

## Altered Pharmacokinetics of Daunorubicin in Rats with CCl<sub>4</sub>-Induced Hepatic Injury

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**ABSTRACT - PURPOSE.** The effect of CCl<sub>4</sub>-induced experimental hepatic injury (CCl<sub>4</sub>-EHI) on the pharmacokinetics of daunorubicin was investigated systemically in rats, in an attempt to elucidate the major determinants of the effect of CCl<sub>4</sub>-EHI on the pharmacokinetics of the drug. **METHODS.** CCl<sub>4</sub>-EHI was induced in rats by a single intraperitoneal injection of CCl<sub>4</sub> (1mL/kg rat), and a 24 h fasting period. Daunorubicin was administered intravenously to control and EHI rats at a dose of 11.3 mg/mL/kg and the *in vivo* pharmacokinetics was studied. The *in vitro* uptake of the drug into isolated hepatocytes and canalicular liver plasma membrane (cLPM) vesicles, as well as the liver microsomal degradation of the drug, were also determined. **RESULTS.** The area under the plasma concentration-time curve (AUC) of daunorubicin was increased by 1.6 times, resulting in a 34% decrease in the systemic clearance (CL) in rats with CCl<sub>4</sub>-EHI. The apparent biliary (CL<sub>bile</sub>) and urinary (CL<sub>urine</sub>) clearance of the drug were unchanged, whereas the AUC of daunorubicinol, the major metabolite of daunorubicin, was decreased by 66% in rats with CCl<sub>4</sub>-EHI. EHI seemed to affect the hepatobiliary elimination of the drug in several ways: the *in vitro* intrinsic sinusoidal uptake clearance was decreased by 20%; the *in vitro* intrinsic canalicular excretion clearance of the drug was increased by 1.7 times; and the *in vitro* liver microsomal degradation of daunorubicin was significantly retarded. **CONCLUSIONS.** CCl<sub>4</sub>-EHI appears to impair the hepatic metabolism of daunorubicin, thereby decreasing the CL and increasing the AUC of daunorubicin.

### INTRODUCTION

Daunorubicin is one of the most important and widely used anticancer drugs and is used to treat

breast and lung carcinoma, lymphoma, and leukemia (1). However, the clinical use of daunorubicin is often limited by serious problems such as chronic cardiomyopathy and congestive heart failure (2). The drug is predominantly metabolized by aldo-keto reductase, glycosidases, and CYP 2B1 in the liver, and the metabolites, as well as daunorubicin, undergo extensive biliary excretion (3,4). In fact, more than 80% of the intravenous dose of the drug is recovered in the bile as parent and metabolite forms, whereas the recovery in urine is limited (*i.e.*, 10% of the dose) (5,6).

Liver diseases likely affect the pharmacokinetics of drugs that are mainly metabolized or excreted in the liver (7,8). To study the pharmacokinetics of drugs in hepatic disease, an experimental hepatic injury (EHI) induced by a single dose of carbon tetrachloride (CCl<sub>4</sub>) is a widely used model of hepatic disease (9,10). CCl<sub>4</sub> leads to the acute, reversible necrosis of centrilobular hepatocytes, followed by liver regeneration (11). In response to hepatotoxicity, inflammation occurs via the activation of Kupffer cells and the release of inflammatory mediators such as cytokines, which are thought to alter the expression and activity of several liver-derived proteins (12,13). Recently, multiple alterations in the expression of organic anion transporters by EHI have been reported. For example, the expression of Na<sup>+</sup>/taurocholate cotransporting polypeptide (ntcp), organic anion transporting polypeptide 1 (Oatp1), and Oatp2 decreases, whereas that Oatp4 and bile salt export pump

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(Bsep) remains unchanged (14,15). The expression of multidrug-resistance related protein (Mrp2) is not affected for hepatic microsomes (14), whereas a significant decrease is observed for the canalicular membrane (15). In addition, our previous studies has revealed that the *in vitro* transport of organic cations across the sinusoidal membrane is decreased as a result of CCl<sub>4</sub>-EHI (16), while the transport activity of P-glycoprotein (P-gp) in the canalicular membrane is increased by CCl<sub>4</sub>-EHI (15).

In addition, CCl<sub>4</sub>-EHI markedly alters the liver enzyme system, changing the pharmacokinetic properties of hepatically eliminated drugs such as propranolol (17). It has been also reported that the expression of cytochrome P450 (CYP) in the rat is significantly reduced in CCl<sub>4</sub>-EHI (18). Jiko et al. (10) have reported that testosterone 6 $\beta$ -hydroxylase activity via CYP3A is reduced by 92% in rat liver microsomes with CCl<sub>4</sub>-EHI, and liver dysfunction caused by hepatic failure affected the pharmacokinetics of paclitaxel *in vivo*. Therefore, to investigate the effects of hepatic injury on the pharmacokinetics of daunorubicin systemically, we examined the effect of CCl<sub>4</sub>-EHI on the unit processes of hepatic disposition of the drug (*e.g.*, systemic pharmacokinetics, sinusoidal uptake, canalicular excretion, and hepatic metabolism) *in vivo* and *in vitro*.

## METHODS AND MATERIALS

### Materials

[<sup>3</sup>H]Daunorubicin (4.4 Ci/mmol) was purchased from Perkin-Elmer Inc. (Wellesley, MA). Daunorubicin hydrochloride, daunorubicinol (metabolite) and doxorubicin hydrochloride were generous gifts from Dong-A Pharmaceutical Co. (Kyounggido, Korea). All other reagents, including the reagents used in the isolated hepatocyte, canalicular liver plasma membrane (cLPM) vesicle, and liver microsome studies, were purchased from Sigma Aldrich (St. Louis, MO).

### Animals

Male Sprague-Dawley rats (250-300 g, Dae-Han Biolink, Daejeon, Korea) were used to prepare

isolated hepatocytes, cLPM vesicles and liver microsomes, and in the pharmacokinetic studies. The rats were divided randomly control and CCl<sub>4</sub>-EHI groups. All rats were provided with food (SamYang Company, Seoul, Korea) and water *ad libitum*, and maintained in a light-controlled room (light: 07:00-19:00, dark: 19:00-07:00) kept at a temperature of 22 $\pm$ 2 °C and a relative humidity of 55 $\pm$ 5% (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, Seoul, Korea). The experimental protocols involving animal study were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, according to National Institutes of Health Guidelines (NIH publication #85-23, revised in 1985).

### Induction of experimental hepatic injury by CCl<sub>4</sub>

Rats were injected intraperitoneally with a single dose of CCl<sub>4</sub> (1 mL/kg) as a 50% (v/v) solution in olive oil and then fasted for 24 h, but with free access to water. The control animals received a corresponding dose of olive oil, followed by the same experimental protocol. The induction of experimental hepatic injury was confirmed by a 4.6-times increase in the activity of alanine aminotransferase (ALT; from 53.7 $\pm$ 8.8 to 247.1 $\pm$ 92 unit/mL serum, n=6) and a 6-times increase in the activity of aspartate aminotransferase (AST; from 107.9 $\pm$ 6.9 to 665.1 $\pm$ 74 unit/mL serum, n=6). The activities of ALT and AST were measured using modified method of Reitman and Frankel (19) with a commercial colorimetric determination kit (Yeoung Dong Pharm. Co., Seoul, Korea).

### Pharmacokinetic study

Under ketamine anesthesia (50 mg/kg, *i.p.* injection), the femoral arteries and veins of rats (n=5) were cannulated with PE-50 polyethylene tubing (Intramedic, Sparks, MD), and a 3% (w/v) mannitol saline solution was infused through the femoral vein cannula at a rate of 10 mL/h/kg to obtain a constant urine flow. After opening the abdomen, the bile duct and ureters were cannulated with PE-10 polyethylene tubing (Intramedic, Sparks, MD). After recovery from the surgery, the animals were given daunorubicin

hydrochloride (11.3 mg/mL/kg, dissolved in distilled water) via the femoral vein. Blood samples (200  $\mu$ L) were collected from the femoral artery at 0, 1, 5, 10, 20, 30, 45, 60, 120, and 180 min, and bile and urine were collected for 30-min periods up to 3 h. The total volume of blood withdrawn was 2 mL. After each blood sampling, 150  $\mu$ L of heparinized saline (20 units/mL) was injected via the catheter to flush the catheter and prevent blood clotting, and the fluid loss was compensated for with an injection of saline at each collection via the femoral vein. Body temperature was maintained using a heat lamp. Blood samples were centrifuged immediately, and 100  $\mu$ L aliquot of each plasma sample was collected and stored at -70 °C until analysis.

#### ***Uptake of daunorubicin into isolated hepatocytes***

Isolated hepatocytes were obtained from control (n=3) and CCl<sub>4</sub>-EHI (n=3) rats according to a previously described procedure (20). The cell suspension (2 mL, 2.5-3.0 $\times$ 10<sup>6</sup> cells/mL) was preincubated in the medium for 5 min at 37 °C and a 20- $\mu$ L aliquot of a [<sup>3</sup>H]daunorubicin solution with various initial radioactivities was added to the suspension to give a final medium concentration of 1-100  $\mu$ M as daunorubicin hydrochloride (0.004-0.4  $\mu$ Ci). While incubating each suspension at 37 °C, 200- $\mu$ L aliquots of the suspension were sampled at 30, 60, 90, and 120 seconds, and the level of radioactivity in the hepatocytes was determined as described previously (20). The amount of daunorubicin in hepatocytes (expressed as pmole/10<sup>6</sup> cells) was then plotted against time for each suspension. The initial uptake rate of the drug into hepatocytes, calculated from the linear portion of the plot, was then plotted against the initial concentration of the drug in the medium.

#### ***Uptake of daunorubicin into cLPM vesicles***

Canalicular liver plasma membrane (cLPM) vesicles were prepared from control (n=3) and CCl<sub>4</sub>-EHI rats (n=3) according to the method of Inoue et al. (21) and characterized as described previously (15). Uptake was initiated by adding 40  $\mu$ L of membrane suspension buffer (MSB, 250 mM sucrose, 10 mM Hepes, 10 mM Tris, 10 mM MgCl<sub>2</sub>, and 0.2 mM CaCl<sub>2</sub>, pH 7.4) containing 0.2

$\mu$ M daunorubicin hydrochloride (0.035  $\mu$ Ci) with an ATP-regenerating system (1.2 mM ATP, 3 mM phosphocreatine, and 3.6  $\mu$ g/100  $\mu$ L creatine phosphokinase) to the preincubated vesicle suspension. At predetermined times, the uptake was quenched by adding 1 mL of ice-cold suspension containing 20  $\mu$ M daunorubicin. The entire sample was then rapidly filtered through an MF-MEMB filter (0.45- $\mu$ m pore size, 25-mm diameter, Seoul Science, Seoul, Korea) that had been presoaked in ice-cold MSB for 2 h. After washing with 10 mL of ice cold MSB, the filter was dissolved in 4 mL of scintillation cocktail (Ultima Gold, Perkin-Elmer), and the radioactivity of the mixture was determined by liquid scintillation counting (Wallac 1409, Perkin-Elmer). The initial uptake rate of the drug was obtained from the linear portion (generally up to 1 min) of the temporal profile, and then plotted against the initial concentration of the drug in the medium. The concentration dependency of the ATP-dependent initial uptake rate of daunorubicin was examined for the concentration range of 5-500  $\mu$ M.

#### ***Liver microsomal degradation of daunorubicin***

Rat liver microsomes were prepared using conventional methods (22). The liver was perfused with ice-cold isolation buffer (0.154 M KCl, 50 mM Tris-HCl, pH 7.4), homogenized in four volumes of isolation buffer, and then centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was centrifuged at 100,000 g for 60 min at 4 °C, and the microsomal pellet was washed by resuspension in fresh buffer and centrifuged again at 100,000 g for 60 min at 4 °C. Liver microsomes were resuspended in two volumes of 0.12 M Tris (pH 7.4), and 1-mL aliquots were stored frozen at -70 °C. The microsomal protein and total CYP content of the liver microsomes were measured using the methods of Lowry et al. (23) and Omura and Sato (24), respectively.

Microsomes (0.5 mg of total protein) were diluted to a final volume of 460  $\mu$ L using 50 mM Tris-HCl buffer (pH 7.4), and 30  $\mu$ L of Tris-HCl buffer containing an NADPH-regenerating system (1 mM NADP<sup>+</sup>, 3 mM glucose-6-phosphate, 3 mM MgCl<sub>2</sub>, and 2 U/mL glucose-6-phosphate dehydrogenase) was added, followed

by a further incubation for 15 min at 37 °C in a shaking water bath (25). After preincubating of the microsomal suspension for 5 min at 37 °C, the microsomal incubation was started by adding 10 µL of 1.5 mM daunorubicin hydrochloride. The reaction was terminated at predetermined times (*i.e.*, 0, 5, 10, and 15 min) by placing the incubation tubes on ice and immediately adding 1 mL of ice-cold acetonitrile and centrifuging at 10,000 rpm for 10 min. A 50-µL aliquot was injected into the HPLC system to determine the residual daunorubicin.

#### **HPLC assay of daunorubicin and daunorubicinol**

The concentrations of daunorubicin in plasma, bile and urine, as well as those of daunorubicinol in plasma, were quantified by HPLC with a fluorescence detector, as previously described (15). Briefly, a 100-µL aliquot of the biological samples (*i.e.*, plasma, bile, and urine) was deproteinized by adding 250 µL of methanol and 1 mL ethyl acetate and centrifuging at 10,000 rpm for 5 min. An 1.2-mL aliquot of the supernatant was evaporated under a gentle stream of nitrogen gas, and the residue was reconstituted in a 50-µL aliquot of the mobile phase (water:acetonitrile:0.1 M phosphoric acid = 61:31:8 v/v/v, pH 3.5). A 30-µL aliquot of the reconstituted solution was injected into the HPLC system, which consisted of a Hitachi L-7110 pump (Hitachi, Japan), a Shimadzu RF 535 fluorescence detector (Shimadzu, Japan; excitation 470 nm, emission 565 nm), a Hitachi D-7500 integrator and a C<sub>18</sub> column (Shisheido, CAPCELL PAK, 4.6 × 250 mm, 5 µm). The flow rate of the mobile phase was set at 1 mL/min. The eluent resulted in sharp, well-resolved peaks corresponding to doxorubicin (internal standard, 6.8 min), daunorubicinol (11.7 min), and daunorubicin (14.4 min). The calibration curves for daunorubicin were linear over the concentration range of 0.05-25.0 µg/mL for plasma, bile, and urine samples, and the inter- and intra-day coefficients of variation for daunorubicin were below 14.5%. The calibration curve for daunorubicinol was linear over the concentration range of 0.05-2.50 µg/mL for plasma, and the inter- and intra-day coefficients of variation of daunorubicinol were below 10.0%.

#### **Data analysis for the *in vitro* uptake into isolated hepatocytes and cLPM vesicles**

To estimate the relevant uptake kinetic parameters for both isolated hepatocyte and cLPM vesicle systems, the initial uptake rate-concentration data from each experimental system were fitted to a modified Michaelis-Menten equation (Eq. 1), using WinNonlin (ver. 5.1., Pharsight, Mountain View, CA):

$$V_0 = \frac{V_{\max} \times S}{K_m + S} + K_d \times S \quad (1)$$

where  $V_0$  is the initial uptake rate of daunorubicin,  $S$  is the initial concentration of daunorubicin in the medium, and  $K_d$  is a passive diffusion constant.  $V_{\max}$  and  $K_m$  represent the maximum uptake rate and medium concentration at half of the maximal uptake rate, respectively. The intrinsic clearance for the uptake ( $CL_{int}$ ) was obtained from  $V_{\max}/K_m$ .

#### **Pharmacokinetic analysis**

The area under the plasma concentration-time curve from time zero to 3 h ( $AUC_{0-3h}$ ) was calculated using a trapezoidal rule and the area from the last datum point to time infinity was estimated by dividing the last measured plasma concentration by the terminal phase rate constant. Standard methods (26) were used to calculate the systemic clearance ( $CL$ ) and apparent volume of distribution at a steady state ( $V_d$ ) using noncompartmental analysis (WinNonlin, ver. 5.1.; Pharsight, Mountain View, CA). The apparent urinary ( $CL_{urine}$ ) and biliary ( $CL_{bile}$ ) clearances were calculated by dividing the total amount of daunorubicin excreted during the first 3 h into the urine ( $Ae_{u,0-3h}$ ) and bile ( $Ae_{b,0-3h}$ ) by the  $AUC$  for 3 h ( $AUC_{0-3h}$ ), respectively.

#### **Statistical analysis**

All data are expressed as the mean±standard deviation (S.D.). A  $p$  value of less than 0.01 was considered statistically significant using the non-parametric Mann-Whitney test between the two means for the unpaired data.

## RESULTS

### *Pharmacokinetics of daunorubicin in CCl<sub>4</sub>-EHI*

The plasma concentration-time profiles of daunorubicin following intravenous administration of the drug as daunorubicin hydrochloride at a dose of 11.3 mg/mL/kg and relevant pharmacokinetic parameters for control and CCl<sub>4</sub>-EHI rats are shown in Figure 1 and Table 1. The plasma concentration of daunorubicin was generally higher in CCl<sub>4</sub>-EHI rats compared to control rats (Figure 1A). As a result, the AUC of daunorubicin was increased by 1.64 times ( $p < 0.01$ ) compared to the control value, and consequently, the systemic clearance (CL) of the drug was decreased by 34% in CCl<sub>4</sub>-EHI rats. No significant change was observed in the apparent volume of distribution at a steady state ( $V_d$ ).

In CCl<sub>4</sub>-EHI rats, no significant changes were observed in the urinary recovery of the drug during the first 3 h (Figure 1C and  $Ae_{u,0-3h}$  in Table 1), whereas the biliary recovery of the drug during the first 3 h was increased by 1.2 times ( $p < 0.01$ , Figure 1D and  $Ae_{b,0-3h}$  in Table 1). The plasma profile of daunorubicinol, the major metabolite of daunorubicin, was generally lower in CCl<sub>4</sub>-EHI rats compared to control rats (Figure 1B), and the  $AUC_{m,0-3h}$  of daunorubicinol was decreased by 34% compared to control rats ( $p < 0.01$ , Table 1). As the consequence of these changes, the apparent urinary ( $CL_{urine}$ ) and biliary ( $CL_{bile}$ ) clearance of the drug remained unchanged (Table 1).

### *In vitro transport of daunorubicin*

For high concentrations of the drug, CCl<sub>4</sub>-EHI decreased the initial uptake rate of daunorubicin into hepatocytes (Figure 2A). The negative slope of the Eadie-Hofstee plot (Figure 2A, inset) suggests the involvement of a carrier-mediated transport mechanism in the sinusoidal uptake of the drug in control and CCl<sub>4</sub>-EHI rats. A nonlinear regression assuming a carrier-mediated process and passive diffusion in the uptake of the drug (Eq. 1) demonstrated a significant decrease in  $V_{max}$  ( $p < 0.01$ ), but not in  $K_m$  (Table 2), suggesting that the amount of relevant transporters for sinusoidal uptake was decreased without

influencing their affinity for daunorubicin in rats with CCl<sub>4</sub>-EHI. As a result, a slight decrease (20%,  $p < 0.01$ ) in the intrinsic clearance ( $CL_{int}$ ) for sinusoidal uptake was observed for rats with CCl<sub>4</sub>-EHI (Table 2). The passive diffusion clearance of the drug across the sinusoidal membrane ( $K_d$ ) also decreased slightly with CCl<sub>4</sub>-EHI (Table 2).

Contrary to the decrease in sinusoidal uptake, CCl<sub>4</sub>-EHI increased the uptake of daunorubicin into cLPM vesicles for all concentrations examined (Figure 2B). The Eadie-Hofstee transformation indicated that carrier-mediated transport was involved in the canalicular excretion of daunorubicin (Figure 2B, inset), which is consistent with previous reports (27). A nonlinear regression using Eq. 1 revealed profound ( $p < 0.01$ , each) increases in the values of  $V_{max}$  (2 times),  $CL_{int}$  (1.7 times), and  $K_d$  (1.7 times), with no change in  $K_m$  (Table 2), suggesting that the canalicular excretion of daunorubicin, contrary to sinusoidal uptake, is significantly increased in rats with CCl<sub>4</sub>-EHI.

### *Liver microsomal degradation of daunorubicin*

To examine the metabolism of daunorubicin, we prepared liver microsomes from control and CCl<sub>4</sub>-EHI rats. The protein yield (23.4±0.79 mg/g liver for control, 21.1±1.2 mg/g liver for CCl<sub>4</sub>-EHI) was not changed, whereas the total CYP content (0.51±0.08 nmole/mg protein for control, 0.35±0.09 nmole/mg protein for CCl<sub>4</sub>-EHI) was decreased in rats with CCl<sub>4</sub>-EHI ( $p < 0.01$ ). Daunorubicin was fairly stable in the absence of an NADPH-regenerating system after incubation at 37 °C for 15 min (Figure 3A). On adding an NADPH-regenerating system, a significant amount of daunorubicin disappeared (60% disappearance in 15 min) for control, whereas this was significantly retarded (*i.e.*, changed to a 30% disappearance in 15 min) in the CCl<sub>4</sub>-EHI rats (Figure 3A), suggesting that the metabolism of daunorubicin is decreased in rats with CCl<sub>4</sub>-EHI. The metabolic activity calculated from the slope of the concentration decline curve (Figure 3A) was 3.66±0.8 and 1.60±0.4 nmole/h ( $p < 0.01$ ) for control and CCl<sub>4</sub>-EHI microsomes, respectively, amounting to a 56% decrease with CCl<sub>4</sub>-EHI (Figure 3B).

**Table 1.** Summary of the effects of CCl<sub>4</sub>-EHI on the pharmacokinetic parameters of daunorubicin after intravenous administration to rats at a dose of 11.3 mg/mL/kg<sup>a</sup>

	Control	CCl <sub>4</sub> -EHI
AUC (μg/mL×min)	243.4±32	399.5±66*
CL (mL/min/kg)	47.1±6.7	31.3±5.6*
V <sub>d</sub> (mL/kg)	1654±284	1532±423
AUC <sub>m,0-3h</sub> (μg/mL×min)	81.5±10.1	54.0±19.1*
Ae <sub>u,0-3h</sub> (% of dose)	6.8±0.7	7.8±1.7
Ae <sub>b,0-3h</sub> (% of dose)	35.5±2.0	43.3±2.3*
CL <sub>urine</sub> (mL/min/kg)	4.3±1.1	3.1±1.5
CL <sub>bile</sub> (mL/min/kg)	22.5±4.7	18.1±9.8

a: All data represent the means ± standard deviation (n=5).

\*: Statistically different from control rats ( $p < 0.01$ ).

**Table 2.** Kinetic parameters for the *in vitro* uptake of daunorubicin into isolated hepatocytes and cLPM vesicles<sup>a</sup>

Function		Control	CCl <sub>4</sub> -EHI
Uptake (isolated hepatocytes)	V <sub>max</sub> (nmole/min/10 <sup>6</sup> cells)	1.98±0.08	1.19±0.09*
	K <sub>m</sub> (μM)	238±42	179±18
	CL <sub>int</sub> <sup>b</sup> (μL/min/10 <sup>6</sup> cells)	8.33±1.7	6.69±0.6*
	K <sub>d</sub> (μL/min/10 <sup>6</sup> cells)	15.46±1.0	13.11±0.8*
Excretion (cLPM vesicles)	V <sub>max</sub> (nmole/min/mg protein)	12.67±1.0	25.08±1.3*
	K <sub>m</sub> (μM)	146.9±27	174.6±33
	CL <sub>int</sub> <sup>b</sup> (μL/min/mg protein)	86.54±14.4	143.8±20.6*
	K <sub>d</sub> (μL/min/mg protein)	23.2±3.8	38.4±3.0*

a: All data represent the means ± standard deviation (n=3).

b: Intrinsic clearance (CL<sub>int</sub>) was calculated by the division of V<sub>max</sub> with K<sub>m</sub>.

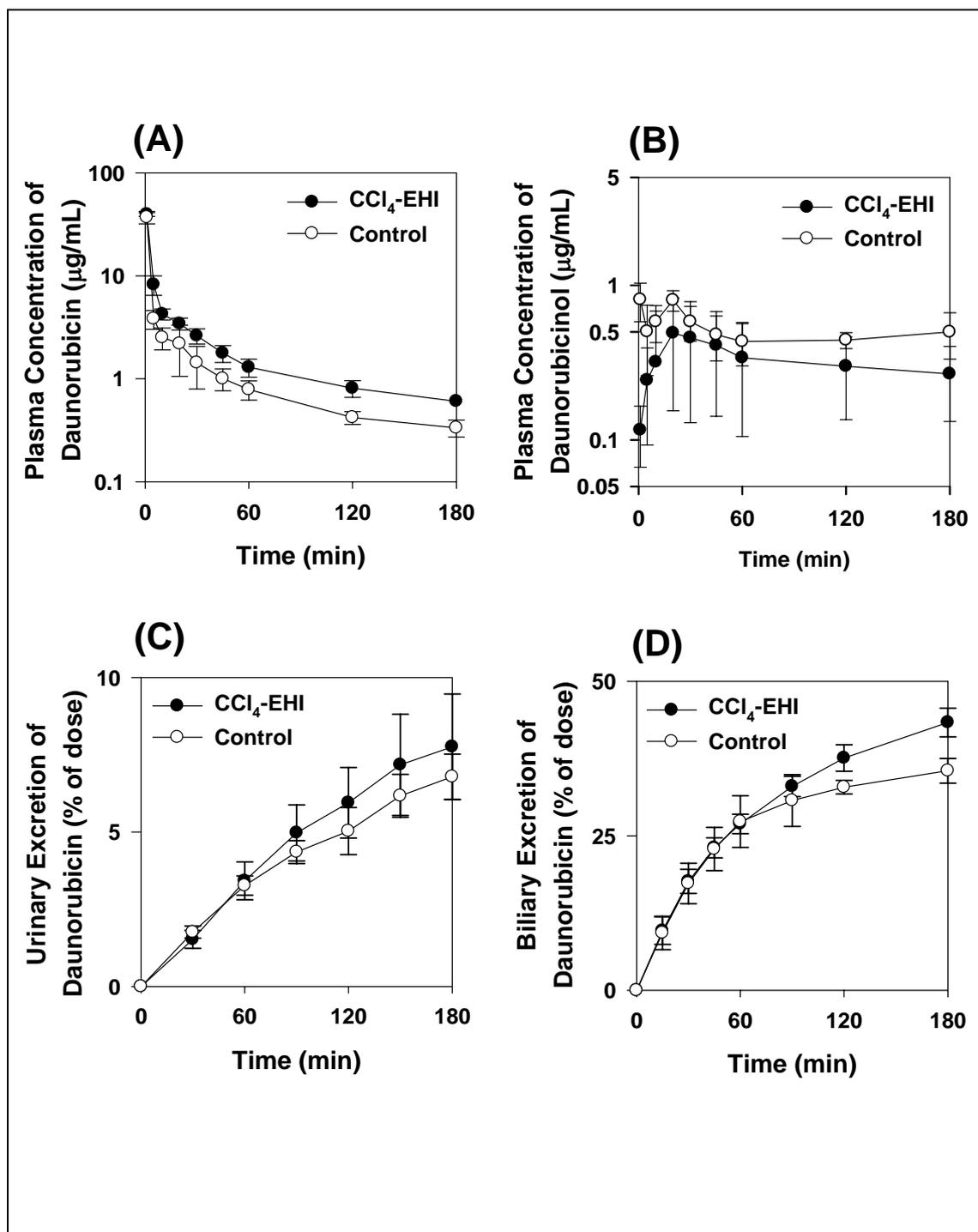
\*: Statistically different from respective controls ( $p < 0.01$ ).

## DISCUSSION

When hepatic injury is induced, changes in the elimination of xenobiotics are expected because the liver plays an important role in the metabolism and excretion of xenobiotics. The results presented in this study indicated that CCl<sub>4</sub>-EHI affects the pharmacokinetics of daunorubicin. That is, after the intravenous administration of daunorubicin, the AUC was increased by 1.6 times, whereas CL was reduced by 34% compared to the controls. The apparent urinary clearance (CL<sub>urine</sub>) was not changed in CCl<sub>4</sub>-EHI rats, indicating that the change in the pharmacokinetics of daunorubicin caused by CCl<sub>4</sub>-EHI is unlikely

to be associated with renal excretion. This suggests that a change in the hepatic disposition is responsible for the change in the pharmacokinetics of daunorubicin.

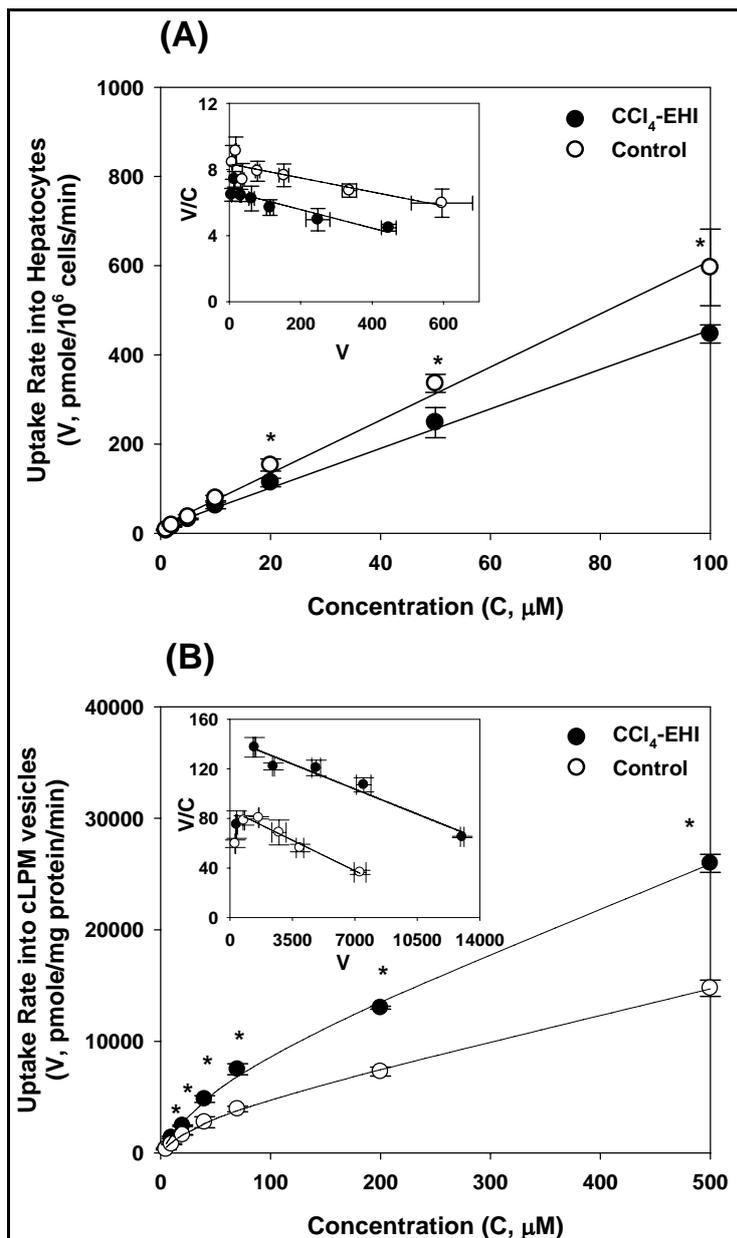
It has been reported that active transport processes are involved in the hepatobiliary elimination of daunorubicin, and CCl<sub>4</sub>-EHI affects the processes (15,27). However, the relevant mechanisms have not been elucidated systemically. The total *in vitro* sinusoidal uptake clearance (*i.e.*, CL<sub>int</sub> + K<sub>d</sub>) was decreased by 17% in CCl<sub>4</sub>-EHI (Table 2). In the sinusoidal uptake of daunorubicin into hepatocytes, passive diffusion appears to play a greater role than active transport because the K<sub>d</sub> is approximately 2 times greater



**Figure 1.** The plasma concentration (A), and urinary (C), and biliary excretion (D) of daurorubicin and the plasma concentration of daurorubicinol (B) in control (○) and CCl<sub>4</sub>-EHI rats (●) following the intravenous administration of 11.3 mg/mL/kg daurorubicin hydrochloride. Each point represents the mean ± standard deviation of five rats

than that of  $CL_{int}$  for both control and CCl<sub>4</sub>-EHI rats. This may be associated with the high lipophilicity of the drug. The active sinusoidal uptake might be mediated by sinusoidal organic

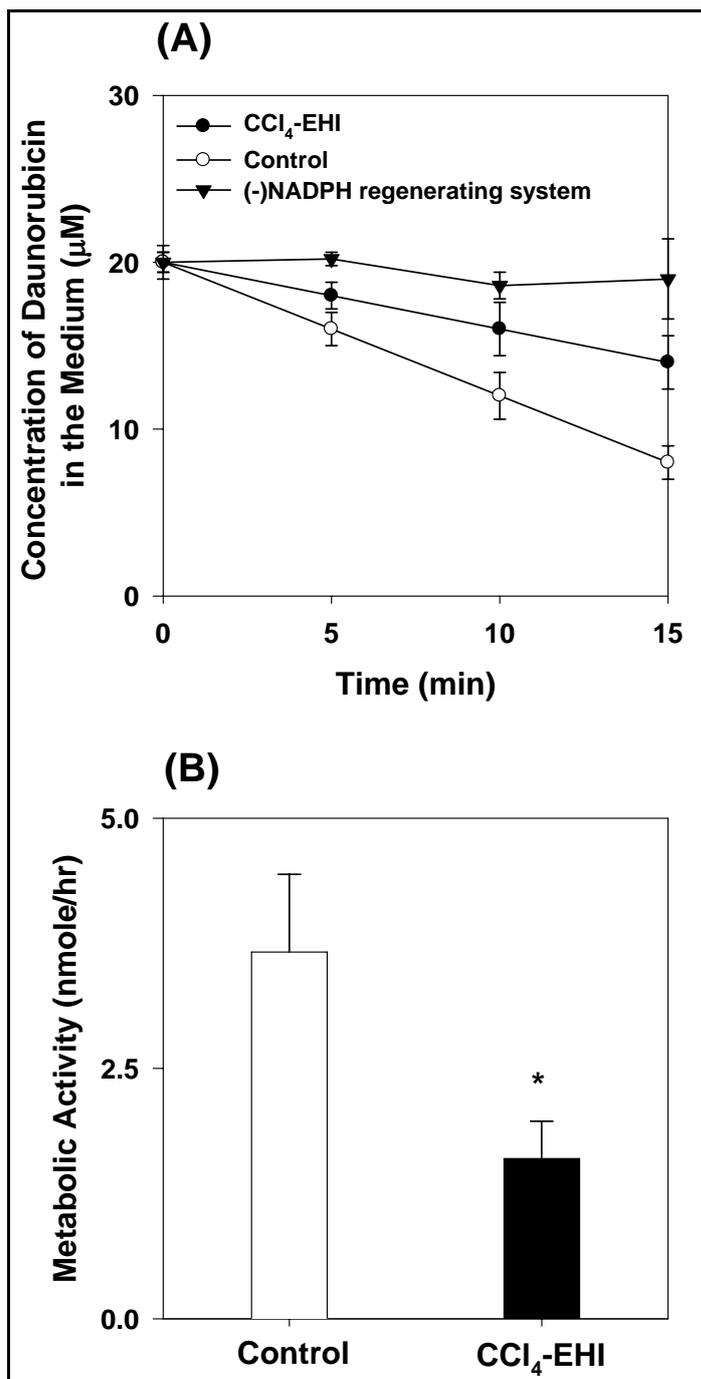
cation transporters (*e.g.*, OCT1) considering that daurorubicin is cationic at physiological pH. In the cLPM vesicles, the  $CL_{int}$  was 3.7 times greater than the  $K_d$  for both control and CCl<sub>4</sub>-EHI rats



**Figure 2.** (A) Concentration dependency for the initial uptake rate of daunorubicin (1-100  $\mu\text{M}$ ) into isolated hepatocytes from control ( $\circ$ ) and  $\text{CCl}_4$ -EHI rats ( $\bullet$ ). (B) Concentration dependency for the initial uptake rate of daunorubicin (5-500  $\mu\text{M}$ ) into cLPM vesicles from control ( $\circ$ ) and  $\text{CCl}_4$ -EHI rats ( $\bullet$ ) in the presence of ATP. The curves were generated from the estimated kinetic parameters in Table 2. Each point represents the mean  $\pm$  standard deviation of triplicate measurements from three different batches of hepatocytes and cLPM vesicle preparations. The Eadie-Hofstee transformation of the initial uptake rate is shown in the inset. \* : Significantly different from control rats ( $p < 0.01$ ).

(Table 2), suggesting a greater contribution of ATP-dependent active transport compared to passive diffusion in the canalicular excretion of daunorubicin. This contrasted the case for

sinusoidal uptake. The total *in vitro* canalicular excretion clearance (*i.e.*,  $\text{CL}_{\text{int}} + K_d$ ) was increased by 1.7 times in  $\text{CCl}_4$ -EHI. Daunorubicin and daunorubicinol (27-29) are P-gp substrates



**Figure 3.** Effect of CCl<sub>4</sub>-EHI on the metabolism of daunorubicin (30 µM as daunorubicin hydrochloride) in rat liver microsomes. (A) The temporal profile of daunorubicin remaining in microsomal suspensions (37 °C) from control (○, ▼) and CCl<sub>4</sub>-EHI (●) rats (n=5, each) in the presence (○, ●) and absence of the NADPH-regenerating system (▼). (B) Metabolic activity was calculated from the rate of degradation of daunorubicin (Figure 3A). The open bar indicates control rats (n=5) and the closed bar indicates CCl<sub>4</sub>-EHI rats (n=5). \* : Statistically different from control rats (*p*<0.01).

(30,31). Therefore, the increased expression of P-gp on canalicular membranes in CCl<sub>4</sub>-EHI rats (15) appears to be consistent with the increases in

$V_{max}$  and  $CL_{int}$  observed here.  $K_d$  was also increased by 1.7 times in CCl<sub>4</sub>-EHI, implying increased fluidity of the canalicular membrane for

the diffusion of daunorubicin. The 1.7 times increase in the total *in vitro* canalicular excretion clearance with CCl<sub>4</sub>-EHI is fairly consistent with the 1.8 times increase in the *in vivo* canalicular excretion clearance of the drug in CCl<sub>4</sub>-EHI rats (15). Previously, we found that hepatic concentration of daunorubicin at a steady state was decreased in CCl<sub>4</sub>-EHI (15). The decrease appears to be associated with the increase in the canalicular excretion under the decreased hepatic uptake (Table 2).

The sinusoidal  $K_d$  was decreased, whereas the canalicular  $K_d$  was increased by CCl<sub>4</sub>-EHI (Table 2), although the underlying mechanism for this discrepancy is currently unknown. Similar results have been reported previously; the pretreatment of CCl<sub>4</sub> increased the membrane fluidity of the mitochondrial outer membrane, but decreased that of the mitochondrial inner membrane (32). Further investigation is necessary to explain this discrepancy.

Although the *in vitro* intrinsic canalicular excretion clearance ( $CL_{int}$ ) of daunorubicin was increased by 1.7 times, it had a negligible effect on the biliary excretion of daunorubicin in CCl<sub>4</sub>-EHI, possibly because of the decrease in the *in vitro* intrinsic sinusoidal uptake clearance ( $CL_{int}$ ). As a result, sinusoidal uptake, rather than canalicular excretion, appears to govern the overall hepatobiliary excretion and pharmacokinetics of daunorubicin. In addition to the sinusoidal uptake into hepatocytes, hepatic metabolism appears to be most affected by the CCl<sub>4</sub>-EHI. The significant decrease in the systemic clearance in the absence of decreases in the apparent urinary ( $CL_{urine}$ ) and biliary ( $CL_{bile}$ ) clearances (Table 1) implies that CCl<sub>4</sub>-EHI reduces the metabolism or degradation of daunorubicin in the liver. This hypothesis is supported by the fact that CCl<sub>4</sub>-EHI significantly retarded the *in vitro* degradation of the drug in the liver microsomes (Figure 3), and decreased the CYP content in the microsomes from 0.51 to 0.35 nmole/mg protein. Moreover, CCl<sub>4</sub> treatment was reported to cause a marked reduction in the expression of CYP 2B1 (18), which is responsible for the biotransformation of daunorubicin (4). Therefore, the reduced expression of the relevant metabolizing enzyme in the liver and subsequently reduced metabolism of the drug in

the liver (Figure 1B and Figure 3), appear to be responsible for the decreased systemic clearance and increased AUC of daunorubicin in rats with CCl<sub>4</sub>-EHI.

CCl<sub>4</sub>-induced hepatic injury is known to accompany hyperbilirubinemia, steatosis, liver cirrhosis, and necrosis (11,33,34). Moreover, CCl<sub>4</sub>, along with acetaminophen and alcohol, has been classically used in rodent models to investigate mechanisms of hepatotoxicity relevant to human exposure (35). Drug or chemical-induced liver injury accounted for more than 50% of all cases of acute liver failure in the United States from 1997 to 2002 (36). Toxicity resulting from these chemicals induced oxidative stress and altered cellular redox status. The dysregulated redox status, in turn, activated adaptive mechanism to change the expression of hepatic transporters and metabolizing enzymes as well as detoxication genes. This coordinated regulation altered the expression to limit the accumulation of chemicals within the hepatocyte (i.e., in general, decreased hepatic uptake and increased canalicular efflux) (33, 35), which is consistent with our results and implicates altered hepatic function and pharmacokinetics of drugs.

Therefore, in patients with chemical or drug induced hepatotoxicity, adjustment of dosage regimens for drugs that are substrates of these transporters or metabolizing enzymes would be needed (35,37), with particular care on that functional changes of transporters are multidirectional as a function of time after the hepatotoxicity induction (15).

## ABBREVIATIONS

$Ae_{b,0-3h}$ , cumulative amount excreted into bile during the first 3 h;  $Ae_{u,0-3h}$ , cumulative amount excreted into urine during the first 3 h; AUC, area under the plasma concentration-time curve;  $AUC_{m,0-3h}$ , area under the plasma concentration-time curve for metabolite from time zero to 3 h; Bsep, bile salt export pump; CCl<sub>4</sub>, carbon tetrachloride; CCl<sub>4</sub>-EHI, CCl<sub>4</sub> -induced experimental hepatic injury; CL, systemic clearance;  $CL_{bile}$ , apparent biliary clearance;  $CL_{urine}$ , apparent urinary clearance;  $CL_{int}$ , intrinsic clearance; cLPM, canalicular liver plasma membrane; CYP, cytochrome P-450; Mrp, multidrug-resistance related protein; ntcp,

Na<sup>+</sup>/taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; V<sub>d</sub>, apparent volume of distribution at a steady state

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## REFERENCES

- [1]. Tidefelt, U., Prenekert, M., Paul, C., Comparison of idarubicin and daunorubicin and their metabolites regarding intracellular uptake and effect on sensitive and multidrug-resistant HL60 cells. *Cancer Chemother Pharmacol*, 38:476-480, 1996.
- [2]. Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., Gianni, L., Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev*, 56:185-229, 2004.
- [3]. Bachur, N.R., and Gee, M., Daunorubicin metabolism by rat tissue preparations. *J Pharmacol Exp Ther*, 177:567-572, 1971.
- [4]. Goeptar, A.R., Te Koppele, J.M., Lamme, E.K., Pique, J.M., Vermeulen, N.P., Cytochrome P450 2B1-mediated one electron reduction of adriamycin: a study with rat liver microsomes and purified enzymes. *Mol Pharm*, 44:1267-1277, 1993.
- [5]. Maniez-Devos, D.M., Baurain, R., Lesne, M., Trouet, A., Doxorubicin and daunorubicin plasmatic, hepatic and renal disposition in the rabbit with or without enterohepatic circulation. *J Pharmacol*, 17:1-13, 1986.
- [6]. Catapano, C.V., Gwaitani, A., Brogini, M., Corada, M., Bartosek, I., Italia, C., Donelli, M.G., Hepatobiliary metabolism and urinary excretion of 4-demethoxydaunorubicin as compared to daunorubicin in rats. *Anticancer Res*, 8:725-731, 1988.
- [7]. Kimura, T., Nakayama, S., Yamao, T., Kurosaki, Y., Nakayama, T., Pharmacokinetics of indocyanin green in rats with experimentally induced hepatic diseases. *Biol Pharm Bull*, 16:1140-1145, 1993.
- [8]. Lee, D.Y., Lee I.C., Lee, M.G., Pharmacokinetics of omeprazole after intravenous and oral administration to rats with liver cirrhosis induced by dimethylnitrosamine. *Int J Pharm*, 330:37-44, 2007.
- [9]. Choi, M.K., Song, I.S., Park, S.R., Hong, S.S., Kim, D.D., Chung, S.J., Shim, C.K., Mechanism of the stationary canalicular excretion of trbutylmethyl ammonium in rats with a CCl<sub>4</sub>-induced acute hepatic injury. *J Pharm Sci*, 94:317-326, 2005.
- [10]. Jiko, M., Yuno, I., Okuda, M., Inui, K.I., Altered pharmacokinetics of paclitaxel in experimental hepatic or renal failure. *Pharm Res*, 22:228-234, 2005.
- [11]. Recknagel, R.O., Carbon tetrachloride hepatotoxicity. *Pharmacol Rev*, 19:145-195, 1967.
- [12]. Whiting, J.F., Green, R.M., Rosenbluth, A.B., Gollan, J.L., Tumor necrosis factor-alpha decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. *Hepatology*, 22:1273-1278, 1995.
- [13]. Denson, L.A., Auld, K.L., Schiek, D.S., McClure, M.H., Mangelsdorf, D.J., Karpen, S.J., Interleukin-1beta suppresses retinoid transactivation of two hepatic transporter genes involved in bile formation. *J Biol Chem*, 275:8835-8843, 2000.
- [14]. Geier, A., Kim, S.K., Gerloff, T., Dietrich, C.G., Lammert, F., Karpen, S.J., Stieger, B., Meier, P.J., Matern, S., Gartung, C., Hepatobiliary organic anion transporters are differentially regulated in acute toxic liver injury induced by carbon tetrachloride. *J Hepatol*, 37:198-205, 2002.
- [15]. Song, I.S., Lee, Y.M., Chung, S.J., Shim, C.K., Multiple alterations of canalicular membrane transport activities in rats with CCl<sub>4</sub>-induced hepatic injury. *Drug Metab Dispos*, 31:482-490, 2003.
- [16]. Hong, S.S., Chung, S.J., Shim, C.K., Functional impairment of sinusoidal membrane transport of organic cations in rats with CCl<sub>4</sub>-induced hepatic failure. *Pharm Res*, 17:833-838, 2000.
- [17]. Kotsiou, A., Tsamouri, M., Anagnostopoulou, S., Tzivras, M., Vairactaris, E., Tesseromatis, C., H<sup>3</sup> propranolol serum levels following lidocaine administration in rats with CCl<sub>4</sub> induced liver damage. *Eur J Drug Metab*

- Pharmacokinet, 31:97-101, 2006.
- [18]. Lupp, A., Lucas, N., Danz, M., Klinger, W., Transplantation of fetal liver tissue suspension into the spleens of adult syngenic rats: effects of different cytotoxins on cytochrome P450 isoforms expression and on glycogen content. *Exp Toxicol Pathol*, 52:381-393, 2000.
- [19]. Reitman, S., and Frankerl, S., A colorimetric method for the determination of serum glutamic-oxaloacetic and glutamic-pyruvic transaminase. *Am J Clin Pathol*, 28:56-61, 1957.
- [20]. Han, Y.H., Chung, S.J., Shim, C.K., Canalicular membrane transport is primarily responsible for the difference in hepatobiliary excretion of triethylmethylammonium and tributylmethylammonium in rats. *Drug Metab Dispos*, 27:872-879, 1999.
- [21]. Inoue, M., Kinne, R., Tran, T., Biempica, L., Arias, I.M., Rat liver canalicular membrane vesicles: isolation and topological characterization. *J Biol Chem*, 258:5183-5188, 1983.
- [22]. Jewell, W.T., and Miller, M.G., Comparison of human and rat metabolism of molinate in liver microsomes and slices. *Drug Metab Dispos*, 27:842-847, 1999.
- [23]. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., Protein measurement with the folin phenol reagent. *J Biol Chem*, 193:265-275, 1951.
- [24]. Omura, T., and Sato, R., The carbon monoxide-binding pigment of liver microsomes. Evidence for its hemoprotein nature. *J Biol Chem*, 239:2370-2385, 1964.
- [25]. Liu, K.H., Kim, M.G., Lee, D.J., Yoon, Y.J., Kim, M.J., Shon, J.H., Choi, C.S., Choi, Y.K., Desta, Z., Shin, J.G., Characterization of ebastine, hydroxyebastine, and carebastine metabolism by human liver microsomes and expressed cytochrome P450 enzymes: major roles for CYP2J2 and CYP3A. *Drug Metab Dispos*, 34:1793-1797, 2006.
- [26]. Gibaldi, M., and Perrier, D., *Pharmacokinetics*. 2nd ed. Marcel-Dekker, New York, NY, USA, 1982.
- [27]. Kwon, Y., Kamath, A.V., Morris, M.E., Inhibitors of P-glycoprotein-mediated daunomycin transport in rat liver canalicular membrane vesicles. *J Pharm Sci*, 85:935-939, 1996.
- [28]. Young, R.C., Ozols, R.F., Myers, C.E., The anthracycline antineoplastic drugs. *N Engl J Med*, 305:139-153, 1981.
- [29]. Kamimoto, Y., Gatnaitan, Z., Hsu, J., Arias, I.M., The function of Gp 170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *J Biol Chem*, 264:11693-11698, 1989.
- [30]. Ross, D.D., Doyle, L.A., Yang, W., Tong, Y., Cornblatt, B., Susceptibility of idarubicin, daunorubicin, and their C-13 alcohol metabolites to transport mediated multidrug-resistance. *Biochem Pharmacol*, 10:1673-1683, 1995.
- [31]. Callies, S., de Alwis, D.P., Mehta, A., Burgess, M., Aarons, L., Population pharmacokinetic model for daunorubicin and daunorubicinol coadministered with zosuquidar (LY335979). *Cancer Chemother Pharmacol*, 54:39-48, 2004.
- [32]. Wu, D.F., Peng, R.X., YE, L.P., Yu, P., The effect of silymarin on hepatic microsomal and mitochondrial membrane fluidity in mice. *Zhongguo Zhong Yao Za Zhi*, 28:870-872, 2003.
- [33]. Tada, S., Nakamoto, N., Kameyama, K., Tsunematsu, S., Kumagai, N., Saito, H., Ishii, H., Clinical usefulness of edaravone for acute liver injury. *J Gastroenterol Hepatol*, 18: 851-857, 2003.
- [34]. Kim, S.W., Schou, U.K., Peters, C.D., de Seigneux, S., Kwon, T.H., Knepper, M.A., Jonassen T.E.N., Froki, J., Nielsen, S., Increased apical targeting of renal epithelial sodium channel subunits and decreased expression of type 2 11-hydroxysteroid dehydrogenase in rats with CCl4-induced decompensated liver cirrhosis. *J Am Soc Nephrol*, 16: 3196-3210, 2005.
- [35]. Aleksunes, L.M., Slitt, A.M., Cherrington, N.J., Thibodeau, M.S., Klaassen, C.D., Manatou, J.E., Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. *Toxicological Sciences* 83: 44-52, 2005.
- [36]. Lee, W.M., Acute liver failure in the United States. *Semin. Liver Dis*. 23: 217-226, 2003.
- [37]. McLean, A.J., Morgan, D.J., Clinical pharmacokinetics in patients with liver disease. *Clin Pharmacokinet* 21: 42-69, 1991.