Combined Contributions of Impaired Hepatic CYP2C11 and Intestinal Bcrp Activities and Expressions to Increased Exposure of Oral Glibenclamide in Streptozotocin-induced Diabetic Rats

Haiyan Liu, Li Liu, Jia Li, Dan Mei, Ru Duan, Nan Hu, Haifang Guo, Zeyu Zhong, Xiaodong Liu

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, China
Running title: Diabetes mellitus changes the pharmacokinetics of glibenclamide

Corresponding Author:

Prof. Xiaodong Liu

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China

E-mail: xdlju@cpu.edu.cn (X.Liu)

Tel: +86-25-83271006

Fax: +86-25-83271060

Number of text pages: 38

Number of tables: 4

Number of figures: 5

Number of references: 41

Number of words in the Abstract: 248

Number of words in the Introduction: 689

Number of words in the Discussion: 1253

Abbreviations: AUC, the area under the concentration-time curve; Bcrp, Breast cancer resistance protein; Cmax, the peak concentration; CYP450s, Cytochrome P450s; GLB, glibenclamide; HPLC, High-performance liquid chromatography; K-H, Krebs-Henseleit; Mrps, multidrug resistance-associated proteins, NADP, β-nicotinamide adenine dinucleotide phosphate; NOV, novobiocin; Peff, apparent permeability coefficient; P-gp, P-glycoprotein; STZ, streptozotocin; SUL, sulfaphenazole; TBST, Tris-buffered saline and 0.05% Tween 20
Abstract

The purpose of the study was to evaluate the contribution of the impaired cytochrome P450s (CYP450s) and breast cancer resistance protein (Bcrp) activity and expression to drug pharmacokinetics under diabetic states. Diabetic rats were induced by intraperitoneal administration of streptozocin (STZ). Glibenclamide (GLB), a substrate of Bcrp, was served as a model drug. The pharmacokinetics of oral GLB (10 mg/kg) was studied. The results showed that diabetes mellitus significantly increased exposure (AUC and Cmax) of GLB following an oral administration. Data from hepatic microsomes suggested impairment of GLB metabolism in diabetic rats. GLB metabolism in hepatic microsomes was significantly inhibited by a selective inhibitor (sulfaphenazole, SUL) of CYP2C11 and CYP2C11 antibody. Western blot further showed the contribution of impaired CYP2C11 expression to the impairment of GLB metabolism. Data from excretion showed that approximately 72% of oral dose was excreted via feces of normal rats, indicating an important role of intestinal Bcrp. Diabetes significantly decreased recovery from feces, which was only 40% of oral dose. Results from in situ single-pass intestine perfusion revealed that diabetes significantly increased the apparent permeability coefficient (Peff) and decreased efflux of GLB via intestine, inferring impairment of intestinal Bcrp function, which may play a role in the increased exposure of oral GLB in diabetic rats. Insulin treatment partly or completely reversed the changes in diabetic rats. All results gave the conclusion that both the impaired hepatic CYP2C11 and intestinal Bcrp expression and activity induced by diabetes contributed to the increased exposure of oral GLB.
Introduction

Both clinical trials and animal experiments have clearly demonstrated that diabetes mellitus markedly alters the expression and activity of the CYP450s (Shimojo et al., 1993; Kataoka et al., 2005; Hu et al., 2011), which further affect pharmacokinetic (PK) behaviors of some drugs. Accumulative studies indicate that alterations in the expression and activity of CYP450s under diabetic states are dependent on types of diabetes and CYP450s isoforms. For example, a study showed that diabetes markedly up-regulated the expression of CYP1B1, 1A2, 2B1 and 2E1 proteins, conversely, decreased expression of CYP2C11 protein in hepatic microsomes of streptozotocin (STZ)-induced diabetic rats (Sindhu et al., 2006). Our previous studies displayed that diabetes increased expression and activity of CYP3A in liver microsomes of rats, resulting in higher clearance of intravenous verapamil in diabetic rats induced by STZ (Hu et al., 2011) or combination of high-fat diet and STZ (Chen et al., 2011). Oltipraz is mainly metabolized via CYP1A1/2, 2B1/2, 2C11, 2D1 and 3A1/2 in rats. The increased expressions and mRNA levels of CYP1A2, 2B1/2 and 3A1 in diabetic rats induced by alloxan or STZ may result in lower values of areas under the curve and higher clearance of oral oltipraz (Bae et al., 2006). Similarly, a previous report showed that greater AUC of intravenous sildenafil in diabetic rats was attributed to suppression of CYP2C11 expression (Ahn et al., 2011). Moreover, increased oral AUC value of metformin in diabetic rats may come from decreased in hepatic CYP2C11 expression (Choi et al., 2008).

In addition to CYP450s, some ATP-binding cassette (ABC) drug transporters including P-glycoprotein (Abcb1/P-gp), multidrug resistance-associated proteins (Abccs/Mrps) and breast cancer resistance protein (Abcg2/Bcrp), were also altered under diabetic states (Liu et al., 2006; Liu et al., 2007; Quezada et al., 2011). Several
studies have showed that high levels of Bcrp mRNA were predominantly observed in kidney, intestine, liver and cerebral cortex of male rats (Tanaka et al., 2005; Zhang et al., 2011). The high level of Bcrp mRNA in kidney suggests an important role of Bcrp in the urinary excretion of the Bcrp substrate (Mizuno et al., 2004). The intestinal Bcrp may limit the uptake of drug and excretion of numerous xenobiotics including topotecan and some dietary carcinogens via the lumen of gastrointestinal tract, which becomes an important reason leading to low bioavailability of oral drugs. Our previous study showed that the function and expression of Bcrp in cerebral cortex of STZ-induced diabetic rats were impaired and the levels of Bcrp mRNA and protein were decreased in the intestine and liver of diabetic rats (Liu et al., 2007; Zhang et al., 2011). These gave the clue that alterations in expression and activity of Bcrp under diabetes may change the pharmacokinetic behaviors of some drugs after oral administration.

Glibenclamide (GLB), a long-acting second generation sulfonylurea antidiabetic agent, is extensively metabolized in the liver (Feldman, 1985). Similarly to other sulphonylureas, CYP2C9 plays a major role in the GLB metabolism in humans (Yin et al., 2005). However, several reports have showed that human CYP3A4 is the major CYP450s involved in GLB metabolism (Naritomi et al., 2004; Zhou et al., 2010). What’s more, a study showed that more than one CYP450s isozymes including CYP3A4, 2C9, 2C8 and 2C19 are involved in the GLB metabolism (Zharikova et al., 2009). Additionally, GLB itself is also a substrate of Bcrp and which has been widely used for assaying Bcrp function (Gedeon et al., 2008a). All of these results suggested that both alterations in expression and activity of CYP450s and Bcrp induced by diabetes appeared to have an important effect on pharmacokinetic behaviors of GLB.

The present study was undertaken to investigate whether STZ-induced diabetic rats...
altered the pharmacokinetic behaviors of oral GLB and whether such alterations came from the changed activities of CYP450s and Bcrp. The activities of hepatic microsomes were measured by the depletion of GLB and the isoform of CYP450s involved in the GLB metabolism were identified by special inhibitor and antibody. Moreover, the expression of the corresponding isoform of CYP450s was measured using western blot analysis. The function of Bcrp to the absorption of GLB was evaluated by the experiments of excretion and in situ single-pass perfusion.

Materials and methods

Chemicals

Glibenclamide (GLB) and diclofenac sodium were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sulfaphenazole (SUL), tolbutamide, novobiocin (NOV), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP) and streptozocin (STZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-hydroxytolbutamide was purchased from Toronto Research Chemicals Inc (Ontario, Canada). Rabbit anti-rat CYP2C11 polyclonal antibody was purchased from Abcam plc (Cambridge, UK). HPLC grade acetonitrile was obtained from Merck (Damstadt, Germany). Protamine zinc insulin was from Wanbang Pharmaceutical Co.(Xuzhou, China). All of the other reagents were of analytical grade and were commercially available.

Animals
Male Sprague Dawley rats, 5-week old (weighing 170–200 g), were supplied by Slac Laboratory Animal Ltd (Shanghai, China). The rats were maintained in an air conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10% with 12-h light/dark cycle. They were fed a standard diet (laboratory rodent chow; Nanjing, China) and water ad libitum. The animals were acclimatized to the facilities for 5 days and fasted with free access to water for 12 h prior to experiment. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University, and every effort was made to minimize stress to the animals.

**Diabetic rats induced by streptozotocin**

The diabetic rats were induced by an intraperitoneal (i.p.) administration of 65 mg/kg of STZ (dissolved in sodium citrate buffer, pH 4.5) according to our method previously described (Liu et al., 2006). Age-matched normal rats were injected with the vehicle (sodium citrate buffer, pH 4.5). On day 7 post STZ injection, the fasting blood glucose levels were measured using commercially available glucose kit (Jian-cheng Biotech Co., Nanjing, China) based on glucose oxidase method. Rats with fasting blood glucose levels in excess of 11.1 mM were considered to be diabetic rats (Liu et al., 2006). The diabetic rats were randomly divided into two groups. Group 1 were served as diabetic control (DM) and only received vehicle. Group 2 were served as insulin treatment (DM+IN) and subcutaneously received protamine zinc insulin (5 U/kg, twice a day) for 4 weeks. The normal control rats (CON) also received vehicle. Fasting blood glucose, body weight and food intake were monitored weekly. All the experiments were carried out on the 35th day after the injection of STZ or vehicle.
Pharmacokinetics of oral administration of GLB in experimental rats

On day 35 after the injection of STZ or vehicle, the experimental rats, fasted overnight, orally received 10 mg/kg of GLB (suspended in 0.5% of carboxymethylcellulose sodium). Oral dosage of GLB was referenced by a previous report (León-Reyes et al., 2009). Blood samples (approximately 250 μL) were collected in heparinized eppendorf tubes via the oculi chorioideae vein under light ether anaesthesia at 0.5, 1, 2, 3, 4, 6, 8, 14 and 24 h after oral administration of GLB. After each 3-4 samplings, the appropriate amount of 0.9% saline was given to the experimental rats via tail vein to compensate blood loss. Plasma samples were immediately obtained by centrifugation at 4000 rpm for 10 min and stored at -20 °C until analysis.

The excretion of GLB into bile, urine and feces

The experimental rats, fasted overnight, orally received 10 mg/kg of GLB and were individually housed in metabolic cage. Urine and feces were collected before dosing and over 6-h intervals through 36 h post-dose. Feces were homogenized in water (1:10, w/v), and urine volumes were recorded. Aliquots of urine and feces samples were stored at -20 °C until analysis.

For biliary excretion, the experimental rats were anaesthetized by intraperitoneal (i.p.) injection of 1% pentobarbital sodium salt dissolved in 0.9% saline (0.5 mL) and a polyethylenetube (PE-19; Clay Adams, USA) was then inserted into the common bile duct. After confirming the bile flow, GLB (10 mg/kg) was orally given to the rats. Bile was collected into containers before dosing and intervals from 0-2, 2-4, 4-6, 6-8 and 8-12 h post-dose. The bile flow was measured and normalized by body weight. All the samples were stored at -20 °C until analysis.
Preparation of rat hepatic microsomes

Hepatic microsomes were prepared freshly from CON, DM and DM+IN rats according to methods previously described (Xie et al., 2010). Rats were sacrificed under light ether anaesthesia, and then livers were quickly harvested. The microsomal pellets were re-suspended in 0.01 M phosphate buffer containing 30% glycerol, and stored at -80 °C. The protein concentration was measured by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

GLB metabolism in rat hepatic microsomes

GLB metabolism was determined by measuring depletion of the drug. Each reaction solution (200 μL of total volume) was composed of the following components at their respective final concentrations: 4 μM GLB, which is approximately to its reported apparent K_m value (Zharikova et al., 2009); 0.5 mg/mL rat hepatic microsomal protein and 0.01 M phosphate buffer (pH 7.4). The GLB was pre-dissolved in methanol and the final volume of methanol in the reaction mixture was less than 1%. The incubation mixture was preincubated for 5 min at 37 °C, initiated by addition of an NADPH regenerating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase and 5 mM MgCl_2). Following designed time (0, 5, 15, 30, 45 and 60 min), the reaction was terminated by addition of 20 μL of 1 M HCl. All the incubations were performed in triplicate.

Tolbutamide metabolism in rat hepatic microsomes

4-hydroxylation of tolbutamide often was used to evaluate the activity of CYP2C9 in human. A report showed that the human CYP2C9 was equivalent to CYP2C
(including CYP2C6 and CYP2C11) in rats (Bogaards et al., 2000). 4-hydroxylation of tolbutamide in rats was mediated via CYP2C6 (Dostalek et al., 2005; Wang et al., 2007). Here the activity of CYP2C6 in rats was also assessed using formation of 4-hydroxytolbutamide according to methods previously reported (Komatsu et al., 2000). Briefly, hepatic microsomes (0.5 mg/mL) were incubated with tolbutamide (5-400 μM) and NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 1U/mL glucose 6-phosphate dehydrogenase and 5 mM MgCl₂) for 45 min at 37 °C in a final volume of 200 μL. The reaction was terminated by addition of 20 μL of 1 M HCl. All the incubations were performed in triplicate.

The effect of sulfaphenazole and CYP2C11 antibody on GLB metabolism in rat hepatic microsomes

To determine the inhibition effect of CYP2C11 on the GLB metabolism, sulfaphenazole (SUL) and CYP2C11 antibody were selected. SUL was considered to be a selective inhibitor of CYP2C11 in rats and used for evaluating the role of CYP2C11 in the GLB metabolism in rats. SUL (10 or 40 μM) was added to incubation mixture (200 μL) containing 0.5 mg/mL rat hepatic microsomes and 4 μM GLB and the process was prepared according to the method described above. After 30 min incubation, the reaction was terminated by addition of 20 μL of 1 M HCl. The effects of CYP2C11 antibody on the GLB metabolism in normal rat hepatic microsomes was documented to further verify the contribution of CYP2C11 to GLB metabolism. The rabbit anti-rat CYP2C11 polyclonal antibody was pre-incubated with hepatic microsomes for 20 min at room temperature followed by addition of GLB. The mixture was obtained and the process was prepared according to the method described above. Following 45 min incubation, the reaction was terminated by
addition of 20 μL of 1 M HCl. The remaining amount of GLB in reacted mixture was determined and the remaining percent was estimated.

**Western blot analysis in rat liver microsomes**

Western blot analysis was used for assessing levels of CYP2C11 protein in hepatic microsomes according to previous methods (Sindhu et al., 2006). Briefly, microsomal proteins (20 μg) of hepatic microsomes were subjected to SDS-polyacrylamide (10%) gel electrophoresis and the separated proteins blotted onto a pure nitrocellulose membrane. The membrane was incubated in fresh blocking buffer (TBST, pH 7.4, containing 5% nonfat dried milk) at room temperature for 3 h. After washing the membrane with TBST four times for 5 min each, it was then incubated with primary antibody (1:2000 dilution; polyclonal rabbit anti-rat CYP2C11) for 5 h at room temperature. The membranes were then washed and incubated with a peroxidase-conjugated goat anti-rabbit goat IgG (secondary antibody, 1:2000 dilution) for 1.5 h. Samples were normalized to β-actin. 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.

**In situ single-pass intestine perfusion of rats**

In situ single-pass perfusion was used for evaluating the absorption of GLB and efflux of GLB via small intestine according to previously described (Yu et al., 2010). Briefly, the CON, DM and DM+IN rats were anesthetized by intraperitoneal (i.p.) injection of
1% pentobarbital sodium salt dissolved in 0.9% saline (0.5 mL). The abdomen was opened through a middle incision and the jejunum was isolated between two cannulas by 10 cm, which were fixed by ligation. The manipulation was practiced carefully to minimize any intestinal blood supply disturbances. Then the intestine was returned to the abdominal cavity and abdomen closed.

For absorption experiment, GLB (10 µg/mL) was resolved in Krebs-Henseleit (K-H) as the temperature was set at 37 °C. Phenol red (Jiancheng Biotech Co., Nanjing, China), a concentration of 20 µg/mL, was employed as the impermeable volume marker for measuring water flux. In general, the isolated jejunum segment was pre-perfused with 0.9% saline at 0.2 mL/min (37 °C) for 20 min to reach a steady state for water and solute absorption, and then was followed by K-H buffer containing GLB (10 µg/mL). After getting to a steady-state (30 min), consecutive effluent (at 15 min intervals) was collected via the distal cannula. At the end of the experiments, the animals were sacrificed; perfused intestinal segments were removed and the areas of absorption were measured. The cumulative fraction of absorption was estimated. The apparent effective permeability \( P_{\text{eff}} \) (cm/min) was also calculated as following equation (Johnson et al., 2003).

\[
P_{\text{eff}} = \frac{-Q \ln(C_{\text{out}} / C_{\text{in}})}{A}
\]

where \( C_{\text{out}} \) and \( C_{\text{in}} \) indicate the outlet concentration and the inlet concentration of GLB which is corrected for volume change in the segment in outlet and inlet tubing. \( A \) (cm\(^2\)) is the area of the perfused intestinal segment and \( Q \) is the flow rate (0.2 mL/min).

For the function of Bcrp to the absorption of GLB, novobiocin (NOV), a specific inhibitor of Bcrp, was selected. The apparent effective permeability \( P_{\text{eff}} \) of GLB was further measured in the presence of Bcrp inhibitor NOV (50 and 200 µM) via intestinal of normal rats. The following progress of in situ single-pass perfusion was
done according to the absorption experiment described above.

For efflux experiment, the isolated intestine segments were pre-perfused with 0.9% saline at 0.2 mL/min (37 °C) for 20 min to reach a steady state for water and solute absorption, followed by K-H buffer free-drug. After intravenous dose of GLB (1 mg/kg), consecutive effluent (at 15 min intervals) was collected via the distal cannula. The cumulative efflux of GLB via the intestine segment was measured.

**Drug assays**

The concentrations of GLB and 4-hydroxytolbutamide were measured by HPLC method. The HPLC system (Shimadzu, Tokyo, Japan) was equipped with an LC-10A pump, a SIL-10ADvp auto injector and a CTO-10ASvp column oven. Separation was performed at a flow rate of 1.0 mL/min on a Waters Symmetry C_{18} column (5.0 μm, 150×4.6 mm i.d., 5 μm).

Levels of GLB in the biological samples were determined by an HPLC method described previously (Al-Dhawailie et al., 1995; Gedeon et al., 2008b) with a minor modification. Every biological sample (100 μL plasma or feces, 200 μL urine or bile samples, 200 μL microsomal incubation, 200 μL intestine perfusate or efflux fluid) was spiked with 20 μL of 1 M HCl and diclofenac sodium (final concentration was 10 μg/mL) was used as internal standard. After vortexing (30 s), 1 mL of ethyl acetate was added to the mixture shaking for 10 min, then centrifuged at 4000 rpm for 10 min. Organic phase was transferred into clean tubes and dried under reduced pressure using a integrated SpeedVac SPD2010-230 (ThermoFisher, USA), reconstituted with 100 μL of mobile phase and 20 μL was injected into the HPLC system. The mobile phase consisted of acetonitrile and 20 mM ammonium acetate buffer pH 4.5 (45:55, v/v). The concentration of GLB was measured with a fluorescence detector (RF-10AXL).
set at an excitation wavelength of 308 nm and an emission wavelength of 360 nm. The recoveries were higher than 70%. The linear range of GLB in plasma and feces was 0.039-2.5 μg/ml. The linear range of GLB in bile, urine, incubation mixture and intestine perfusate was 0.0195-2.5 μg/ml, respectively. The intra-day and inter-day assay coefficients of variation of the assay were less than 10%.

Measurement of 4-hydroxytolbutamide in incubation mixture was carried out according to methods previously reported (Palamanda et al., 2000) with a slight modification. Briefly, 20 μL of phenacetin (internal standard, 25 μg/mL) and 1 mL of ethyl acetate were added to 200 μL microsomal incubation mixture and centrifuged at 4000 rpm for 10 min. After evaporated to dryness the residues were resuspended in 100 μL of mobile phase and 20 μL was injected into the HPLC system. The mobile phase consisted of acetonitrile and 20 mM ammonium acetate buffer pH 4.5 (22:78, v/v). The analysis was quantified by a SPD-10Avp ultraviolet detector set at the wavelength of 230 nm. The lowest detection limit of 4-hydroxytolbutamide was 0.078 μg/mL. The recovery was higher than 75%. Both the intra-day and inter-day assay coefficients of variation were less than 10%.

**Pharmacokinetic analysis**

The concentration-time data for GLB in each rat were individually analyzed using non-compartmental analysis operated on Phenix WinNonlin Version 6.1 (Pharsight CO., Ltd., USA). The area under the plasma concentration versus time profile (AUC₀-∞) was calculated by trapezoidal rule with extrapolation to infinity. The peak concentration (Cₘₐₓ) and the time to reach Cₘₐₓ (Tₘₐₓ) were obtained directly from the plasma concentration-time profiles. The terminal elimination constant (K) was obtained from the least-square linear regression slope of ln-concentration versus time.
and terminal elimination half-life (T1/2) was calculated as 0.693/K. The Mean residence time (MRT) was calculated as the area under the first moment curve divided by AUC0-∞. The oral clearance (Cl/F) and volume distribution (V/F) were defined as Dose/AUC0-∞ and Dose/AUC0-∞/K, respectively, where F represents absolute bioavailability.

In hepatic microsome incubation, the area under GLB concentration versus time curve (AUC0-t) was measured by linear trapezoidal rule. The clearance (Clapp,h) of GLB in hepatic microsomes was estimated as initial dose divided by AUC0-t. The terminal elimination constant in hepatic micorsomes (kh) was obtained from least-square linear regression slope of ln-concentrations versus time and terminal elimination half-life (T1/2,h) was calculated as 0.693/kh.

Michaelis-Menten equation was used for charactering kinetics of 4-hydroxytolbutamide formation from tolbutamide in rat hepatic microsomes. Michaelis-Menten constant (Km) and maximum rate of 4-hydroxytolbutamide formation (Vmax) were estimated by non-linear least-square regression using programming solver (Microsoft Excel, 2003). The intrinsic clearance for the formation of 4-hydroxytolbutamide (Clint) was calculated as Vmax/Km.

**Statistical analysis**

Data were represented as mean ± standard deviation (S.D). Statistical differences between groups were evaluated by one-way of analysis of variance. If analysis was significant, the differences between groups were estimated using Student Newman-Keuls multiple comparison test for post hot analysis. A p value of less than 0.05 was considered to be statistically significant.
Results

Physiological and biochemical parameters of experiment rats

The STZ-injected rats exhibited symptoms of diabetes mellitus such as polyphagia, polyuria, polydipsia and low body weight during the 5-week study. Body weight, fasting blood glucose level and liver weight were measured on day 35 following injection of STZ (Table 1). Significantly higher fasting glucose level and relative liver weight and lower body weight were observed in DM rats. Insulin treatment might partly ameliorate the symptoms induced by diabetes, but did not affect the increased liver weight induced by diabetes.

Pharmacokinetics of GLB in experimental rats after oral administration

The plasma concentrations of GLB in CON, DM and DM+IN rats after oral administration of GLB (10 mg/kg) were measured (Figure 1A) and the corresponding pharmacokinetic parameters were estimated (Table 2). Compared with CON rats, DM rats had markedly higher plasma concentrations of GLB, accompanied by higher exposure (AUC_{0-24} and C_{max}) and lower oral clearance. The AUC_{0-24} and C_{max} values of DM rats were 564.7% and 357.0% of CON rats, respectively. The oral clearance (Cl/F) value of DM rats was only 18% of CON rats. The rate of absorption of GLB in DM rats was slower accompanied with longer T_{max}. Insulin therapy may almost abolish the alteration of pharmacokinetic profile of oral GLB induced by diabetes.

After oral administration, the recoveries of GLB from 36 h urine were 0.004-0.023% of dose (Table 2). Similarly, the excreted amount of GLB in 12 h bile was less than 0.1% (Table 2), though DM rats showed higher recoveries in bile than that of CON rats. In addition, DM rats had higher bile flow compared with CON rats (Figure 1B). However, high recovery of GLB from 36 h feces of normal rats was found (Table 2),
which was about 72% of the oral dose GLB. Diabetes may significantly decrease recovery of GLB from 36 h feces after oral dose, which was only 40% of oral dose, indicating enhancement of GLB absorption. Insulin treatment reversed the increased bile flow induced by diabetes mellitus. These results suggested that the enhanced absorption of GLB via intestine was one of the reasons that contributed to the increased exposure of oral GLB in DM rats.

**GLB metabolism in rat hepatic microsomes**

GLB is considered to be mainly eliminated in liver (Feldman, 1985). To further investigate whether the increased exposure of oral GLB in vivo was from impairment of rat hepatic microsomes activity, the characteristics of GLB metabolism in hepatic microsomes of rats were measured using the depletion of GLB (Figure 2A, Table 3). Significant increases in both $C_{60\text{min}}$ and $AUC_{0-60\text{min}}$ of GLB in hepatic microsomes mixture of DM rats were observed, which induced 7.4-fold and 2.9-fold of CON rats, respectively. It was also found that intrinsic clearance ($Cl_{\text{app-h}}$) of GLB in hepatic microsomes of DM rats was significantly decreased, which was only 25% of hepatic microsomes of CON rats. The $T_{1/2-h}$ was prolonged from 12.4 min in CON rats to 31.5 min in DM rats. All these results indicated that the GLB metabolism in hepatic microsomes of DM rats was impaired and that the suppression of GLB metabolism observed in vitro hepatic microsomes was directly related to the increased in the exposure of oral GLB in diabetic rats.

**Metabolism of tolbutamide in rat hepatic microsomes**

The 4-hydroxylation of tolbutamide in rat hepatic microsomes was used for assaying activity of CYP2C6. In contrast to our expectation, increased formation rate of
4-hydroxytolbutamide was found in hepatic microsome of DM rats (Figure 2B, Table 4). The estimated values of $V_{\text{max}}$ and intrinsic clearance of tolbutamide in hepatic microsomes of DM rats were 2.0-fold and 2.2-fold of CON rats, respectively, suggesting that the activity of CYP2C6 was induced rather than suppressed in DM rats. Insulin treatment partly reversed the increase of CYP2C6 activity induced by diabetes.

**Effect of sulfaphenazole and CYP2C11 antibody on GLB metabolism in rat hepatic microsomes**

Since the structure of human two enzymes, CYP2C9 and CYP2C8, are closely related to rat CYP2C11 and CYP2C13 (Wang et al., 2009), CYP2C11 was taken into consideration to explore the mechanism of decreased GLB metabolism. To determine roles of CYP2C11 in GLB metabolism, depletion of GLB in rat hepatic microsomes was investigated in the presence of chemical inhibitor (SUL) and CYP2C11 antibody. The amount of GLB in the incubation mixture before reaction was set as 100%, the remaining fractions of GLB in incubation mixture following 30 min reaction was calculated. The results showed that SUL concentration-dependently inhibited GLB metabolism (Figure 3A) in hepatic microsomes of CON, DM and DM+IN rats. In comparison with the absence of SUL, addition of 40 $\mu$M of SUL could significantly increase the remaining fractions of GLB in hepatic microsomes of CON, DM and DM+IN rats which from 8%, 38% and 18% to 65%, 91% and 66%, respectively. The inhibitory effect of SUL in CON rats was much stronger than that in DM rats, which was 812.5% and 239.5% respectively compared with groups without SUL. Addition of 10 $\mu$M of SUL also significantly increased remaining fractions of GLB in hepatic microsomes of the rats. These results indicated that CYP2C11 contributed...
approximately 50%-60% to the total GLB depletion and GLB metabolism could be inhibited by CYP2C11 inhibitor SUL. The contribution of CYP2C11 to the depletion of GLB was further confirmed by immunoinhibition in hepatic microsomes of normal rats (Figure 3B). The results showed that antibody against CYP2C11 inhibited GLB metabolism in a concentration-dependent manner. Remaining percent of GLB in presence of antibody (60 μL/mg microsomal protein) against CYP2C11 was 70%, which was significantly higher than that (15%) in the absence of CYP2C11 antibody. Antibody addition (20 μL/mg microsomal protein) also caused a significant increase in remaining percent of GLB. The results from CYP2C11 antibody addition were in line with the findings using SUL. The results verified roles of CYP2C11 in the GLB metabolism in rats, which indicated that decrease in GLB metabolism in DM rats was partly due to impairment of CYP2C11 activity under diabetic rats.

**Western blot**

Levels of CYP2C11 protein in hepatic microsomes of CON, DM and DM+IN rats were performed by western blot (Figure 4). The level of CYP2C11 in hepatic microsomes of DM rats was significantly ($p = 0.0004, p < 0.001$) lower than that in CON rats, which was only 20% of CON rats, inferring impairment of CYP2C11 expression. The impaired expression of CYP2C11 in hepatic microsomes of DM rats was partially reversed by insulin therapy. The decrease in level of CYP2C11 in hepatic microsomes of DM rats was in parallel with impairment of GLB metabolism, indicating that the suppression of CYP2C11 expression in hepatic microsomes induced by diabetes contributed to the decrease in GLB metabolism.
The intestine absorption and efflux of GLB in the experimental rats

High recovery of GLB was found in feces of CON rats after an oral dose of GLB (10 mg/kg), but diabetes significantly decreased recovery of GLB in feces, inferring enhancement of GLB absorption. The intestinal apparent effective permeability (P_{eff}) (Figure 5A) and accumulative amount of absorbed GLB (Figure 5B) were further measured using an in situ single-pass jejunum perfusion. The results clearly demonstrated that the amount of accumulative absorption of GLB in DM rats was higher than that in CON rats, and significant increases were found in both 90 min and 120 min perfusion (35% increase in 90 min perfusion and 42% increase in 120 min perfusion, respectively). Significantly increased P_{eff} values of GLB were found in intestine of DM rats at 30, 45, 75 and 90 min of perfusion. The increase in P_{eff} values of GLB was in parallel with the decrease in recovery of GLB in feces of DM rats. Insulin treatment may partly attenuate these increases induced by diabetes.

To investigate whether the increased absorption of GLB was associated with decreased in efflux of GLB across intestine, GLB efflux via intestine (Figure 5C) was measured following intravenous dose (1mg/kg, dissolved in saline and 0.2% DMSO) using an in situ single-pass jejunum perfusion. The amount of accumulative efflux of GLB during 60 min in the experiment rats was only 0.02-0.03% of intravenous dose. Significantly decreased efflux of GLB during 15-30 min interval was found (Figure 5C) in intestine of DM rats. All these results demonstrated the contribution of decrease in efflux and increase in absorption via intestine to higher exposure of GLB after oral dose.

A selective inhibitor NOV (Su et al., 2007) was selected to assess the role of Bcrp in GLB intestinal absorption, and jejunum was perfused in the presence or absence of NOV (50 and 200 μM) in normal rats. It was found that NOV increased the intestinal
apparent effective permeability ($P_{\text{eff}}$) (Figure 5D) in a concentration-dependent manner. The addition of 200 $\mu$M NOV led to a 25% increase at 45 min of perfusion and 32% increase at 90 min of perfusion, respectively. The data demonstrated that inhibition of intestinal Bcrp by NOV significantly promoted the absorption of GLB.

**Discussion**

The present study was conducted to study the altered pharmacokinetics of the model drug GLB under diabetic states, and to investigate whether it was from impaired activity and expression of CYP450s together with Bcrp. The main finding was that diabetes significantly increased the exposure (AUC and $C_{\text{max}}$) of GLB after oral dose. GLB is extensively metabolized in the liver and therefore GLB metabolism in hepatic microsomes of experimental rats was analyzed. The result showed that in vitro clearance of GLB in hepatic microsomes of DM rats was significantly decreased, which was in parallel with the in vivo increased exposure of GLB. The conclusion demonstrated that the increased oral exposure of GLB under diabetic states was partly due to the impairment of GLB metabolism. Insulin treatment partly reversed the alterations in the pharmacokinetic parameters induced by diabetes.

In human, data from GLB metabolism showed that several hepatic CYP450s were involved in GLB metabolism, including CYP2C9 (Yin et al., 2005), CYP3A4, 2C8 and 2C19 (Naritomi et al., 2004; Zharikova et al., 2009). Zhou et al. (2010) reported that CYP3A4 was the major enzyme for in vitro metabolism of GLB in humans, but our previous reports demonstrated that the activity of hepatic CYP3A (Chen et al., 2011; Hu et al., 2011) in diabetic rats was induced rather than suppressed, which excluded the contribution of CYP3A isoforms to the decreased clearance of GLB under diabetes. Therefore, we focused on roles of CYP2C9. A report showed that the
human CYP2C9 was equivalent to CYP2C (including CYP2C6 and CYP2C11) in rats (Bogaards et al., 2000). Accumulating studies have showed that 4-hydroxylation of tolbutamide in rats is mediated via CYP2C6 and was also used for assaying activity of CYP2C6 (Dostalek et al., 2005; Wang et al., 2007). Hence, 4-hydroxylation of tolbutamide was used to reflect the activity of CYP2C6 in hepatic microsomes of rats. Contrary to our expectation, the amount of 4-hydroxytolbutamide formed in hepatic microsomes of DM rats was increased rather than decreased, which indicated that activity of CYP2C6 was induced by diabetes. The increased formation rate of 4-hydroxytolbutamide was consistent with the increase in expression of CYP2C6 protein in STZ-induced diabetic rats (Shimojo et al., 1993) whereas this result did not explain the decreased GLB metabolism in hepatic microsomes of diabetic rats. 

CYP2C11 in rats showed very similar 3-dimensional structures of human CYP2C9 (Wang et al., 2009). A report showed that the protein homology between human CYP2C9 and rat CYP2C11 was 77% (Lewis, 1996). In rats, CYP2C11 was considered to be the major CYP450, and the level of CYP2C11 accounted for 54% of total CYP contents in rat. Several studies revealed the impaired CYP2C11 expression in liver of diabetic rats (Shimojo et al., 1993; Iber et al., 2001; Sindhu et al., 2006). All of these results implied that CYP2C11 may be involved in the GLB metabolism in rats. In order to verify this hypothesis, inhibitory effects of CYP2C11 antibody and SUL, known to be a specific inhibitor of CYP2C11, on GLB metabolism in hepatic microsomes were documented. The results manifested that SUL (10 and 40 μM) markedly inhibited the GLB metabolism (Figure 3A) in hepatic microsomes of the rats. A report showed that sulfaphenazole (IC₅₀ ≥ 100 μM) did not affect the tolbutamide 4-hydroxylation (Eagling et al., 1998), inferring that different CYP2C isoforms catalyzed tolbutamide and GLB. Results from using polyclonal anti-CYP2C11
antibody further verified the contribution of CYP2C11 to the GLB metabolism in rats. The contribution of the CYP2C11 to the total GLB metabolism was approximately 50%-60%. Data from western blot clearly stated that diabetes significantly down-regulated expression of CYP2C11 protein in hepatic microsomes. Thus, these results revealed that impairment of CYP2C11 expression and activity induced by diabetes could partly contribute to the increased exposure of GLB after oral administration. The mechanism in suppression of CYP2C11 expression under diabetic states was not fully understood. Some reports showed that glucagon may down-regulate CYP2C11 and its expression was more sensitive to suppression by glucagon at low level insulin than at high level (Iber et al., 2001), which indicated that low level of insulin in DM rats may be a reason of suppressed CYP2C11 expression. This deduction was partly supported by the present finding that insulin treatment reversed the suppression. In addition, diabetes was often associated with high levels of cytokines and cytokine-like inflammation markers such as TNF-α and interleukin-6, and these cytokines were reported to suppress CYP expression in primary hepatocyte culture (Iber et al., 2000), referring the role of higher level of cytokines in suppression of CYP2C11. Other metabolic enzyme(s) or CYP450 isoforms may also have effect on the metabolism of GLB and further studies are required.

Experiments on GLB excretion showed that only 0.004-0.02% amount of oral GLB was detected in 36-h urine and less than 1% in 12-h bile, which suggested a slight role of bile and urine excreted to exposure of GLB and this was consistent with a previous report (Naraharisetti et al., 2007). However, the excreted total amounts of GLB in 36-h feces was up to 72% of the oral dose in CON rats, which was consistent with the previous study done by using 14C-GLB (Christ et al., 1969). The significantly decreased amount of GLB in feces of DM rats was observed, which was only 55.6%
of CON rats, indicating that diabetes enhanced absorption of GLB. Data from in situ single pass perfusion of jejunum clearly demonstrated that the diabetes significantly increased apparent effective permeability ($P_{\text{eff}}$) and decreased efflux of GLB via intestine, showing contribution to enhancement of GLB absorption.

GLB itself is a substrate of Bcrp and often used for assaying function of Bcrp (Gedeon et al., 2008a). Bcrp was highly expressed in intestine of rats (Tanaka et al., 2005), which become a reason leading to high recovery of GLB in feces after oral dose. Our previous study showed that diabetes may down-regulate protein levels and mRNA levels of intestinal Bcrp (Zhang et al., 2011), the levels of Bcrp mRNA and protein in intestine of diabetic rats were only 75% (significantly decreased) and 34% of CON rats, respectively, and this was in line with enhanced absorption of GLB under diabetes. This indicated that impairment of intestinal Bcrp expression may be another reason leading to increased exposure of GLB after oral dose. The presence of Bcrp inhibitor (50 and 200 μM NOV) significantly increased the apparent effective permeability ($P_{\text{eff}}$) which showed that GLB had an important effect on the absorption of GLB. GLB was reported to be a substrate of P-gp, but there was also a report showed that GLB could possibly be transported by Bcrp and Mrp3, but not by P-gp and Mrp2 (Gedeon et al., 2006). Moreover, our previous study (Yu et al., 2010) showed that diabetes also impaired P-gp function and expression in intestine. Bcrp exhibits partially an overlap in substrate specificity with P-gp, which indicated that the impairment of intestinal P-gp may partly contribute to higher exposure of oral GLB under diabetic states. Other drug transporters such as Mrps and organic anion transporters may affect pharmacokinetics of GLB under diabetic states.

In summary, the present study demonstrated that the increased exposure of GLB after oral dose under diabetic states partly resulted from a combination of the suppression
of both hepatic CYP2C11 and intestinal Bcrp activity and expression. Insulin treatment can partly or completely reverse alteration in the pharmacokinetics of oral GLB induced by diabetes. Further studies are required to identify the reason behind the down-regulation of CYP2C11, also the physiological meaning of the combined effect of CYP450s and efflux transporters.
Authorship contributions

Participated in research design: X. Liu, H. Liu

Conducted experiments: H. Liu, Li, Mei, Duan, Hu, Guo, Zhong

Data analysis: H. Liu, L. Liu, Mei, Hu

Contributed to writing: H. Liu, X. Liu, L. Liu
DMD #43513

References


León-Reyes MR, Castañeda-Hernández G and Ortiz MI (2009) Pharmacokinetic of


Zhou L, Naraharisetti SB, Liu L, Wang H, Lin YS, N Isoheranen, JD Unadkat,
Footnotes:

The project was supported by the National Science Foundation of People’s Republic of China [Grants 81072693, 81102503, 30873123 ].

H. Liu and L. Liu contributed equally to this work.
Legends for figures:

Figure 1. Plasma concentration of GLB (A, n=6-7 rats) and bile flow (B, n=4-5 rats) after oral administration (10 mg/kg) in CON (○), DM (▲) and DM+IN (●) rats. Data represent the mean ± S.D., * p <0.05, ** p <0.01, versus CON rats; # p <0.05, ## p <0.01, versus DM rats.

Figure 2. The metabolism of GLB and tolbutamide in vitro hepatic microsomes of CON (○), DM (▲) and DM+IN (●) rats. A. In vitro the depletion of GLB (4 μM) with the incubation time of 0, 5, 15, 30, 45 and 60 min; B. Formation rate of 4-hydroxytolbutamide in hepatic microsomes. Data represent the mean ± S.D. of 4 rats, * p <0.05, ** p <0.01, versus CON rats; # p <0.05, ## p <0.01, versus DM rats.

Figure 3. A. The effect of sulfafenazole (SUL) on the depletion of GLB in hepatic microsomes of CON, DM and DM+IN rats. Data represent the mean ± S.D. of 4-5 rats, * p <0.05, ** p <0.01, versus without inhibitor; ## p <0.01 versus CON rats. B. Effect of antibody of CYP2C11 on the depletion of GLB in hepatic microsomes of normal rats. The amount of GLB in the incubation time of 0 min (the absence of NADPH) was set 100%. Depletion of GLB was measured in the absence or presence of SUL or antibody of CYP2C11. Data represent the mean ± S.D. of three experiments, * p <0.05, ** p <0.01, versus without antibody.

Figure 4. A. CYP2C11 protein expression in hepatic microsomes of CON, DM and
DM+IN rats. Representative western blot stains of CYP2C11 (20 μg of loading per lane). B. Ratios of relative staining intensity for CYP2C11 levels are presented in comparison with those of control rats. Data represent the mean ± S.D. of 4 rats, **p <0.01, ***p <0.001, versus CON rats.

**Figure 5.** In situ single-pass of jejunum perfusion in experimental rats. A. The corresponding apparent effective permeability ($P_{eff}$); B. Accumulative absorption of GLB. The perfusion medium (K-H buffer) contained the GLB (10 μg/mL). C. Accumulative efflux of GLB via jejunum over 60 min collection at a 15min-interval and the intravenous dose of GLB was 1 mg/kg. Data represent the mean ± S.D. of 5 rats, *p <0.05, **p <0.01, versus CON rats; # p <0.05, ## p <0.01, versus DM rats. D. The corresponding apparent effective permeability ($P_{eff}$) of GLB in the absence or presence of novobiocin (NOV) in normal rats. Data represent the mean ± S.D. of 5 rats, *p <0.05, versus the absence of NOV.
Table 1. Physiological and biochemical characteristics in CON, DM and DM+IN rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DM</th>
<th>DM+IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>173.67 ± 6.53</td>
<td>180.4 ± 6.24</td>
<td>179.5 ± 2.59</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>333.83 ± 22.17</td>
<td>213.83 ± 12.75***</td>
<td>270.83 ± 20.44**</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>5.37 ± 0.24</td>
<td>26.99 ± 2.17***</td>
<td>5.40 ± 1.77###</td>
</tr>
<tr>
<td>Wet liver weight (g)</td>
<td>10.53 ± 1.65</td>
<td>8.67 ± 1.00*</td>
<td>11.25 ± 1.49##</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td></td>
<td>39.83 ± 6.11**</td>
<td>41.54 ± 3.01**</td>
</tr>
<tr>
<td>(mg liver/g body weight)</td>
<td>31.45 ± 3.58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. of 6-7 rats. * p <0.05, ** p <0.01, *** p <0.001 versus CON rats; ## p <0.01, ### p <0.001 versus DM rats.
Table 2. Pharmacokinetic parameters of GLB after oral administration (10 mg/kg) to CON, DM and DM+IN rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DM</th>
<th>DM+IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AUC_{0-\infty}(\mu g \text{ h/mL}))</td>
<td>2.61 ± 0.44</td>
<td>15.23 ± 4.79**</td>
<td>3.00 ± 1.41***</td>
</tr>
<tr>
<td>(AUC_{0-t}(\mu g \text{ h/mL}))</td>
<td>2.35 ± 0.29</td>
<td>13.27 ± 3.94**</td>
<td>2.38 ± 0.96**</td>
</tr>
<tr>
<td>(T_{1/2}) (h)</td>
<td>4.55 ± 1.23</td>
<td>5.11 ± 1.08</td>
<td>8.18 ± 6.43</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>7.23 ± 1.70</td>
<td>9.28 ± 1.57</td>
<td>8.15 ± 2.30</td>
</tr>
<tr>
<td>Cl/F (L/h/kg)</td>
<td>3.93 ± 0.68</td>
<td>0.72 ± 0.26**</td>
<td>3.98 ± 1.76**</td>
</tr>
<tr>
<td>Vd/F (L/kg)</td>
<td>25.72±8.84</td>
<td>6.36±0.63**</td>
<td>36.8±17.10**</td>
</tr>
<tr>
<td>(C_{max}) (\mu g/mL)</td>
<td>0.35±0.11</td>
<td>1.25±0.24**</td>
<td>0.25±0.06**</td>
</tr>
<tr>
<td>(T_{max}) (h)</td>
<td>1.50±1.00</td>
<td>7.2±1.79**</td>
<td>1.0±0.61**</td>
</tr>
<tr>
<td>Bile excretion (%)</td>
<td>0.01±0.007</td>
<td>0.097±0.130</td>
<td>0.018±0.017</td>
</tr>
<tr>
<td>Urine excretion (%)</td>
<td>0.0042±0.001</td>
<td>0.023±0.009**</td>
<td>0.019±0.005**</td>
</tr>
<tr>
<td>Feces excretion (%)</td>
<td>72±10</td>
<td>40±9**</td>
<td>50±21**</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. of 6-7 rats, * \(p < 0.05\), ** \(p < 0.01\) versus CON rats; # \(p < 0.05\), ## \(p < 0.01\), versus DM rats.
Table 3. Pharmacokinetic parameters of GLB depletion in CON, DM and DM+IN rats hepatic microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DM</th>
<th>DM+IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0$ ($\mu$g/mL)</td>
<td>2.06 ± 0.12</td>
<td>2.00 ± 0.13</td>
<td>2.06± 0.27</td>
</tr>
<tr>
<td>$C_{60\text{min}}$ ($\mu$g/mL)</td>
<td>0.07 ± 0.02</td>
<td>0.52 ± 0.12**</td>
<td>0.09 ± 0.02##</td>
</tr>
<tr>
<td>AUC$_{0-60\text{min}}$ ($\mu$g min/mL)</td>
<td>18.40 ± 2.04</td>
<td>54.96 ± 6.68**</td>
<td>21.40 ± 2.76###</td>
</tr>
<tr>
<td>$T_{1/2}$ (min)</td>
<td>12.44 ± 1.94</td>
<td>31.54 ± 5.41**</td>
<td>12.66 ± 2.02###</td>
</tr>
<tr>
<td>$Cl_{app,h}$ (mL/min/mg protein)</td>
<td>0.205±0.025</td>
<td>0.052 ± 0.0089**</td>
<td>0.176 ±0.028###</td>
</tr>
</tbody>
</table>

The initial GLB concentration of the incubation mixture was 4 $\mu$M (1.98 $\mu$g/mL) in the volume of 200 $\mu$L. Data represent the mean ± S.D. of 4-5 rats, ** $p$ <0.01, versus CON rats; ## $p$ <0.01, versus DM rats.
Table 4. Kinetic parameters for 4-hydroxytolbutamide formation in CON, DM and DM+IN rats hepatic microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON (nmol/min/mg protein)</th>
<th>DM (nmol/min/mg protein)</th>
<th>DM+IN (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.04±0.006</td>
<td>0.08±0.005*</td>
<td>0.06±0.007 #</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>127.8±20.12</td>
<td>118.1±12.94</td>
<td>132.6±23.56</td>
</tr>
<tr>
<td>$Cl_{\text{int}}$ (μL/min/mg protein)</td>
<td>0.31±0.07</td>
<td>0.67±0.05**</td>
<td>0.47±0.04** #</td>
</tr>
</tbody>
</table>

$V_{\text{max}}$, maximum enzyme velocity; $K_m$, Michaelis–Menten constant; $Cl_{\text{int}}$, intrinsic clearance; Data represent the mean ± S.D. of 4 rats, ** $p < 0.01$, * $p < 0.05$ versus CON rats; # $p < 0.05$, versus DM rats.
Figure 2

A

GLB concentration (μM)

CON
DM
DM+IN

T (min)
0 15 30 45 60

B

V (nmol/min/mg protein)

CON
DM
DM+IN

Tolbutamide concentration (μM)
0 100 200 300 400

** * # ##
Figure 3

A

![Graph showing % GLB remaining](image)

- CON
- DM
- DM+IN

NO inhibitor | 10 μM SUL | 40 μM SUL
---|---|---

B

![Graph showing % GLB remaining](image)

Anti-rat CYP2C11 serum (μL/mg microsomal protein)
- 0
- 20
- 60

**Significance Levels:**
- *P < 0.05
- **P < 0.01
- ### P < 0.001
Figure 5

A: Graph showing the efflux of Peff \times 10^{-3} (cm/min) over time (T) for different groups: CON, DM, and DM+IN.

B: Graph showing the accumulative absorption (µg/cm²) over time (T) for different groups: CON, DM, and DM+IN.

C: Graph showing the accumulative efflux of GLB (µg) over time (T) for different groups: CON, DM, and DM+IN.

D: Graph showing the efflux of Peff \times 10^{-3} (cm/min) over time (T) for different groups: GLB, GLB+50 µM NOV, and GLB+200 µM NOV.