Opposite Effect of Diabetes Mellitus Induced by Streptozotocin on Oral and Intravenous Pharmacokinetics of Verapamil in Rats

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Abbreviations:

P-gp, P-glycoprotein; NADP, β-nicotinamide adenine dinucleotide phosphate; PI,

protease inhibitor; STZ, streptozotocin; HPLC, High-performance liquid

chromatography; i.g., intragastric; i.v., intravenous; S.D., standard deviation; AUC,

the area under the concentration-time curve; GAPDH, glyceraldehyde-3-phosphate

dehydrogenase; CYP, Cytochrome P450; CL_{int}, intrinsic clearance.

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ABSTRACT

The aim of this study was to report the effect of diabetes mellitus on pharmacokinetics of verapamil in route-dependent manner. Diabetic rats were induced by streptozotocin. Plasma concentration of verapamil and its metabolite, norverapamil, were measured following oral (10 mg/kg) or intravenous (1 mg/kg) administration. The concentrations of verapamil in portal plasma after oral administration were also determined. Norverapamil formation was used for assessing CYP3A activity in hepatic and intestinal microsomes of diabetic rats. The protein level of CYP3A1 and CYP3A2 in liver and intestine were measured using western blot. It was found that the diabetes significantly increased plasma concentration of verapamil and norverapmil after oral administration, which resulted in a 74% increase in the AUC of verapamil, but the ratio of $AUC_{(norverapamil)}/AUC_{(verapamil)}$ was significantly decreased by 38%. In contrast, diabetes significantly decreased the AUC of verapamil by 22% following intravenous administration. Diabetes also resulted in increased AUC of verapamil in portal vein by 3.8-fold compared with control rats. The absolute bioavailability of verapamil was higher than that of control rats. In vitro study showed that increased CYP3A activity in the hepatic microsome and decreased CYP3A activity in the intestinal microsome were accompanied by the increase and decrease of the protein expression of CYP3A1/2 in liver and intestine of diabetic rats, respectively. In conclusion, diabetes mellitus revealed a tissue specific effect on CYP3A activity and expression (induced in liver and inhibited in intestine), resulting in the opposite pharmacokinetic behaviors of verapamil following oral and intravenous

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administration to diabetic rats.

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INTRODUCTION

Several reports showed that the expressions of some hepatic cytochromes P450 (CYP450) isozymes were changed in diabetic patients and experimental diabetic animals (Shimojo, 1994; Lee et al., 2010; Kim et al., 2005a). Many phase II metabolic enzymes, including glucuronidase and sulfurlase and glutathione S-transferase, were also profoundly affected by diabetes mellitus (Price and Jollow, 1986). Furthermore, the increase of hepatic blood flow rate (Sato et al., 1991), decrease of bile flow rate, alteration of bile composition (Carnovale et al., 1986), impairment of kidney function (Nadai et al., 1990) and hepatotoxicity (Watkins and Sherman, 1992) were also observed under diabetic state. All these findings indicate that diabetic conditions may alter pharmacokinetic behavior of some drugs.

The CYP3A enzymes are the most important drug-metabolizing enzymes which are primarily expressed in the liver, metabolizing more than 50% of the marketed drugs. It was reported that the enzyme activity, protein expression and/or mRNA level of hepatic CYP3A1 (Kim et al., 2005b; Borbás et al., 2006) and CYP3A2 in streptozotocin (STZ)-induced diabetic rats (Borbás et al., 2006; Shimojo et al., 1993) were higher than those in control rats. Several reports showed that the up-regulation of hepatic CYP3A in diabetic rats may contribute to the faster clearance and lower AUC of drugs, such as telithromycin and clarithromycin (Lee and Lee, 2008; Kim et al., 2005b). It is known that small intestine also plays an important role in first-pass extraction of orally ingested xenobiotics. A significant amount of CYP3A is expressed

in the intestine to metabolize drugs during their transit across the intestinal epithelium (Lin et al., 1999). Opposite to the findings in liver, diabetes significantly decreased (50.7% decrease) the intestinal testosterone 6β-hydroxylase (a CYP3A1/2 marker) activity (Borbás et al. 2006). It was reported that the AUC of cyclosporine in diabetic rats was approximately 65.5% greater than that in control rats after oral administration (Ogata et al., 1996). Our previous study demonstrated that diabetes mellitus significantly increased the exposure of baicalin following oral dose, while decreased the exposure of baicalin following intravenous dose (Liu et al., 2010). All the results suggest that the effect of diabetes mellitus on pharmacokinetics of drugs is route-dependent.

Verapamil, a phenylamine calcium channel blocking agent, is widely used for the treatment of hypertension, ischemic heart disease, supraventricular tachyarrhythmias, and hypertrophic cardiomyopathy (McTavish and Sorkin, 1989). Cardiovascular disorder is one of the major complications of diabetes mellitus, so verapamil is also a common drug for diabetic patients (García Donaire and Ruilope, 2007). Verapamil is known to be a dual substrate of CYP3A and P-glycoprotein (P-gp). Both liver and intestine were considered to be the major sites of biotransformation and first-pass extraction of verapamil. Verapamil is mainly metabolized by CYP3A in both liver and small intestine to form a primary N-demethylation metabolite, norverapamil (Kroemer et al., 1993). Human CYP3A4 and rat CYP3A1 have 73% protein homology (Lewis, 1996). It was reported that CYP3A4 was mainly responsible for the metabolism of

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verapamil in human, while CYP3A1/2 govern the metabolism of verapamil in rats (Tracy et al., 1999; Choi and Burm, 2008; Hanada et al., 2008). Verapamil undergoes extensive first-pass metabolism, with low oral bioavailability (10% to 20%) in various animal species (Schomerus et al., 1976; Woodcock et al., 1981). The hepatic extraction ratio of verapamil was calculated to be 0.437 in the study. The orally administered verapamil is probably metabolized to an appreciable extent (approximately 30%) in the intestinal mucosa (Fromm et al., 1998). The intestinal extraction ratio is 0.323 and 0.687 for (S)- and (R)- verapamil (Hanada et al., 2008). It has been reported that intestinal CYP3A was down regulated in diabetic rats (Borbás et al. 2006).

The aim of the present study was to investigate whether the effect of diabetes mellitus on pharmacokinetics was route-dependent using verapamil as the model drug. The alterations in activities and protein expression of CYP3A in liver and intestine of diabetic rats were also investigated.

MATERIALS AND METHODS

Materials

Verapamil and propranolol hydrochloride were purchased from the National Institute for the Normal of Pharmaceutical and Biological Products (Beijing, China). Norverapamil hydrochloride, protease inhibitor cocktail P8340 (PI), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, β-nicotinamide adenine

dinucleotide phosphate (NADP) and streptozotocin (STZ) were purchased from Sigma Corporation (Shanghai, China). HPLC grade acetonitrile was obtained from Merck (Damstadt, Germany). Verapamil injectable-solution (2ml: 5mg) was obtained from Hefeng Pharmaceutical Group (Shanghai, China). Mouse anti-rat CYP3A1 monoclonal antibody and rabbit anti-rat CYP3A2 polyclonal antibody were purchased from Abcam plc (Cambridge, UK). All the other reagents were of analytical grade and were commercially available.

Animals

5-week-old male Sprague-Dawley rats (weighing 180-200g) were supplied by Slac Laboratory Animal Ltd (Shanghai, China). The rats were maintained under controlled conditions of temperature ($22\pm2°C$) and relative humidity ($50\%\pm10\%$) with 12h light-dark cycle. Water and food (laboratory rodent chow; Nanjing, China) were allowed ad libitum. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

Induction of diabetes mellitus in rats by STZ injection

Diabetic rats were induced by intraperitoneal administration of STZ (65mg/kg, 1mL/100g) dissolved in 0.05M citrate buffer (PH 4.5) immediately before administration. Age-matched control rats only received citrate buffer. Development of diabetes was confirmed by fasting blood glucose levels using glucose reagent kit (Jiancheng Biotech Co., Nanjing, China). Animals were considered to be diabetic only

when their fasting blood glucose levels exceeded 11.1 mM (200mg/dL) after 1 week of STZ injection. All experiments were done on the 35th day after STZ injection because it was reported that complications from any toxic effect of STZ were minimized by carrying out the experiments 4 to 5 weeks after initial STZ injection (Watkins and Sherman, 1992).

Pharmacokinetics of verapamil in diabetic rats and control rats after oral and intravenous administration

On day 35, after fasted overnight, diabetic and age-matched control rats were orally administrated 10 mg/kg of verapamil. Blood samples (300 μL) were collected in heparinized Eppendorf tubes via the oculi chorioideae vein at 5, 10, 20, 30, 60, 120, 240, 360 and 480 min following the oral dose of verapamil under light ether anesthesia, and the appropriate amount of heparinized blood collected from the untreated rats was injected intravenously at the appropriate time. After being centrifuged at 5,000 rpm for 5min, the plasma samples were obtained and frozen at -20°C until analysis. For intravenous administration, verapamil (1 mg/kg) was given to diabetic and control rats over 1 min via caudal vein administration. Blood samples (300 μL) were collected at 5, 10, 20, 30, 60, 120, 180 and 300 min following the intravenous dose under light ether anesthesia. The collection and the treatment of blood samples were performed by the same method as described for oral administration.

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Concentrations of verapamil in plasma of portal vein

Portal vein cannulation was performed to diabetic and age-matched control rats fasted for 12h under sodium pentobarbital (60 mg/kg, i.p.) anesthesia, according to the method described previously (Yu et al., 2010b). Portal blood samples (300 μL) were collected via cannulation at 5, 10, 15, 30, 60 and 90 min after oral administration of verapamil (10 mg/kg). After each sample was taken, the catheter was flushed with 300 μL of saline containing 50U of heparin to compensate for blood loss. Plasma samples were obtained and stored at -20°C for analysis.

Preparation of rat hepatic and intestinal microsomes

Hepatic and intestinal microsomes were prepared freshly from diabetic and control rats. After ether anaesthesia, rat blood was bled through aorta, and then liver and small intestine were excised quickly. Rat hepatic and intestinal microsomes were prepared according to the methods described previously (Xie et al., 2010; Bruyère1 et al., 2009). The microsomal pellets were re-suspended in PBS (PH 7.4) containing PI and 20% glycerol, and stored at -80°C. The protein concentration of the microsomes was measured by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

***In vitro* metabolism of verapamil**

Verapamil is mainly metabolized into norverapamil via CYP3A-mediated N-dealkylation, and the formation of norverapamil was used as a marker to assess

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CYP3A activity in this study. The incubation mixture (final volume: 200 μL) consisted of 40 μL of rat hepatic microsomes (0.2 mg/mL) or intestinal microsomes (1 mg/mL), 40 μL of NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$), and phosphate buffer(pH 7.4). After a 5-min pre-incubation at 37°C, the reaction was initiated by adding 10 μL of verapamil solution. The reaction was terminated by adding 20 μL of 2 M NaOH after incubation at 37°C for 10 min. All of the above microsomal incubation conditions were in the linear range of the reaction rate (Xie et al., 2010). The final concentrations of verapamil were set to be 5, 10, 20, 40, 100, 200 and 300 μM in hepatic incubation mixture or 5, 20, 40, 100, 200, 300, 400, 800 and 1600 μM in intestinal incubation mixture respectively.

Measurement of CYP3A Protein in Rat Liver and Intestine

The protein levels of CYP3A1 and CYP3A2 in liver and intestine were evaluated using Western blot, as described previously (Kim et al., 2005a). Intestinal (duodenal and jejunal) homogenate was prepared by a method described previously (Mitschke et al., 2008). Twenty micrograms proteins of hepatic microsome or intestinal homogenate were resolved by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis. CYP3A1 and 3A2 were detected using mouse anti-rat CYP3A1 monoclonal antibody and rabbit anti-rat CYP3A2 polyclonal antibody respectively. Then, immune complexes were revealed by secondary antibody (peroxidase-conjugated goat anti-rabbit or anti-mouse whole molecule IgG). Samples

were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Immunoreactive proteins were visualized by chemiluminescence (ECL Western Blotting Detection Reagents; Applygen Technologies Inc., Beijing, China), and band density was measured by densitometry using Gel-Pro Analyzer 4.0.

HPLC analysis of verapamil and norverapamil

Verapamil and norverapamil in plasma or incubation mixture were determined by HPLC method described previously (Xie et al., 2010). The HPLC system was equipped with an LC-10A pump, a CTO-10AS$_{vp}$ columen oven, a RF-10AXL fluorescence detector (Shimadzu) set at an excitation wavelength of 280 nm and emission wavelength of 310 nm. The mobile phase consisted of 0.02 M KH$_2$PO$_4$: acetonitrile (72: 28, v/v), and the flow rate was set to 1.0 mL/min. The retention times were as follows: internal standard, 3.8 min, norverapamil, 9.0 min and verapamil, 10.4 min.

10 μL of propranolol solution (2 μg/mL) was added to 100 μL of plasma as internal standard, followed by 10 μL of 2 M NaOH. Sample was extracted with 2 mL of ether and vortex mixed, followed by centrifugation (4, 000 rpm, 10min). The organic layer was transferred and evaporated to dryness under a stream of nitrogen gas in a water-bath at 45°C and the residue was reconstituted in 100 μL of mobile phase and centrifuged (20, 000 rpm, 10 min). 20 μL of the supernatant was injected onto the HPLC system. The limit of quantification for both verapamil and norverapamil was 7.8 ng/mL. Both the intraday and interday assay coefficients of variation were <10%.

For the incubation mixture preparation, 2 mL ether was added to 200 µL microsomal suspension and prepared according to the procedure described above. The assay for norverapamil in the hepatic and intestinal incubation mixture was linear over the range 0.26-4.2 µM and 0.032-2.1 µM, respectively. The limit of quantification for norverapamil in both hepatic and intestinal incubation mixture was 0.016 µM.

Data analysis and statistical analysis

Pharmacokinetic parameters were estimated by noncompartmental analysis using DAS 2.0 software package (Wannan Medical College). The peak plasma concentration (C_{max}) and the time to C_{max} (T_{max}) values were directly obtained from the experimental data. The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule. $T_{1/2}$ was calculated as $T_{1/2} = 0.693/k$. The clearance (CL) was calculated as dose/$AUC_{0-\infty}$; Absolute bioavailability (F%) = $(AUC_{oral}/AUC_{iv}) \times (Dose_{iv}/Dose_{oral}) \times 100$.

Enzyme kinetic parameters were estimated by non-linear least-squares regression using programming solver (Microsoft excel 2003). Eadie-Hofstee plots were used to check for kinetic characteristics. Michaelis-Menten equation (equation 1) was used to calculate apparent K_m and V_{max} values.

$$v = \frac{V_{max} \cdot S}{K_m + S} \quad (1)$$

Where v is the velocity of formation of norverapamil; S, the concentration of verapamil in the incubation mixture; V_{max}, maximum metabolic velocity; K_m, Michaelis-Menten constants;

The extended Michaelis-Menten equation (equation 2) was used to estimate kinetic parameters for two enzymes systems.

$$v = \frac{V_{max,1}S}{K_{m,1} + S} + \frac{V_{max,2}S}{K_{m,2} + S} \quad (2)$$

Where the subscript 1 and 2 denoted the high- and low affinity components of the reaction, respectively. S was the concentration of verapamil. Intrinsic clearance ($CL_{int,i}$) was defined to be $V_{max,i}/K_{m,i}$.

Results are expressed as the mean ± standard deviation (S.D.). Statistical differences among groups were evaluated by the two-tailed Student's t test. A p value of less than 0.05 indicated a significant difference.

RESULTS

Physiological and biochemical examination in control and STZ-induced diabetic rats

Body weight, fasting blood glucose level and liver weight were measured on day 35 following injection of STZ (Table 1). STZ induced rats became diabetic with symptoms of polydipsia, polyuria, polyphagia, hyperglycaemia and decrease in body weight gain. Control rats had blood glucose levels of approximately 7 mM, while diabetic rats displayed about a four-fold increase in blood glucose levels. Diabetic rats had decrease in body weight and increase in wet liver weight compared to controls, which resulted in significantly increase of relative liver weight in diabetic rats (30.40±3.44 mg liver/g body weight of control rats *v.s.* 50.15±7.66 mg liver/g body weight of diabetic rats).

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Pharmacokinetics of verapamil in diabetic rats and control rats after oral and intravenous administration

The plasma concentration of verapamil and its metabolite, norverapamil in diabetic and control rats were measured (Figure 1A and 1B) after oral administration of verapamil (10mg/kg). The corresponding pharmacokinetic parameters were estimated (Table 2). Compared with control rats, diabetic rats had higher plasma concentration of verapamil, resulting in higher exposure (C_{max} and $AUC_{0-480min}$) and lower oral clearance. The mean $AUC_{0-480min}$ and C_{max} of diabetic rats were 1.7 folds and 3.3 folds of control rats, respectively. It was also found that diabetes enhanced plasma concentration of norverapamil. Significant increase was found in C_{max} of norverapamil. However, the ratio of $AUC_{(nor)}/AUC_{(ver)}$ was significantly lower, which was only 62% of control rats. The results indicated that diabetes enhanced the exposure of verapamil after oral administration.

In order to investigate whether higher oral exposure of verapamil in plasma of diabetic rats resulted from decrease in system clearance, the pharmacokinetics of verapamil (Figure 1C) were studied in diabetic and control rats after intravenous administration of verapamil (1mg/kg). Contrary to our expectation, diabetic rats showed lower plasma concentration of verapamil accompanied by lower exposure ($AUC_{0-300min}$) and higher system clearance. The results indicated that diabetes increased rather than decreased system clearance of verapamil, which excluded the

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possibility that higher exposure of verapamil after oral administration came from decrease in system clearance in diabetic rats. It was also noted that diabetic rats had higher absolute bioavailability of verapamil (29.88% in diabetic rats vs. 15.16% in control rats).

Concentrations of verapamil in plasma of portal vein

To further investigate whether the higher exposure of verapamil in diabetic rats resulted from the decrease of pre-system metabolism and exclude the liver effects, the plasma concentrations of verapamil in portal vein were measured after oral administration to diabetic rats and control rats (Figure 1D). Higher concentrations of verapamil were found in portal plasma of diabetic rats. The C_{max} and $AUC_{0-90min}$ in portal plasma of diabetic rats were estimated to be 5.83±3.78 μg/mL and 421.99±11.07 μg·min/mL respectively, which were significantly higher than those in control rats (C_{max} 1.44±0.60 μg/mL and AUC 87.48±40.11 μg·min/mL), inducing 3.0-fold and 3.8-fold increase, respectively. However, diabetes did not affect T_{max} of verapamil (30.0±22.9 min in diabetic rats *vs.* 37.5±15.0 min in control rats). All the results indicated that the increase in exposure of verapamil after oral administration in diabetic rats may result from alteration of pre-system metabolism on intestinal wall.

***In vitro* metabolism of verapamil**

To further confirm *in vivo* results, metabolisms of verapamil in both hepatic and intestinal microsomes were documented using formation of norverapamil (Figure 2

and Tabel 3). The formation rates of norverapamil were measured in different concentration of verapamil. Compared with control rats, higher formation rate of norverapamil were found in hepatic microsome of diabetic rats. The estimated K_m values were similar in both diabetic rats and control rats, but the V_{max} in hepatic microsome of diabetic rats was significantly higher than that in control rats, resulting higher intrinsic clearance (V_{max}/K_m) in diabetic rats.

Contrast to hepatic microsome, the formation rate of norverapamil in intestinal microsome of diabetic rats was significantly lower than that in control rats. It was found that the intestinal metabolism of verapamil showed biphasic characteristics, suggesting that two components were involved in the metabolism of verapamil in intestine. Although the apparent maximum velocity ($V_{max,2}$) for low-affinity component was larger than that ($V_{max,1}$) for the high-affinity component, the intrinsic clearance ($V_{max,2}/K_{m2}$) of low-affinity component was significantly lower than that ($V_{max,1}/K_{m1}$) for the high-affinity component. Diabetes significantly decreased apparent maximum velocity (V_{max1}) for high- affinity component, accompanied by a trend to increase K_{m1}, which led to significantly decreased intrinsic clearance of high-affinity components. The intrinsic clearance of high-affinity components in intestinal microsomes of diabetic rats was only 30% of that in control rats. However, diabetes showed a trend to decrease intrinsic clearance of low-affinity components, but no significance was found. All the results demonstrated diabetes increased intrinsic clearance of verapamil in hepatic microsomes but decreased intrinsic

clearance of verapamil in intestinal microsome, which was consisted with *in vivo* findings.

Measurement of CYP3A Protein on rat liver and intestine

Metabolism of verapamil was catalyzed mainly by CYP3A1 and CYP3A2 in rats. The protein levels in liver and intestine were measured using western blot (Figure 3). It was found that diabetes increased both CYP3A1 and CYP3A2 protein level in liver. The effect of diabetes on hepatic CYP3A protein levels was CYP3A isoform-dependent. The increased extent of CYP3A2 protein was larger than that of CYP3A1, which induced increase by 151% and 50% of control rats, respectively. Contrast to liver, diabetes decreased both CYP3A1 and CYP3A2 in intestine. It was observed that the effect of diabetes on intestinal CYP3A protein levels was CYP3A isoform- and regional- dependent. In duodenum, diabetes showed stronger decrease in CYP3A1 than in CYP3A2, which was 32% and 67% of control rats, respectively. In jejunum, diabetes also decreased CYP3A1 protein level, but did not affect CYP3A2. The CYP3A protein levels were increased in liver and decreased in intestine of diabetes rats, which was in agreement with *in vivo* finding and *in vitro* results using microsomes.

DISCUSSION

The main finding of the study was the opposite effects of diabetes on pharmacokinetics of verapamil after oral and intravenous administration. *In vivo* study

showed that C_{max} and AUC of verapamil after oral administration in diabetic rats were significantly higher than those in control rats. In order to investigate whether the higher exposure of verapamil came from decreased system clearance, the pharmacokinetics of verapamil was also studied after intravenous administration. The results were contrast to our expectations. The system clearance of verapamil in diabetic rats was increased rather than decreased. The results excluded the possibility that higher exposure of verapamil following oral administration in diabetic rats came from decrease of system clearance, which indicated that higher exposure of verapamil in diabetic rats may be due to the decrease of pre-system metabolism, especially in intestinal mucosa. Similar phenomena were also found in other drugs. Our previous study showed that the exposure of baicalin was increased following oral administration in diabetic rats, but decreased after intravenous administration (Liu et al., 2010). A report demonstrated that cyclosporine A in diabetic rats after intravenous administration was significantly lower than that in control rats (Brunner et al., 1989). On the contrast, the AUC of cyclosporine A in diabetic rats after oral dose was approximately 65% greater than that in control rats (Ogata et al., 1996). Similarly, AUC of telithromycin was lower in diabetic rats following intravenous dose than that in control rats. However, the AUC was comparable between diabetic and control rats after oral administration (Lee and Lee, 2008).

In order to verify the above hypothesis and exclude liver effect, the plasma concentration of verapamil in portal vein after oral administration were measured.

Higher exposure of verapamil in portal vein of diabetic rats, similar to the finding in peripheral vein, indicated that diabetes enhanced plasma concentration and high oral bioavailability via decreasing metabolism or extrusion of verapamil in intestine. A report showed that contribution of intestinal metabolism to first-pass extraction of verapamil following oral administration was 30% (Fromm et al., 1998). Another report showed that the intestinal extraction ratios were 0.323 for (S)- verapamil and 0.687 for (R)- verapamil (Hanada et al., 2008). All the results indicated that intestine plays an important role in first-pass extraction of verapamil. It is well-known that verapamil is a dual substrate of CYP3A and P-gp. CYP3A is expressed abundantly in intestinal wall, which becomes one of the reasons resulting in lower bioavailability of verapamil. It indicated that decrease in metabolism of verapamil in intestinal mucosa may partly contribute to higher exposure of verapamil in diabetic rats. Our previous study (Yu et al., 2010a) showed that diabetes may induce impairment of P-glycoprotein (P-gp) function and expression in intestine. This indicated that the changes in the gastrointestinal absorption caused by the down-regulation of P-gp function and expression in intestine under diabetic state may also partly contribute to higher exposure of verapamil after oral administration. It was also found that extent of C_{max} and $AUC_{0-90min}$ increase in portal vein of diabetic rats were higher than those in peripheral vein, which can be partly explained by the increase of clearance in liver.

To further confirm above findings, the metabolism of verapamil in hepatic and intestinal microsomes was measured and intrinsic clearances were calculated. The

intestinal metabolism of verapamil showed biphasic kinetic characteristics: high-affinity and low-affinity components. The CL_{int} of both high- and low- affinity components in intestinal microsomes of diabetic rats were lower than those in control rats. It was noted that the total CL_{int} in diabetic rats was only 30% of control rats, which was in line with the increase (3.8-fold) of AUC of verapamil in portal vein of diabetic rats. It is known that metabolism of verapamil is mainly mediated by CYP3A, which indicate that diabetes may decrease CYP3A activity in intestine. The result was consistent with the report that diabetes significantly decreased intestinal testosterone 6β-hydroxylase (a CYP3A marker) activity (Borbás et al. 2006). Oppositely, higher intrinsic clearance of verapamil was noted in hepatic microsome of diabetic rats. In general, hepatic clearance of high hepatic extraction ratio drug is independent of intrinsic clearance and plasma protein binding. However, we tried to estimate hepatic extraction ratio of verapamil in rats using "parallel-tube" model $E = 1 - \exp(-f_u \cdot CL_{int}/Q_h)$. The hepatic blood flow (Q_h), content of microsome protein and liver weight were assumed to be 0.95 ml/min/g liver, 44.8 mg/g liver, 40g/ kg body weight (Iwatsubo et al., 1997). Verapamil belonged to high binding to plasma drug and its free fraction (f_u) was approximately 10% (McTavish et al., 1989). The value of $f_u \cdot CL_{int}/Q_h$ was calculated to be only 0.575 for control rats, that indicated that the contribution of CL_{int} to hepatic clearance of verapamil was not negligible. The estimated CL_{int} in liver of diabetic rats was 1.22-folds of that in liver of control rats, which was near to the ratio (1.32) of system clearance in diabetic rats to control rats. The results indicated that increase of microsomal enzyme activity may partly explain

the increase in system clearance under diabetic condition. Of course, other factors such as hepatic blood flow, protein binding, function of P-gp and clearance in kidney may partly be reasons inducing increase in system clearance under diabetic condition.

To confirm whether the metabolic activity of verapamil was associated with the protein expression of CYP3A, the hepatic and intestinal CYP3A protein expressions in both groups were determined. The result was positive. The protein expression of CYP3A was increased in liver and decreased in intestine of diabetic rats, which was in accordance with the results of metabolism of verapamil. The protein expression of CYP3A in liver was increased in diabetic rats, which supports the conclusion that the induction of liver CYP3A by diabetes contributed to the smaller AUC and faster clearance of verapamil after intravenous administration. The down-regulation of intestinal CYP3A in diabetic rats was confirmed to take part in the increased AUC of verapamil after oral administration.

Diabetic animal model induced by STZ has been widely used in pharmacokinetic study (Shimojo et al., 1993; Sato et al., 1991; Lee et al., 2010; Liu et al., 2010; Yu et al., 2010a; Ogata et al., 1996), but real mechanism altering pharmacokinetic of drug is unclear. Several studies demonstrated that the changes of pharmacokinetics in diabetic rats induced by STZ are mainly due to the indirect effect of STZ (Ioannides, 1996; Lee et al., 2010). The complication of any toxic effects of STZ was negligible 4-5 weeks after STZ injection (Watkins and Sherman, 1992). In diabetic rats induce by

STZ, insulin deficiency caused by destruction of pancreatic β-cells by STZ provokes various disorders of metabolism involving glucose, ketones and lipids. In addition, impaired secretion of other hormones, such as growth hormone, testosterone and tetraiodothyronine, has been demonstrated in these animals (Kenneth and John, 1989). Some hepatic cytochromes P450 isozymes of diabetic rats were reversed, or partly, to the level of the controls after insulin treatment (Yamazoe et al., 1989). It has been reported that insulin and ketone body participated in the regulation of hepatic CYP2E1 in diabetic rats (Woodcroft et al., 2002; Abdelmegeed et al., 2005). However, the mechanisms involved in the regulation of CYP3A in liver and intestine by diabetes mellitus were not reported, which need to be further investigated.

In conclusion, the higher metabolic activity of verapamil in hepatic microsome of diabetic rats was associated with higher protein levels of CYP3A in liver, whereas the lower metabolism activity of verapamil in intestinal microsome of diabetic rats was associated with lower protein levels of CYP3A in intestine. These results may contribute to opposite alteration of verapamil pharmacokinetics after oral and intravenous administration in diabetic rats. It was indicated that diabetes mellitus revealed a route-dependent effect on the pharmacokinetics of verapamil in diabetic rats, partly resulting from a tissue specific effect on CYP3A in liver and intestine under diabetic condition.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hu N, Liu XD, Liu L

Conducted experiments: Hu N, Xie SS, Wang XT, Liu HY, Liu X, Chen GM

Contributed new reagents and analytical tools: Liu XD, Xie L, Wang GJ

Data analysis: Hu N, Liu L, Pan X

Contributed to writing: Hu N, Liu XD, Liu L

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FOOTNOTE:

Legends for figures:

Figure 1 Semi-log plot of systemic plasma concentration of verapamil (A) and norverapamil (B) after *i.g.* 10 mg/kg of verapamil, systemic plasma concentration of verapamil (C) after *i.v.* 1 mg/kg of verapamil, and portal plasma concentration of verapamil (D) after *i.g.* 10 mg/kg of verapamil in control (open circle) and diabetic (closed circel) rats. *P<0.05, **P<0.01. Data represent the mean ± S.D. of five or six rats

Figure 2 Formation rate of norverapamil from verapamil in hepatic (A) and intestinal (B) microsomes of control (open circle) and diabetic (closed circle) rats. Insets represent the corresponding Eadie-Hofstee plots. Data represent the mean ± S.D. of four rats.

Figure 3 Protein levels of CYP3A1 and CYP3A2 in liver, duodenum and jejunum of the age-matched control and diabetic rats. Representative Western blot stains of CYP3A1 (A) and CYP3A2 (B), relative stain intensity for CYP3A1 (C) and CYP3A2 (D) levels are presented in comparison with those of control rats. Data represent the mean ± S.D. of four rats, * p<0.05 vs control rats

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Table 1 Physiological and biochemical characteristics in control and diabetic rats induced by STZ

Parameter	Control rats	Diabetic rats
Initial body weight (g)	174.87±3.80	184.26±3.58
Final body weight (g)	390.8±19.55	249.33±31.16 [***]
Blood glucose (mM)	6.82±0.52	28.68±2.88[***]
Wet liver weight (g)	10.53±0.81	11.68±1.47
Relative liver weight (mg liver/g body weight)	30.40±3.44	50.15±7.66[***]

Data represent the mean ± SD of ten to twelve rats. ***P<0.001 vs control rats

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Table 2 Pharmacokinetic parameters of verapamil and/or norverapamil after *i.g.*

(10mg/kg) and *i.v.* (1mg/kg) administration of verapamil to control and diabetic rats

Parameters	Control rats	Diabetic rats
Oral administration		
Verapamil		
C_{max} (ng/ml)	134.31±39.66	442.10±147.90[**]
T_{max} (min)	34.0±15.2	21. 7±4.1
$AUC_{0-480min}$ (µg min/ml)	22.02±6.24	38.31±8.49[**]
$AUC_{0-\infty}$ (µg min/ml)	27.09±7.91	41.56±7.85[**]
$T_{1/2}$ (min)	224.7±42.9	196.1±40.2
Norverapamil		
C_{max} (ng/ml)	68.14±20.22	112.33±15.91[**]
T_{max} (min)	36.0±13.4	25. 0±5.5
$AUC_{0-480min}$ (µg min/ml)	14.58±2.75	16.12±1.80
$AUC_{0-\infty}$ (µg min/ml)	19.48±2.33	20.17±2.52
$T_{1/2}$ (min)	232.9±46.6	224.7±42.9
AUC($_{Nor}$)/AUC($_{Ver}$)	0.69±0.13	0.43±0.09[**]
F (%)	15.16	29.88
Intravenous administration		
Verapamil		
$AUC_{0-300min}$ (µg min/ml)	15.37±1.82	11.91±2.76[*]
$AUC_{0-\infty}$ (µg min/ml)	17.87±1.53	13.91±3.07[*]

$T_{1/2}$ (min)	22.3±3.4	18.4±4.7
CL (ml/min/kg)	56.28±4.60	74.24±13.53[*]

AUC, area under concentration-time curve; C_{max}, peak plasma concentration; T_{max}, time to C_{max}; $T_{1/2}$, terminal half life; CL, plasma clearance; F, absolute bioavailability; Nor, norverapamil; Ver, verapamil. Data represent the mean ± S.D. of five or six rats.
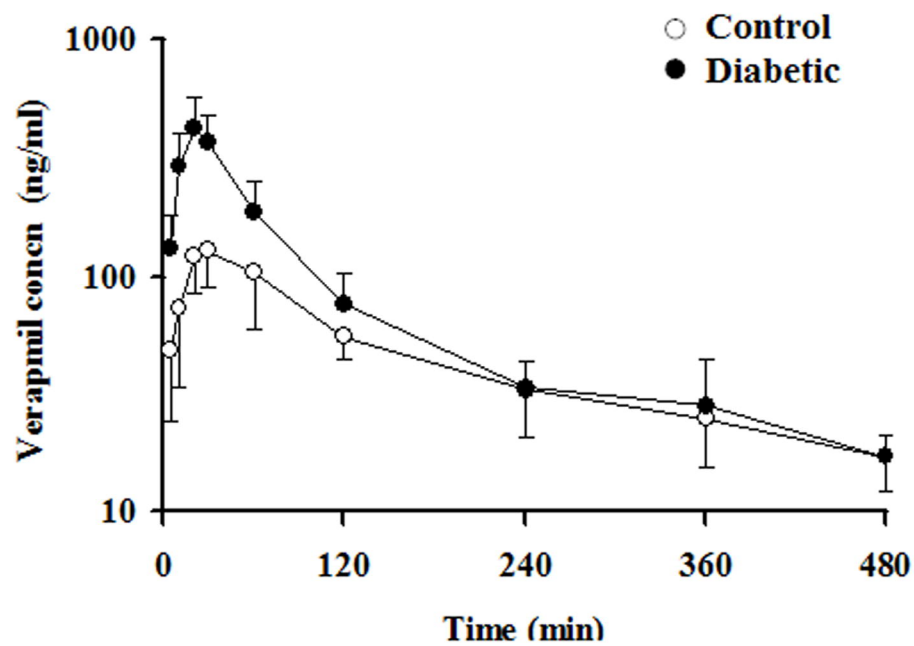
*P<0.05, **P<0.01 vs control rats

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Table 3 Kinetic parameters for norverapamil formation in hepatic or intestinal microsomes of control and diabetic rat (n=4)

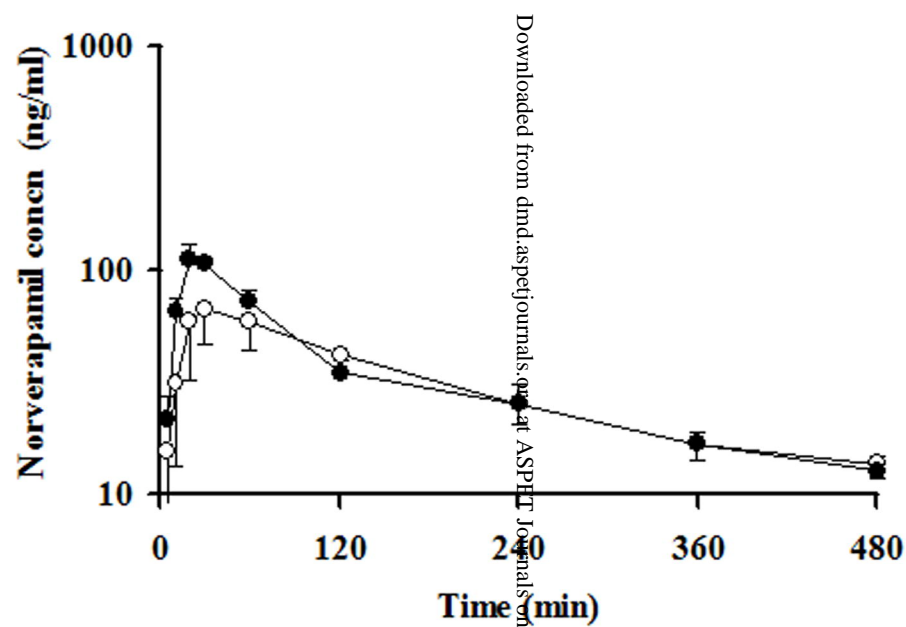| Parameters	Control rats	Diabetic rats
Hepatic microsome		
V_m (nmol/min/mg protein)	1.60±0.30	2.38±0.32[*]
K_m (μM)	13.21±1.97	16.09±2.32
CL_{int} (ml/min/mg protein)	0.122±0.016	0.149±0.015[*]
CL_{int} (ml/min/g liver)	1.110±0.273	1.261±0.206
Intestinal microsome		
V_{m1} (pmol/min/mg protein)	49.04±15.78	22.70±5.07[*]
K_{m1} (μM)	34.06±5.19	55.37±22.22
CL_{int1} (μl/min/mg protein)	1.42±0.28	0.43±0.08[**]
V_{m2} (pmol/min/mg protein)	122.41±32.04	139.23±10.75
K_{m2} (μM)	1041.37±88.47	1388.66±157.69[*]
CL_{int2} (μl/min/mg protein)	0.12±0.04	0.10±0.02
Total CL_{int} (μl/min/mg protein)	1.54±0.24	0.53±0.10[**]
Total CL_{int} (μl/min/g intestinal mucosa)	5.382±1.106	1.130±0.235[**]

K_m, Michaelis–Menten constant; V_{max}, maximum enzyme velocity; CL_{int}, intrinsic clearance; the subscript 1 and 2 denoted the high- and low- affinity components of the reaction, respectively. * p<0.05 vs control rats
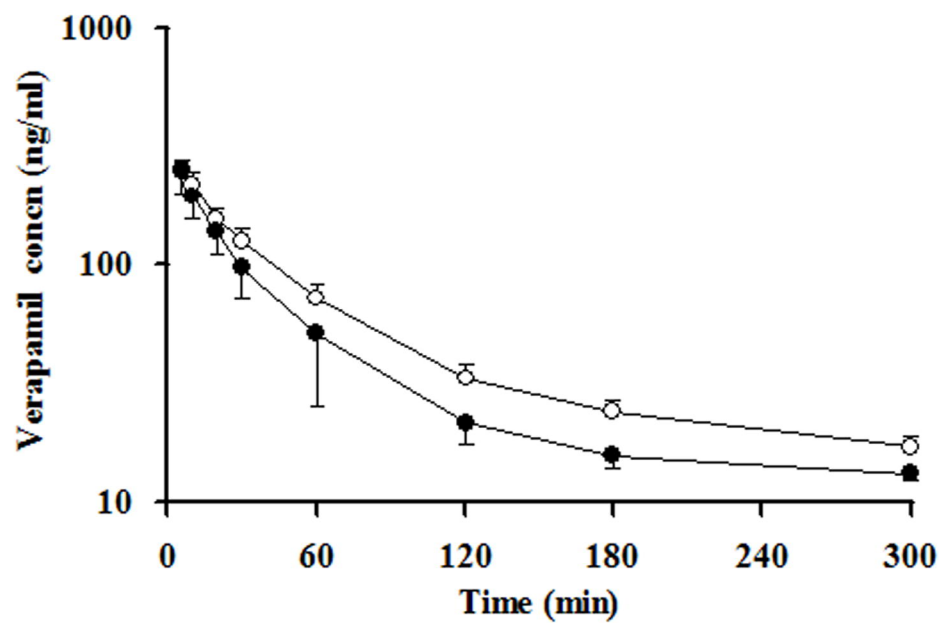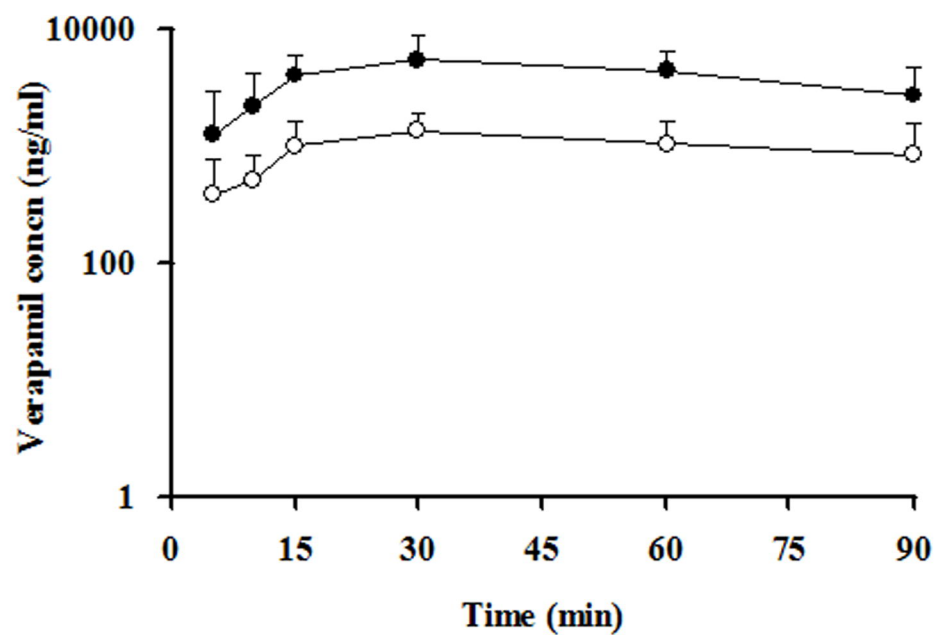
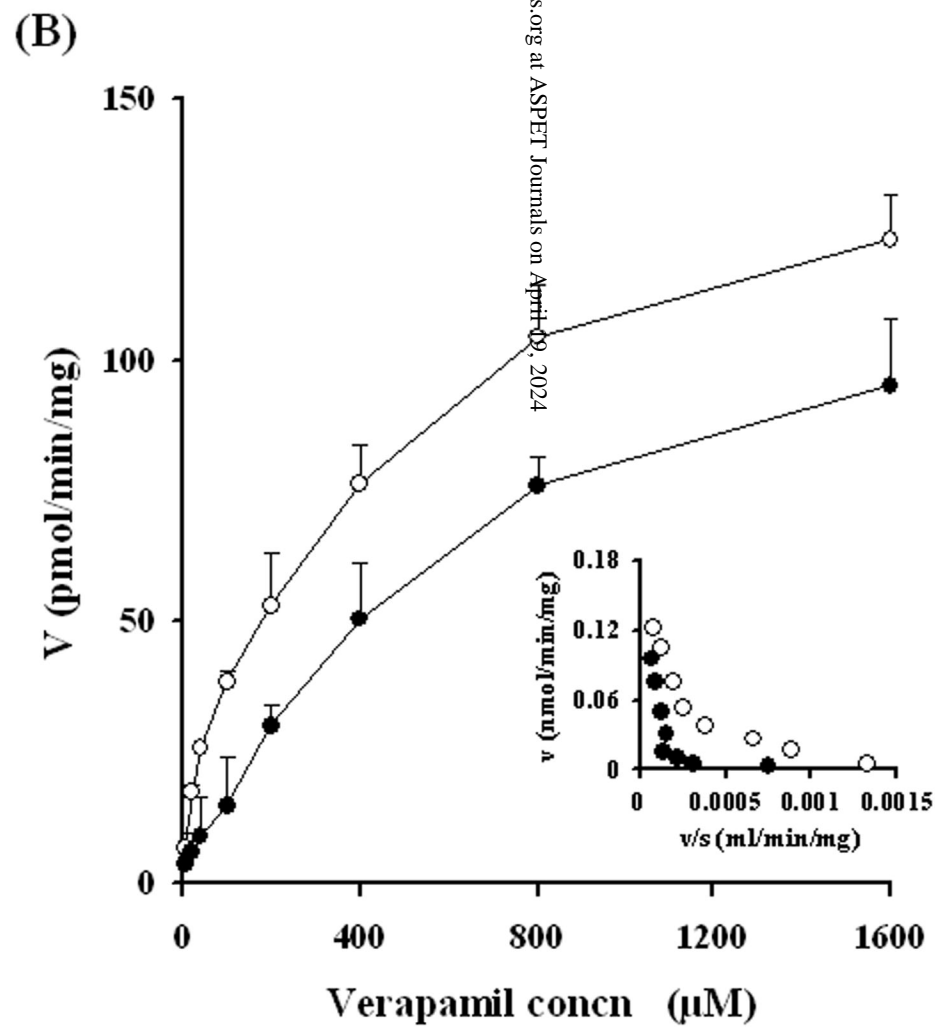# Figure 1

(A)

(B)

(C)

(D)

Figure 2

Figure 3