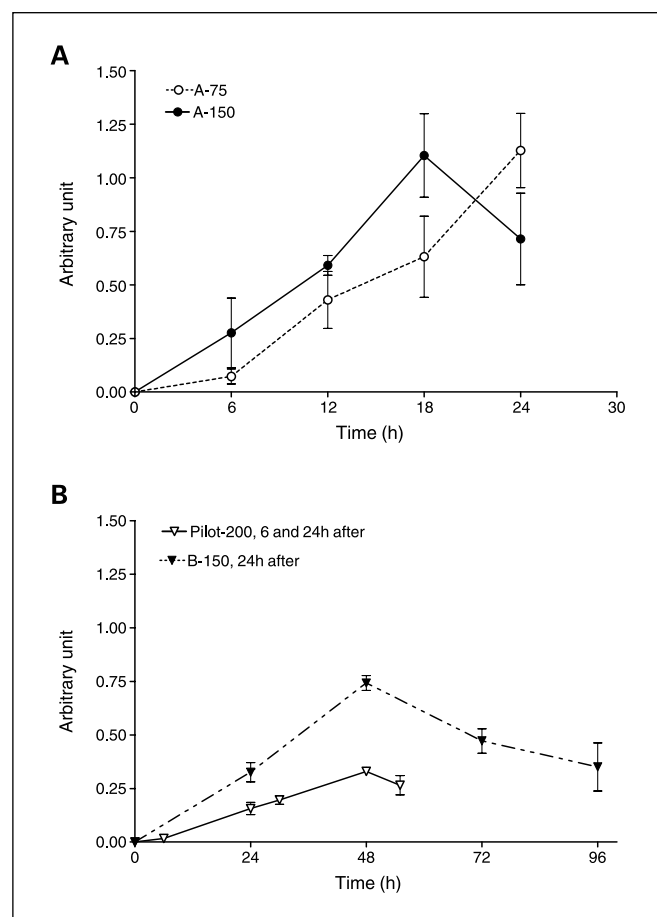
**Microsomal activity.** The CYP2B-dependent microsomal activity, as measured by CPA 4-hydroxylation, is presented in Fig. 5. As for the CYP2B protein levels, the 4-hydroxylation of CPA increased in the microsomal fraction obtained from treated rats compared with that obtained from untreated rats. CYP2B activity increased with number of doses in both A-75 and A-150 by a maximum of 3.6-fold and 6-fold, respectively (Fig. 5A). In group B-150, autoinduction was observed until the third dose in rats treated at 24-h intervals ( $\sim 4$ -fold). However, after the fourth dose, the amount of the formed 4-OH-CPA was decreased (Fig. 5B). In the pilot study (pilot-200), the rate of 4-OH-CPA formation was twice as high using 0.1 mmol/L CPA compared with that observed using 1 mmol/L CPA, possibly due to substrate inhibition by the high concentration.

**Pharmacokinetic study.** The plasma concentrations of both CPA and 4-OH-CPA were determined individually from samples taken at 30 min after each administration (Fig. 6). A decrease in the plasma CPA concentrations after repeated dosing was observed in all groups, indicating autoinduction of CPA metabolism. Moreover, the 30-min after dose concentrations of 4-OH-CPA increased significantly with repeated dosing in group A-75, with 2-fold higher concentrations after the fourth injection compared with that found after the first dose. In groups treated with high doses of CPA (A-150 and B-150), no significant increase in 4-OH-CPA concentrations was observed until the third injection. However, there was a reduction in 4-OH-CPA levels after the last injection in groups A-150 and B-150 (Fig. 6B). Furthermore, a significant



**Fig. 3.** mRNA induction levels for CYP2B1 (●) and CYP2B2 (○) after repeated doses of 200 mg/kg CPA in the pilot study. Points, mean of three rats; bars, SD.





**Fig. 4.** The effect of repeated doses of CPA on CYP2B protein expression. *A*, protein levels were measured at 6 h after administration of CPA in groups A-75 (○) and A-150 (●). *B*, protein levels were measured at 6 and 24 h after the dosage in groups B-150 (▼) and pilot-200 (▽). Points, mean of three rats; bars, SD.

( $P < 0.05$ ) increase in the ratio of 4-OH-CPA/CPA was observed with repeated CPA doses in group A-75 but not in group A-150 (Fig. 6C). The ratio of concentrations of 4-OH-CPA/CPA was actually lower after the fourth injection in group B-150 (Fig. 6C), as well as 6 h after the third injection in pilot-200.

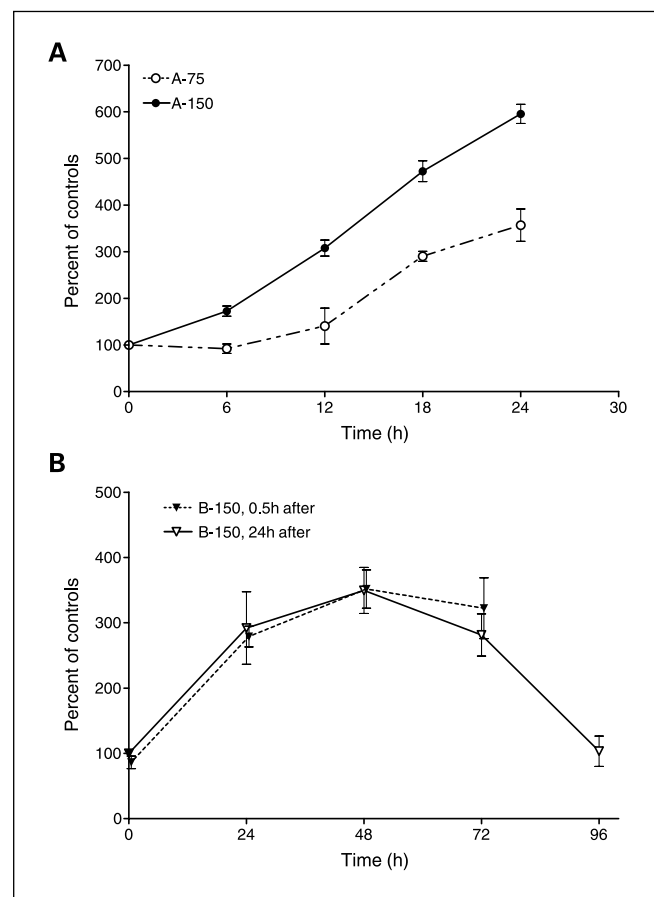
## Discussion

CPA has been in clinical use for more than 40 years. However, the complete mechanism of its action is still not fully understood. Several investigations have shown that the repeated administration of CPA induces its own metabolism (13, 14, 18). Recently, it was shown that the administration of a single dose of CPA (25) resulted in an increase of mRNA levels for CYP1B1/2 and CYP3A with a maximum at ~6 h (4-8 h) postadministration. In the present study, we investigated the effect of repeated administration of CPA on CYP2B1 and CYP2B2 mRNA and protein levels, as well as CPA 4-hydroxylation.

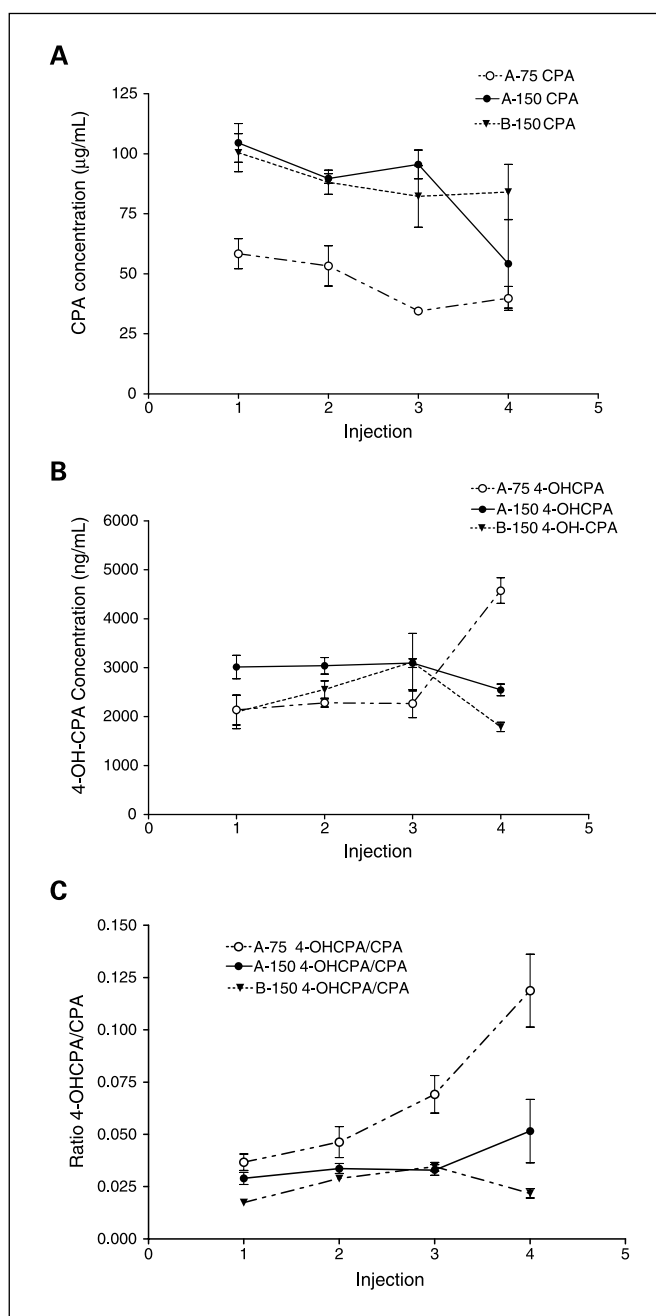
High doses of CPA are an important part of the conditioning regimen in SCT settings. SCT recipients receive doses of 50 to 100 mg/kg every 24 h for 2 or 4 consecutive days. Several studies have shown wide variability in high-dose CPA kinetics and an autoinduction after repeated doses (16). Other studies

have reported the correlation between CPA dose and the adverse effects in patients receiving high doses of CPA (30). The elimination half-life is ~5 to 10 h in human, whereas the elimination half-life in rats is ~1 h. In the present investigation, we have chosen to administer four doses at 6-h intervals (i.e., four to six elimination half-lives) in similarity to the clinical setting in transplanted patients. We found that the mRNA levels continuously increased over a 24-h period in the low-dose group (A-75), whereas the induction of mRNA in the high dose group (A-150) was observed after the first and second doses only. After the third dose, the mRNA levels decreased which, possibly, is due to toxicity and/or down-regulation caused by CPA and/or CPA metabolites. The CYP2B mRNA levels after repeated administration at 24-h intervals (B-150) were lower compared with the levels of mRNA detected when the doses were repeated at shorter intervals.

It has been shown that numerous CYP proteins are inducible by a variety of drugs (31, 32). In our investigation, the CYP2B1/2 protein levels, like the mRNA levels, continued to increase over 24 h in A-75, i.e., 6 h after the last dose. The increase in protein levels in A-150 reached its maximum at 18 h compared with the maximum level of mRNA levels at 12 h. This delay of



**Fig. 5.** CPA 4-hydroxylation activity measured *in vitro*. *A*, using liver microsomes from rats treated with 75 mg/kg at 6 h intervals; group A-75 (○) and group A-150 (●). Both groups received the CPA dose at 6-h intervals. Livers used for this experiment were removed 6 h after each dose. *B*, group B-150, received CPA (150 mg/kg) at 24-h intervals. Liver samples were obtained at 0.5 h (▼) and 24 h (▽) after each administration (all groups have been defined in Fig. 1). Points, mean of three rats; bars, SD.



**Fig. 6.** Plasma concentrations of CPA (A), 4-OH-CPA (B), and metabolic ratio (C) expressed as the concentrations of 4-OHCPA/CPA in groups A-75 (○), A-150 (●), and B-150 (▼). Plasma samples were collected 0.5 h after each dose. Points, mean of three rats; bars, SD.

~ 6 h may reflect protein/mRNA differences in synthesis and degradation rates. The decrease in CYP2B protein (A-150), found at 24 h most probably, is caused by toxic effect of CPA and/or its metabolites on the apoproteins or by the lack of induction in mRNA after repeated administration of high doses of CPA. A similar effect was observed in group B-150, in which protein levels reached a maximum at 48 h followed by a decrease which perhaps is a consequence of toxicity or inhibition effect of CPA on CYPs. These effects are similar to these reported in other studies, in which the authors reported

a late toxicity (6-14 days) after a single high dose of CPA (130-200 mg/kg; refs. 4, 20-24).

In the present study, we observed an early effect on CYP2B mRNA and protein levels in all animals treated with repeated doses of CPA, in which Anglely et al. (23) did not find any decrease and/or increase in mRNA or protein for CYP2B1 using repeated doses of 40 mg/kg/day for 5 days. This discrepancy is possibly, due to the low dose and the long time interval between doses (24 h) used by Anglely et al.

The increased enzyme activity *in vivo* in group A-75, as measured by the formation of 4-OH-CPA in rat plasma, correlates with the induction of CYP2B mRNA and protein levels. These results were confirmed *ex vivo* by measuring microsomal activity. However, in the high-dose group (A-150) both CPA and 4-OH-CPA declined after the third administration, in parallel with the decrease in protein levels, which could be due to saturated activation site of CPA by repeated administration. Other mechanisms that might be responsible for the declined levels of CPA and its 4-OH-CPA are toxic effect of CPA and/or its metabolites on hepatocytes; because in the groups treated with 150 mg/kg, the liver weight, as well as the total microsomal protein per gram liver, decreased after the second dose (data not shown).

In group B-150, the *in vivo* kinetics of CPA and 4-OH-CPA were consistent with the changes in CYP2B protein levels and the *in vitro* activity of liver microsomes. There was an increase in CPA 4-hydroxylation followed by a decrease after the longer interval of administration, because the enzyme turn-over time may be <24 h in rats (15). When CPA induces its own metabolism, higher amounts of the toxic metabolite (4-OH-CPA) is formed and, therefore, exerts more cytotoxic effects on the cells during transcription and/or protein synthesis. An indication for this toxicity is the decrease in liver weight with repeated administration observed in animals treated with 150 mg/kg.

In our previous study (25), we showed that the expression of different CYP enzymes was normalized at 5 days after injection of a single dose of CPA into rats, whereas other investigators have reported that CPA treatment may result in the suppression of CYPs and activity 6 to 14 days postadministration (4, 20-24, 33).

Due to the short half-life of CPA (1 h) and its metabolite (30 min) in rats (7, 26), undetectable levels of both compounds are expected 6 h postadministration, i.e., five half-lives of CPA. This can be compared with three to four half-lives in patients treated at 24-h intervals (15).

Recently, a group of nuclear receptors involved in transcriptional regulation of CYP genes have been identified. These receptors include the constitutive androstane receptor, the pregnane receptor, the retinoid X receptor, and the glucocorticoid receptor (34). Moore et al. reported that pregnane receptor is more likely involved in the regulation of CYP3A, whereas the constitutive androstane receptor is involved in the regulation of CYP2B (35). However, recent publication (36) showed evidence for cross-talk between these pathways and suggested that these receptors are involved in the induction of CYP2B and CYP3A. Our data show that CPA could induce its own metabolism through CYP2B1 induction after repeated doses. However, this autoinduction was more prominent with repeated low doses (A-75). The induction was observed at mRNA level as well as at protein and activity levels. However, this induction was of different magnitudes, which probably

indicate that variation in dosing regimes, such as different dosing intervals and different doses, may affect the transcriptional regulation of the *CYP2B* genes. Therefore, the induction caused by CPA might be due to direct activation of some nuclear receptors, such as constitutive androstane receptor and/or pregnane receptor that regulate CYP expression. Recently, Faucette et al. have shown that CPA is a significant activator of human constitutive androstane receptor 3 in hepatocytes (36). However, further studies are needed to investigate the exact mechanism behind this induction and to find the optimum dosing regimen that certainly may enhance therapeutic efficacy.

In summary, we conclude that CPA autoinduction is time dependent and dose dependent. We found that repeated low

doses of CPA at 6-h intervals increased the levels of *CYP2B* mRNA, protein levels, and both the *in vivo* and *in vitro* CPA 4-hydroxylation activity. However, repeated high doses at 6-h intervals resulted in an induction of mRNA with a maximum after two doses, whereas both protein levels, as well as the *in vivo* and *in vitro* activities, increased until the third dose. Moreover, repeated high doses at 24-h intervals do not alter the induction of *CYP2B* at the protein level as well as the *in vivo* and *in vitro* activities. This may be used in clinical settings to optimize both dose and time interval between doses to achieve maximum induction of CYP with minimum required doses. This, in turn, will increase the therapeutic efficacy and minimize drug-related toxicity.

## References

- Binotto G, Trentin L, Semenzato G. Ifosfamide and cyclophosphamide: effects on immunosurveillance. *Oncology* 2003;65:17–20.
- Lindley C, Hamilton G, McCune JS, et al. The effect of cyclophosphamide with and without dexamethasone on cytochrome P450 3A4 and 2B6 in human hepatocytes. *Drug Metab Dispos* 2002;30:814–22.
- Sladek NE. Metabolism of oxazaphosphorines. *Pharmacol Ther* 1988;37:301–55.
- Chang TK, Weber GF, Crespi CL, Waxman DJ. Differential activation of cyclophosphamide and ifosfamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Res* 1993;53:5629–37.
- Ren S, Yang JS, Kalhorn TF, Slattery JT. Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes. *Cancer Res* 1997;57:4229–35.
- Roy P, Yu LJ, Crespi CL, Waxman DJ. Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. *Drug Metab Dispos* 1999;27:655–66.
- Yu LJ, Drewes P, Gustafsson K, Brain EG, Hecht JE, Waxman DJ. *In vivo* modulation of alternative pathways of P-450-catalyzed cyclophosphamide metabolism: impact on pharmacokinetics and antitumor activity. *J Pharmacol Exp Ther* 1999;288:928–37.
- Delillers GL, Annaloro C, Marconi M, et al. Harvesting of autologous blood stem cells after a mobilising regimen with low-dose cyclophosphamide. *Leuk Lymphoma* 2002;43:1957–60.
- Ahn JS, Park S, Im SA, et al. High-dose versus low-dose cyclophosphamide in combination with G-CSF for peripheral blood progenitor cell mobilization. *Korean J Intern Med* 2005;20:224–31.
- McDonald GB, Slattery JT, Bouvier ME, et al. Cyclophosphamide metabolism, liver toxicity, and mortality following hematopoietic stem cell transplantation. *Blood* 2003;101:2043–8.
- Graham MI, Shaw IC, Souhami RL, Sidau B, Harper PG, McLean AE. Decreased plasma half-life of cyclophosphamide during repeated high-dose administration. *Cancer Chemother Pharmacol* 1983;10:192–3.
- Fasola G, Lo Greco P, Calori E, et al. Pharmacokinetics of high-dose cyclophosphamide for bone marrow transplantation. *Haematologica* 1991;76:120–5.
- Schuler U, Ehninger G, Wagner T. Repeated high-dose cyclophosphamide administration in bone marrow transplantation: exposure to activated metabolites. *Cancer Chemother Pharmacol* 1987;20:248–52.
- Ren S, Kalhorn TF, McDonald GB, Anasetti C, Appelbaum FR, Slattery JT. Pharmacokinetics of cyclophosphamide and its metabolites in bone marrow transplantation patients. *Clin Pharmacol Ther* 1998;64:289–301.
- Hassan M, Svensson US, Ljungman P, et al. A mechanism-based pharmacokinetic-enzyme model for cyclophosphamide autoinduction in breast cancer patients. *Br J Clin Pharmacol* 1999;48:669–77.
- Moore MJ, Hardy RW, Thiessen JJ, Soldin SJ, Erlichman C. Rapid development of enhanced clearance after high-dose cyclophosphamide. *Clin Pharmacol Ther* 1988;44:622–8.
- McGuire TR, Gwilt P, Manouvilov K, et al. High-dose cyclophosphamide in multiple sclerosis patients undergoing autologous stem cell transplantation. *Int Immunopharmacol* 2003;3:279–83.
- Chang TK, Yu L, Maurel P, Waxman DJ. Enhanced cyclophosphamide and ifosfamide activation in primary human hepatocyte cultures: response to cytochrome P-450 inducers and autoinduction by oxazaphosphorines. *Cancer Res* 1997;57:1946–54.
- Gervot L, Rochat B, Gautier JC, et al. Human *CYP2B6*: expression, inducibility and catalytic activities. *Pharmacogenetics* 1999;9:295–306.
- LeBlanc GA, Waxman DJ. Mechanisms of cyclophosphamide action on hepatic P-450 expression. *Cancer Res* 1990;50:5720–6.
- McClure MT, Stupans I. Investigation of the mechanism by which cyclophosphamide alters cytochrome P450 in male rats. *Biochem Pharmacol* 1992;43:2655–8.
- McClure MT, Stupans I. Hormonal perturbation as a possible mechanism for the alteration of cytochrome P450 by cyclophosphamide. *Biochem Pharmacol* 1995;49:1827–36.
- Angley MT, Sansom LN, Stupans I. Cyclophosphamide given repeatedly to the male rat and as a single dose to the female rat. Its effects on hepatic and pulmonary P450 and associated enzymes. *Xenobiotica* 1995;25:1051–62.
- Kraner JC, Morgan ET, Poet TS, Born SL, Burnett VL, Halpert JR. Suppression of rat hepatic microsomal cytochromes P450 by cyclophosphamide is correlated with plasma thyroid hormone levels and displays differential strain sensitivity. *J Pharmacol Exp Ther* 1996;276:258–64.
- Xie H, Afsharian P, Terelius Y, et al. Cyclophosphamide induces mRNA, protein and enzyme activity of cytochrome P450 in rat. *Xenobiotica* 2005;35:239–51.
- Xie HJ, Broberg U, Griskevicius L, et al. Alteration of pharmacokinetics of cyclophosphamide and suppression of the cytochrome p450 genes by ciprofloxacin. *Bone Marrow Transplant* 2003;31:197–203.
- von Bahr C, Groth CG, Jansson H, Lundgren G, Lind M, Glaumann H. Drug metabolism in human liver *in vitro*: establishment of a human liver bank. *Clin Pharmacol Ther* 1980;27:711–25.
- Griskevicius L, Meurling L, Hassan M. Simple method based on fluorescent detection for the determination of 4-hydroxycyclophosphamide in plasma. *Ther Drug Monit* 2002;24:405–9.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- Ayash LJ, Wright JE, Tretyakov O, et al. Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response. *J Clin Oncol* 1992;10:995–1000.
- Iwanari M, Nakajima M, Kizu R, Hayakawa K, Yokoi T. Induction of *CYP1A1*, *CYP1A2*, and *CYP1B1* mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. *Arch Toxicol* 2002;76:287–98.
- Edwards RJ, Price RJ, Watts PS, et al. Induction of cytochrome P450 enzymes in cultured precision-cut human liver slices. *Drug Metab Dispos* 2003;31:282–8.
- Laslett TJ, Alvarez F, Nation RL, Evans AM, Scott SD, Stupans I. Effect of cyclophosphamide administration on the activity and relative content of hepatic P4502D1 in rat. *Xenobiotica* 1995;25:1031–9.
- Pascucci JM, Gerbal-Chaloin S, Drocourt L, Maurel P, Vilarem MJ. The expression of *CYP2B6*, *CYP2C9* and *CYP3A4* genes: a tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* 2003;1619:243–53.
- Moore LB, Parks DJ, Jones SA, et al. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 2000;275:15122–7.
- Faucette SR, Zhang TC, Moore R, et al. Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of *CYP2B6* and *CYP3A4* inducers. *J Pharmacol Exp Ther* 2007;320:72–80.

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