Combined Contributions of Impaired Hepatic CYP2C11 and Intestinal Breast Cancer Resistance Protein Activities and Expression to Increased Oral Glibenclamide Exposure in Rats with Streptozotocin-Induced Diabetes Mellitus

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ABSTRACT:

The purpose of this study was to evaluate the contributions of impaired cytochrome P450 and breast cancer resistance protein (BCRP) activity and expression to drug pharmacokinetics under diabetic conditions. Diabetes was induced in rats with the intra-peritoneal administration of streptozocin. Glibenclamide (GLB), a substrate of BCRP, served as a model drug. The pharmacokinetics of orally administered GLB (10 mg/kg) were studied. The results showed that diabetes mellitus significantly increased exposure (area under the curve and peak concentration) to GLB after 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) of CYP2C11 and an anti-CYP2C11 antibody. Western blotting further indicated the contribution of impaired CYP2C11 expression to the impairment of GLB metabolism. Excretion data showed that ~72% of the orally administered dose was excreted in the feces of normal rats, which indicates an important role for intestinal BCRP. Diabetes significantly decreased the recovery from feces, which was only 40% of the orally administered dose. Results from in situ, single-pass, intestinal perfusion experiments revealed that diabetes significantly increased the apparent effective permeability and decreased the efflux of GLB through the intestine; this suggests impairment of intestinal BCRP function, which may play a role in the increased exposure to orally administered GLB in diabetic rats. Insulin treatment partly or completely reversed the changes in diabetic rats. All results yielded the conclusion that impaired hepatic CYP2C11 and intestinal BCRP expression and activity induced by diabetes contributed to the increased exposure of orally administered GLB.

Introduction

Both clinical trials and animal experiments have clearly demonstrated that diabetes mellitus markedly alters the expression and activity of cytochromes P450 (P450s) (Shimojo et al., 1993; Kataoka et al., 2005; Hu et al., 2011), which affects the pharmacokinetic behaviors of some drugs. Studies indicate that alterations in the expression and activity of P450s under diabetic conditions are dependent on types of diabetes and P450 isoforms. For example, one study showed that diabetes markedly up-regulated the expression of CYP1B1, CYP1A2, CYP2B1, and CYP2E1 proteins, with decreased expression of CYP2C11 protein, in hepatic microsomes of rats with streptozotocin (STZ)-induced diabetes (Sindhu et al., 2006). Our previous studies indicated that diabetes increased the expression and activity of CYP3A in liver microsomes of rats, which resulted in greater clearance of intravenously administered verapamil in rats with diabetes induced by STZ (Hu et al., 2011) or a combination of a high-fat diet and STZ (Chen et al., 2011). Oltipraz is metabolized mainly by CYP1A1/2, CYP2B1/2, CYP2C11, CYP2D1, and CYP3A1/2 in rats. The increased expression and mRNA levels of CYP1A2, CYP2B1/2, and CYP3A1 in rats with diabetes induced by alloxan or STZ result in lower values for the area under the curve (AUC) and higher clearance values for orally administered oltipraz (Bae et al., 2006). Similarly, a previous report showed that the greater AUC of intravenously administered sildenafil in diabetic rats was attributable to suppression of CYP2C11 expression (Ahn et al., 2011). Increased oral AUC values for metformin in diabetic rats may result from decreases in hepatic CYP2C11 expression (Choi et al., 2008).

In addition to P450s, some ATP-binding cassette drug transporters, including P-glycoprotein (P-GP), multidrug resistance-associated proteins, and breast cancer resistance protein (BCRP), are altered under diabetic conditions (Liu et al., 2006, 2007; Quezada et al., 2011). In addition to P450s, some ATP-binding cassette drug transporters, including P-glycoprotein (P-GP), multidrug resistance-associated proteins, and breast cancer resistance protein (BCRP), are altered under diabetic conditions (Liu et al., 2006, 2007; Quezada et al., 2011).

ABBR EVIATIONS: P450, cytochrome P450; AUC, area under the concentration-time curve; BCRP, breast cancer resistance protein; Cmax, peak concentration; GLB, glibenclamide; HPLC, high-performance liquid chromatography; NOV, novobiocin; Peff, apparent effective permeability; P-GP, P-glycoprotein; STZ, streptozotocin; SUL, sulfaphenazole; DM, diabetes mellitus; IN, insulin; CON, control.
Several studies showed that high levels of BCRP mRNA were predominantly observed in the kidney, intestine, liver, and cerebral cortex of male rats (Tanaka et al., 2005; Zhang et al., 2011). The high levels of BCRP mRNA in kidney suggest an important role for BCRP in urinary excretion of the BCRP substrate (Mizuno et al., 2004). The intestinal BCRP may limit the uptake of drugs and the excretion of numerous xenobiotics, including topotecan and some dietary carcinogens, through the lumen of the gastrointestinal tract, which becomes an important reason for low bioavailability of orally administered drugs. Our previous study showed that the function and expression of BCRP in the cerebral cortex of rats with STZ-induced diabetes 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 findings suggested that alterations in the expression and activity of BCRP in diabetes may change the pharmacokinetic behaviors of some drugs after oral administration.

Glibenclamide (GLB), a long-acting, second-generation, sulfonylurea, anti-diabetic agent, is extensively metabolized in the liver (Feldman, 1985). Like other sulfonylureas, CYP2C9 plays a major role in GLB metabolism in humans (Yin et al., 2005). However, several reports showed that human CYP3A4 is the major P450 involved in GLB metabolism (Naritomi et al., 2004; Zhou et al., 2010). Furthermore, a study showed that more than one P450 isoform, including CYP3A4, CYP2C9, CYP2C8, and CYP2C19, is involved in GLB metabolism (Zharikova et al., 2009). GLB itself is a substrate of BCRP and has been widely used to assay BCRP function (Gedeon et al., 2008a). All of these results suggested that alterations in the expression and activity of P450s and BCRP induced by diabetes seemed to have important effects on the pharmacokinetic behaviors of GLB.

The present study was undertaken to investigate whether STZ-induced diabetes in rats altered the pharmacokinetic behaviors of orally administered GLB and whether such alterations resulted from the changed activities of P450s and BCRP. The activities of hepatic microsomes were measured through the depletion of GLB, and the isoform of P450 involved in GLB metabolism was identified by using a special inhibitor and antibody. Expression of the corresponding isoform of P450 was measured by using Western blot analysis. The function of BCRP in the absorption of GLB was evaluated with studies of excretion and in situ single-pass perfusion.

Materials and Methods

Chemicals. 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, NADP, and streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxytolbutamide was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Rabbit anti-rat CYP2C11 polyclonal antibody was purchased from Abcam plc (Cambridge, UK). HPLC-grade acetonitrile was obtained from Watanag Pharmaceutical Co. (Xuzhou, China). All other reagents were of analytical grade and were commercially available.

Animals. Five-week-old, male, Sprague-Dawley rats, weighing 170 to 200 g, were supplied by SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The rats were maintained in air-conditioned animal quarters with a temperature of 22 ± 2°C, a relative humidity level of 50 ± 10%, and a 12-h light/dark cycle. They were fed a standard diet (laboratory rodent chow; Xietong, Nanjing, China) and water ad libitum. The animals were acclimated to the facility for 5 days and were fasted, with free access to water, for 12 h before experiments. The studies were approved by the animal ethics committee of China Pharmaceutical University, and every effort was made to minimize stress to the animals.

Diabetes Induced with Streptozotocin. Diabetes was induced in rats through intraperitoneal administration of 65 mg/kg STZ (dissolved in sodium citrate buffer, pH 4.5), according to our previously described method (Liu et al., 2006). Age-matched normal rats received injections of the vehicle (sodium citrate buffer, pH 4.5). On day 7 after STZ injection, fasting blood glucose levels were measured by using a commercially available glucose kit (Jiancheng Biotech Co., Nanjing, China) based on the glucose oxidase method. Rats with fasting blood glucose levels in excess of 11.1 mM were considered to be diabetic (Liu et al., 2006). The diabetic rats were randomly divided into two groups. Group 1 served as the control diabetes mellitus (DM) group and received only vehicle. Group 2 represented DM rats with insulin (IN) treatment and received subcutaneously administered protamine zinc insulin (5 U/kg) twice per day for 4 weeks. The normal control (CON) rats also received vehicle. Fasting blood glucose levels, body weight, and food intake were monitored weekly. All experiments were performed on the 35th day after the injection of STZ or vehicle.

Pharmacokinetics of Orally Administered GLB in Experimental Rats. On day 35 after the injection of STZ or vehicle, the experimental rats were fasting overnight and received 10 mg/kg GLB (suspended in 0.5% sodium carboxymethylcellulose), administered orally. The oral dosage of GLB was based on a previous report (León-Reyes et al., 2009). Blood samples (−250 μl) were collected through the ocular choroidae vein into heparin-containing Eppendorf tubes (Axxygen, Inc., Union City, CA), under light ether anesthesia, at 0.5, 1, 2, 3, 4, 6, 8, 14, and 24 h after oral administration of GLB. After 3 or 4 samplings, the appropriate amount of 0.9% saline solution was administered to the experimental rats through the tail vein, to compensate for blood loss. Plasma samples were immediately obtained through centrifugation at 4000 rpm for 10 min and were stored at −20°C until analysis.

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

For biliary excretion, the experimental rats were anesthetized with intraperitoneal injection of 1% pentobarbital sodium salt dissolved in 0.9% saline solution (0.5 ml), and a polyethylene tube (PE-19; Clay Adams, Parsippany, NJ) was then inserted into the common bile duct. After confirmation of bile flow, GLB (10 mg/kg) was administered orally to the rats. Bile was collected into containers before dosing and at intervals of 0 to 2 h, 2 to 4 h, 4 to 6 h, 6 to 8 h, and 8 to 12 h after dosing. The bile flow was measured and normalized with respect to body weight. All samples were stored at −20°C until analysis.

<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 level, mM</td>
<td>5.37 ± 0.24</td>
<td>26.99 ± 2.17***</td>
<td>5.40 ± 1.73**</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, mg liver/g body weight</td>
<td>31.45 ± 3.58</td>
<td>39.83 ± 6.11**</td>
<td>41.54 ± 3.01**</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, *** p < 0.001, versus CON rats; * p < 0.01, ** p < 0.001, versus DM rats.
Preparation of Rat Hepatic Microsomes. Hepatic microsomes were prepared freshly from CON, DM, and DM+IN rats, according to methods described previously (Xie et al., 2010). Rats were killed under light ether anesthesia, and livers were quickly harvested. The microsomal pellets were resuspended in 0.1 M phosphate-buffered saline (pH 7.4) containing 30% glycerol and were stored at −80°C. Protein concentrations were measured by using the method described by Bradford (1976), with bovine serum albumin as the standard.

GLB Metabolism in Rat Hepatic Microsomes. GLB metabolism was determined by measuring depletion of the drug. Each reaction solution (total volume, 200 μl) was composed of the following components at their respective final concentrations: 4 μM GLB (which is approximately its reported apparent K_m value) (Zharikova et al., 2009), 0.5 mg/ml rat hepatic microsomal protein, and 0.1 M phosphate-buffered saline (pH 7.4). GLB was predissolved 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, and incubation was initiated with the 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 MgCl₂). After the designated time (0, 5, 15, 30, 45, or 60 min), the reaction was terminated with the addition of 20 μl of 1 M HCl. All incubations were performed in triplicate.

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

Effects of Sulfaphenazole and Anti-CYP2C11 Antibody on GLB Metabolism in Rat Hepatic Microsomes. To determine the inhibitory effect of CYP2C11 on GLB metabolism, SUL and an anti-CYP2C11 antibody were selected. SUL was considered to be a selective inhibitor of CYP2C11 in rats and was used for evaluation of the role of CYP2C11 in GLB metabolism in rats. SUL (10 or 40 μM) was added to an incubation mixture (200 μl) containing 0.5 mg/ml rat hepatic microsomes and 4 μM GLB, and the process was performed according to the method described above. After 30 min of incubation, the reaction was terminated with the addition of 20 μl of 1 M HCl.

The effects of the anti-CYP2C11 antibody on GLB metabolism in normal rat hepatic microsomes were documented to verify the contribution of CYP2C11 to GLB metabolism. The rabbit anti-rat CYP2C11 polyclonal antibody was preincubated with hepatic microsomes for 20 min at room temperature, followed by the addition of GLB. The mixture was prepared and the process was performed according to the methods described above. After 45 min of incubation, the reaction was terminated with the addition of 20 μl of 1 M HCl. The amount of GLB remaining in the reaction mixture was determined, and the proportion remaining was estimated.

Western Blot Analysis of Rat Liver Microsomes. Western blot analysis was used to assess the levels of CYP2C11 protein in hepatic microsomes, according to previously published methods (Sindhu et al., 2006). Briefly, microsomal proteins (20 μg) in hepatic microsomes were subjected to sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis, and the separated proteins were blotted onto a pure nitrocellulose membrane. The membrane was incubated in fresh blocking buffer (Tris-buffered saline with 0.05% Tween 20, pH 7.4, containing 5% nonfat dried milk) at room temperature for 3 h. After the membrane was washed four times (5 min each) with Tris-buffered saline with 0.05% Tween 20, the membrane was incubated with primary antibody (polyclonal rabbit anti-rat CYP2C11, 1:2000 dilution) for 5 h at room temperature. The membranes were then washed and incubated with a peroxidase-conjugated goat anti-rabbit IgG (secondary antibody, 1:2000 dilution) for 1.5 h. Sample values were normalized to β-actin levels. Immunoreactive proteins were observed through chemiluminescence (enhanced chemiluminescence Western blotting detection reagents; Applygen Technologies, Beijing, China), and band densities were measured densitometrically by using a Gel-Pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD).

In Situ, Single-Pass, Intestinal Perfusion of Rats. In situ, single-pass perfusion was used to evaluate the absorption of GLB and the efflux of GLB through the small intestine, as described previously (Yu et al., 2010). In brief, the CON, DM, and DM+IN rats were anesthetized through intraperitoneal injection of 1% pentobarbital sodium salt dissolved in 0.9% saline solution (0.5 ml). The abdomen was opened with a midline incision and the jejunum was

FIG. 1. Plasma concentrations of GLB (n = 6 or 7 rats) (A) and bile flow (n = 4 or 5 rats) (B) 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.
isolated between two cannulas, which were separated by 10 cm and were fixed through ligation. The manipulation was performed carefully, to minimize any intestinal blood supply disturbances. The intestine was then returned to the abdominal cavity and the abdomen was closed.

For absorption experiments, GLB (10 μg/ml) was dissolved in Krebs-Henseleit buffer as the temperature was set to 37°C. Phenol red (Jian-cheng Biotech Co.), at a concentration of 20 μg/ml, was used as an impermeable volume marker for measurement of water flux. In general, the isolated jejunal segment was perfused with 0.9% saline solution (37°C) at 0.2 ml/min for 20 min, to reach a steady state for water and solute absorption; the saline solution was followed by Krebs-Henseleit buffer containing (10 μg/ml). After a steady state was achieved (30 min), consecutive effluent samples were collected at 15-min intervals through the distal cannula. At the end of the experiments, the animals were killed, perfused intestinal segments were removed, and the areas of absorption were measured. The cumulative fraction of absorption was estimated. The apparent effective permeability (Papp) (in centimeters per minute) was calculated according to the following equation (Johnson et al., 2003): 

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

where \( C_{in} \) and \( C_{out} \) indicate the inlet and outlet concentrations of GLB, respectively, corrected for volume changes in the segment in the inlet and outlet tubing. \( A \) (in square centimeters) is the area of the perfused intestinal segment, and \( Q \) is the flow rate (0.2 ml/min).

For assessment of the function of BCRP in the absorption of GLB, nobiletin (NOV), a specific inhibitor of BCRP, was selected. The \( P_{app} \) of GLB was measured in the presence of the BCRP inhibitor NOV (50 and 200 μM) in the intestine of normal rats. The progress of in situ, single-pass perfusion was monitored as in the absorption experiment described above.

For efflux experiments, the isolated intestinal segments were perfused with 0.9% saline solution (37°C) at 0.2 ml/min for 20 min, to reach a steady state for water and solute absorption, followed by drug-free Krebs-Henseleit buffer. After intravenous dosing of GLB (1 mg/kg), consecutive effluent samples were collected at 15-min intervals through the distal cannula. The cumulative efflux of GLB through the intestinal segment was measured.

**Drug Assays.** The concentrations of GLB and 4-hydroxytolbutamide were measured with HPLC methods. The HPLC system (Shimadzu, Tokyo, Japan) was equipped with an LC-10A pump, a SIL-10ADvp auto-injector, and a CTO-10A Asyp column oven. Separation was performed at a flow rate of 1.0 ml/min with a Waters Symmetry C18 column (5.0 μm, 150 × 4.6 mm i.d.; Waters, Milford, MA).

Levels of GLB in the biological samples were determined with an HPLC method described previously (Al-Dhawali et al., 1995; Gedeon et al., 2008a), with a minor modification. Every biological sample (100-μl sample of plasma or feces, 200-μl sample of urine or bile, 200-μl sample of intestinal perfusate or efflux fluid) was spiked with 20 μl of 1 M HCl, and diclofenac sodium (final concentration, 10 μg/ml) was used as an internal standard. After vortex-mixing (30 s), 1 ml of ethyl acetate was added to the mixture with shaking for 10 min, and the mixture was then centrifuged at 4000 rpm for 10 min. The organic phase was transferred into clean tubes, dried under reduced pressure by using an integrated SpeedVac SPD2010-230 system (Thermo Fisher Scientific, Waltham, MA), and reconstituted with 100 μl of mobile phase; 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; Shimadzu) set at an excitation wavelength of 308 nm and an emission wavelength of 360 nm. The recoveries were greater than 70%. The linear range of GLB in plasma and feces was 0.039 to 2.5 μg/ml. The linear range of GLB concentrations in bile, urine, incubation mixtures, and intestine perfusate was 0.0195 to 2.5 μg/ml. The intraday and interday coefficients of variation for the assay were less than 10%.

Measurement of 4-hydroxytolbutamide in incubation mixtures was performed according to previously reported methods (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 of microsomal incubation mixture, and the mixture was centrifuged at 4000 rpm for 10 min. After the mixture was 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 with a SPD-10Avp ultraviolet detector (Shimadzu) set at a wavelength of 230 nm. The detection limit for 4-hydroxytolbutamide was 0.078 μg/ml. Recovery was greater than 75%. The intraday and interday coefficients of variation for the assay were less than 10%.

**Pharmacokinetic Analyses.** The concentration-time data for GLB for each rat were individually analyzed through noncompartmental analysis (Phenix WinNonlin 6.1; Pharsight, St. Louis, MO). The area under the plasma concentration-time profile (AUC0–\( t_{\text{last}} \)) was calculated with the trapezoidal rule, with extrapolation to infinity. The peak concentration (\( C_{\text{max}} \)) and the time to reach \( C_{\text{max}} \) were obtained directly from the plasma concentration-time profiles. The terminal elimination constant (\( k_{\text{e}} \)) was obtained from the least-squares linear regression slope of the natural logarithm of concentration versus time, and the terminal elimination half-life (\( t_{1/2 \text{e}} \)) was calculated as 0.693/\( k_{\text{e}} \). The mean residence time was calculated as the area under the first moment curve divided by \( AUC_{0–\infty} \). The clearance (CL/F) and volume of distribution (\( V/F \)) were defined as dose/AUC0–\( t_{\text{last}} \) and dose/AUC0–\( t_{\text{last}} \) · \( k_{\text{e}} \), respectively, where F represents absolute bioavailability.

In hepatic microsomal incubations, the area under the GLB concentration-time curve (AUC0–\( t_{\text{last}} \)) was measured by using the linear trapezoidal rule. The clearance of GLB in hepatic microsomes (CL/Fexp) was estimated as the initial dose divided by AUC0–\( t_{\text{last}} \). The terminal elimination constant in hepatic microsomes (\( k_{h} \)) was obtained from the least-squares linear regression slope of the natural logarithm of concentration versus time, and the terminal elimination half-life (\( t_{1/2 \text{h}} \)) was calculated as 0.693/\( k_{h} \).

The Michaelis-Menten equation was used for characterization of the kinetics of 4-hydroxytolbutamide formation from tolbutamide in rat hepatic microsomes. The Michaelis-Menten constant (\( K_{m} \)) and the maximal rate of 4-hydroxytolbutamide formation (\( V_{\text{max}} \)) were estimated through nonlinear least-squares regression with Excel Solver (Microsoft, Redmond, WA). The intrinsic clearance for the formation of 4-hydroxytolbutamide was calculated as \( V_{\text{max}}/K_{m} \). The intrinsic clearance for tolbutamide in rat hepatic microsomes was estimated as the initial dose divided by AUC0–\( t_{\text{last}} \). The terminal elimination half-life (\( t_{1/2 \text{h}} \)) was calculated as 0.693/\( K_{h} \). The intraday and interday coefficients of variation for the assay were less than 10%.

**Statistical Analyses.** Data are presented as mean ± S.D. Statistical differences between groups were evaluated through one-way of analysis of variance. If analysis results were significant, then the differences between groups were estimated by using the Student-Newman-Keuls multiple-comparison test for post hoc analysis. A p value of less than 0.05 was considered statistically significant.

**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>AUC0–( t_{\text{last}} ) μg-h/ml</td>
<td>2.61 ± 0.44</td>
<td>15.23 ± 4.79**</td>
<td>3.00 ± 1.41**</td>
</tr>
<tr>
<td>AUC0–( t_{\text{last}} ) μg-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 \text{h}} ), h</td>
<td>4.55 ± 1.23</td>
<td>5.11 ± 1.08</td>
<td>8.18 ± 6.43</td>
</tr>
<tr>
<td>Mean residence time, 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⁻¹ · kg⁻¹)</td>
<td>3.93 ± 0.68</td>
<td>0.27 ± 0.26**</td>
<td>3.89 ± 1.76**</td>
</tr>
<tr>
<td>( V/F ), l/kg</td>
<td>25.72 ± 8.84</td>
<td>6.36 ± 0.63**</td>
<td>36.8 ± 17.09**</td>
</tr>
<tr>
<td>( C_{\text{max}} ), μg/ml</td>
<td>0.35 ± 0.11</td>
<td>1.25 ± 0.24**</td>
<td>0.25 ± 0.06**</td>
</tr>
<tr>
<td>Time to reach ( C_{\text{max}} ), h</td>
<td>1.50 ± 1.00</td>
<td>7.2 ± 1.79**</td>
<td>1.0 ± 0.61**</td>
</tr>
<tr>
<td>Biliary excretion, % of dose</td>
<td>0.09 ± 0.07</td>
<td>0.097 ± 0.130</td>
<td>0.019 ± 0.017</td>
</tr>
<tr>
<td>Urinary excretion, % of dose</td>
<td>0.0042 ± 0.001</td>
<td>0.023 ± 0.009**</td>
<td>0.019 ± 0.005**</td>
</tr>
<tr>
<td>Fecal excretion, % of dose</td>
<td>72 ± 10</td>
<td>40 ± 9**</td>
<td>50 ± 21**</td>
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**p < 0.01, versus CON rats; **p < 0.05, ***p < 0.01, versus DM rats.**
Results

Physiological and Biochemical Parameters for Experimental 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 weights, fasting blood glucose levels, and liver weights were measured on day 35 after injection of STZ (Table 1). Significantly higher fasting blood glucose levels and relative liver weights and lower body weights were observed in DM rats. Insulin treatment partly ameliorated 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 (Fig. 1A), and the corresponding pharmacokinetic parameters were estimated (Table 2). Compared with CON rats, DM rats had markedly higher plasma concentrations of GLB, which were accompanied by higher exposure (AUC0–24h and Cmax) and lower oral clearance values. The AUC0–24h and Cmax values for DM rats were 564.7 and 357.0%, respectively, of the values for CON rats. The oral clearance (CL/F) value for DM rats was only 18% of that for CON rats. Absorption of GLB in DM rats was slower, with longer times to reach Cmax. Insulin therapy may almost abolish the alterations of the pharmacokinetic profile for orally administered GLB induced by diabetes.

After oral administration, the recoveries of GLB from 36-h urine samples were 0.004 to 0.023% of the dose (Table 2). The amount of GLB excreted in 12-h bile samples was less than 0.1% (Table 2), although DM rats showed higher levels of recovery in bile, compared with CON rats. In addition, DM rats had higher bile flow rates, compared with CON rats (Fig. 1B). However, a high level of recovery of GLB from 36-h fecal samples from normal rats, i.e., 72% of the oral dose of GLB, was found (Table 2). Diabetes significantly decreased the recovery of GLB from 36-h fecal samples after an oral dose; the value was only 40% of the oral dose, which indicates enhancement of GLB absorption. Insulin treatment reversed the increased bile flow induced by diabetes mellitus. These results suggested that the enhanced absorption of GLB through the intestine was one of the factors that contributed to the increased exposure to orally administered GLB in diabetic rats.

GLB Metabolism in Rat Hepatic Microsomes. GLB is thought to be eliminated mainly in the liver (Feldman, 1985). For investigation of whether the increased exposure to orally administered GLB in vivo was attributable to impairment of rat hepatic microsomal activity, the characteristics of GLB metabolism in rat hepatic microsomes were measured by using the depletion of GLB (Fig. 2A; Table 3). Significant increases in both C60min and AUC0–60min for GLB in hepatic microsomal mixtures from DM rats were observed (7.4 and 2.9 times CON rat values, respectively). It was found that the intrinsic clearance of GLB in hepatic microsomes from DM rats was significantly decreased (25% of the value for hepatic microsomes from CON rats). The t1/2,η was prolonged from 12.4 min in CON rats to 31.5 min in DM rats. All of these results indicated that GLB metabolism in hepatic microsomes from DM rats was impaired and the suppression of GLB metabolism observed in vitro with hepatic microsomes was directly related to the increase in the exposure to orally administered GLB in diabetic rats.

Metabolism of Tolbutamide in Rat Hepatic Microsomes. The 4-hydroxylation of tolbutamide in rat hepatic microsomes was used to assay the activity of CYP2C6. Contrary to our expectations, an increased rate of formation of 4-hydroxytolbutamide was found in hepatic microsomes from DM rats (Fig. 2B; Table 4). The estimated values for Vmax and the intrinsic clearance of tolbutamide in hepatic microsomes from DM rats were 2.0 and 2.2 times those for CON rats, respectively, which suggests that the activity of CYP2C6 was induced rather than suppressed in DM rats. Insulin treatment partly reversed the increase in CYP2C6 activity induced by diabetes.

Effects of Sulfaphenazole and Anti-CYP2C11 Antibody on GLB Metabolism in Rat Hepatic Microsomes. Because the struc-
tures of two human enzymes, CYP2C9 and CYP2C8, are closely related to those of rat CYP2C11 and CYP2C13 (Wang et al., 2009). CYP2C11 was taken into consideration to explore the mechanism of decreased GLB metabolism. To determine the role of CYP2C11 in GLB metabolism, depletion of GLB in rat hepatic microsomes was investigated in the presence of a chemical inhibitor (SUL) and an anti-CYP2C11 antibody. The amount of GLB in the incubation mixture before the reaction was set as 100%, and the fractions of GLB remaining in the incubation mixture after 30 min of reaction were calculated. The results showed that SUL concentration-dependently inhibited GLB metabolism (Fig. 3A) in hepatic microsomes from CON, DM, and DM+IN rats. In comparison with the absence of SUL, addition of 40 μM SUL significantly increased the fractions of GLB, and DM+IN rats, which ranged from 8 to 65%, 38 to 91%, and 18 to 65%, respectively. The inhibitory effect of SUL in CON rats was much stronger than that in DM rats, with values of 812.5 and 239.5%, respectively, compared with groups without SUL. Addition of 10 μM SUL also significantly increased the fractions of GLB remaining in rat hepatic microsomes. These results indicated that CYP2C11 contributed approximately 50% to 60% to the total GLB depletion and GLB metabolism could be inhibited by the CYP2C11 inhibitor SUL.

The contribution of CYP2C11 to the depletion of GLB was confirmed through immunoinhibition in hepatic microsomes from normal rats (Fig. 3B). The results showed that an antibody against CYP2C11 inhibited GLB metabolism in a concentration-dependent manner. The proportion of GLB remaining in the presence of antibody against CYP2C11 (60 μM microsomal protein) was 70%, which was significantly greater than that (15%) remaining in the absence of the anti-CYP2C11 antibody. Antibody addition at 20 μM microsomal protein also caused a significant increase in the proportion of GLB remaining. The results with anti-CYP2C11 antibody addition were in line with the findings obtained by using SUL. The results verified the role of CYP2C11 in GLB metabolism in rats, which indicated that the decreases in GLB metabolism in DM rats were partly attributable to impairment of CYP2C11 activity in diabetic rats.

Western Blotting. The levels of CYP2C11 protein in hepatic microsomes from CON, DM, and DM+IN rats were assessed with Western blotting (Fig. 4). The level of CYP2C11 in hepatic microsomes from DM rats was significantly (p < 0.001) lower than that in microsomes from CON rats; the level was only 20% of that for CON rats, which suggests impairment of CYP2C11 expression. The impaired expression of CYP2C11 in hepatic microsomes from DM rats was partially reversed by insulin therapy. The decrease in the level of CYP2C11 in hepatic microsomes from DM rats in parallel with impairment of GLB metabolism, which indicates that the suppression of CYP2C11 expression in hepatic microsomes induced by diabetes contributed to the decrease in GLB metabolism.

### Intestinal Absorption and Efflux of GLB in Experimental Rats

High levels of recovery of GLB were found in the feces of CON rats after an oral dose of GLB (10 mg/kg), but diabetes significantly decreased the recovery of GLB in feces, which suggests enhancement of GLB absorption. The intestinal P_{eff} (Fig. 5A) and the amount of absorbed GLB (Fig. 5B) were measured by using in situ, single-pass, jejunal 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 with both 90 min and 120 min of perfusion (35% increase with 90-min perfusion and 42% increase with 120-min perfusion). Significantly increased P_{eff} values for GLB were found in the intestine of DM rats after 30, 45, 75, and 90 min of perfusion. The increase in P_{eff} values for GLB was in parallel with the decrease in the recovery of GLB in the 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 efflux of GLB across the intestine, GLB efflux through the intestine was measured after an intravenously administered dose (1 mg/kg, dissolved in saline solution and 0.2% dimethylsulfoxide) by using in situ, single-pass, jejunal perfusion (Fig. 5C). The amount of GLB efflux in 60 min in the experimental rats was only 0.02 to 0.03% of the intravenous dose. Significantly decreased efflux of GLB during a 15- to 30-min interval was found in the intestine of DM rats (Fig. 5C). All of these results demonstrated the contributions of the decrease in efflux and the increase in absorption through the intestine to greater exposure to GLB after an oral dose.

The selective inhibitor NOV (Su et al., 2007) was selected for assessment of the role of BCRP in GLB intestinal absorption, and the 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 P_{eff} in a concentration-dependent manner (Fig. 5D). The addition of 200 μM NOV led to a 25% increase at 45 min of perfusion and a 32% increase at 90 min of perfusion. The data demonstrated that inhibition of intestinal BCRP by NOV significantly promoted the absorption of GLB.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DM</th>
<th>DM+IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial concentration, μg/ml</td>
<td>2.06 ± 0.12</td>
<td>2.00 ± 0.13</td>
<td>2.06 ± 0.27</td>
</tr>
<tr>
<td>Concentration at 60 min, μg/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} min, μg·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>t_{CL}</td>
<td>0.205 ± 0.025</td>
<td>0.052 ± 0.0089**</td>
<td>0.176 ± 0.028**</td>
</tr>
</tbody>
</table>

* ** 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 rat hepatic microsomes

Data represent the mean ± S.D. for four rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DM</th>
<th>DM+IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max}, nmol·min⁻¹·mg protein⁻¹</td>
<td>0.04 ± 0.006</td>
<td>0.08 ± 0.005*</td>
<td>0.06 ± 0.007*</td>
</tr>
<tr>
<td>K_{cat}, μM</td>
<td>127.8 ± 20.12</td>
<td>118.1 ± 12.94</td>
<td>132.6 ± 23.56</td>
</tr>
<tr>
<td>Intrinsic clearance, μl·min⁻¹·mg protein⁻¹</td>
<td>0.31 ± 0.07</td>
<td>0.67 ± 0.05**</td>
<td>0.47 ± 0.04***</td>
</tr>
</tbody>
</table>

* * p < 0.01, † p < 0.05, versus CON rats; ‡ p < 0.05, versus DM rats.
The present study was conducted to study the altered pharmacokinetic characteristics of the model drug GLB in diabetic states and to investigate whether the changes were attributable to impaired activity and expression of P450s together with BCRP. The main finding was that diabetes significantly increased GLB exposure (AUC and \(C_{\text{max}}\)) after oral dosing. GLB is extensively metabolized in the liver; therefore, GLB metabolism in hepatic microsomes from experimental rats was analyzed. The results showed that in vitro clearance of GLB in hepatic microsomes from DM rats was significantly decreased, which was in parallel with the increased exposure of GLB in vivo. The conclusion demonstrated that the increased oral GLB exposure in diabetic states was partly attributable to the impairment of GLB metabolism. Insulin treatment partly reversed the alterations in the pharmacokinetic parameters induced by diabetes.

In humans, data on GLB metabolism showed that several hepatic P450s were involved in GLB metabolism, including CYP2C9 (Yin et al., 2005), CYP3A4, CYP2C8, and CYP2C19 (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 a contribution of CYP3A isoforms to the decreased clearance of GLB in diabetes. Therefore, we focused on the role of CYP2C9. A report showed that human CYP2C9 was equivalent to CYP2C (including CYP2C6 and CYP2C11) in rats (Bogaards et al., 2000). Accumulating studies have shown that 4-hydroxylation of tolbutamide in rats is mediated by CYP2C6, and this reaction has been used to assay the activity of CYP2C6 (Dostalek et al., 2005; Wang et al., 2007). Therefore, the 4-hydroxylation of tolbutamide was used to reflect the activity of CYP2C6 in rat hepatic microsomes. Contrary to our expectation, the amount of 4-hydroxytolbutamide formed in hepatic microsomes from DM rats was increased rather than decreased, which indicated that the activity of CYP2C6 was induced by diabetes. The increased rate of formation of 4-hydroxytolbutamide was consistent with the increase in the expression of CYP2C6 protein in rats with STZ-induced diabetes (Shimojo et al., 2010).

**FIG. 3.** A, effects of SUL on the depletion of GLB in hepatic microsomes from CON, DM, and DM+IN rats. Data represent the mean ± S.D. for four or five rats. *, \(p < 0.05\); **, \(p < 0.01\), versus without inhibitor; ##, \(p < 0.01\), versus CON rats. B, effect of antibody to CYP2C11 on the depletion of GLB in hepatic microsomes from normal rats. The amount of GLB at the incubation time of 0 min (the absence of NADPH) was set as 100%. Depletion of GLB was measured in the absence or presence of SUL or antibody to CYP2C11. Data represent the mean ± S.D. of three experiments. *, \(p < 0.05\); **, \(p < 0.01\), versus without antibody.

**FIG. 4.** A, CYP2C11 protein expression in hepatic microsomes from CON, DM, and DM+IN rats, as indicated in representative Western blots for CYP2C11 (loading, 20 μg per lane). B, relative staining intensity for CYP2C11, in comparison with control rats. Data represent the mean ± S.D. for four rats. ***, \(p < 0.001\); ***, \(p < 0.01\); **, \(p < 0.01\), versus CON rats.
and its expression was more sensitive to suppression by glucagon at low levels of insulin than at high levels (Iber et al., 2001), which indicated that low levels of insulin in DM rats may be a reason for suppressed CYP2C11 expression. This deduction was partly supported by the present finding that insulin treatment reversed suppression. In addition, diabetes is often associated with high levels of cytokines and cytokine-like inflammation markers such as tumor necrosis factor α and interleukin 6, and these cytokines were reported to suppress P450 expression in primary hepatocyte cultures (Iber et al., 2000), which suggests a role for higher levels of cytokines in the suppression of CYP2C11. Other metabolic enzymes or P450 isoforms may also have effects on the metabolism of GLB, and additional studies are required.

Experiments on GLB excretion showed that only 0.004 to 0.02% of orally administered GLB was detected in 36-h urine samples and less than 1% in 12-h bile samples, which suggested a slight role for excretion in bile and urine in GLB exposure, and this was consistent with a previous report (Naraharissetti et al., 2007). However, the total amount of GLB excreted in 36-h fecal samples was up to 72% of the oral dose in CON rats, which was consistent with the previous study performed by using [14C]GLB (Christ et al., 1969). A significantly decreased amount of GLB in the feces of DM rats was observed (only 55.6% of the amount for CON rats), which indicates that diabetes enhanced the absorption of GLB. Data from in situ, single-pass perfusion of the jejunum clearly demonstrated that diabetes significantly increased the \( P_{\text{eff}} \) and decreased the efflux of GLB through the intestine, which indicates a contribution to the enhancement of GLB absorption.

GLB is a substrate of BCRP and often is used to assay the function of BCRP (Gedeon et al., 2008a). BCRP was highly expressed in the intestine of rats (Tanaka et al., 2005), which became a factor leading to high levels of recovery of GLB in feces after an oral dose. Our previous study showed that diabetes may down-regulate protein and mRNA levels for intestinal BCRP (Zhang et al., 2011); the levels of BCRP mRNA and protein in the intestines of diabetic rats were only 75% (significantly decreased) and 34%, respectively, of the values for CON rats, which was in line with enhanced absorption of GLB in diabetes. This indicated that impairment of intestinal BCRP expression may be another factor leading to increased GLB exposure after oral dosing. The presence of a BCRP inhibitor (50 and 200 \( \mu M \) NOV) significantly increased the \( P_{\text{eff}} \), 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 that GLB could possibly be transported by BCRP and multidrug resistance-associated protein 3 (Yu et al., 2010). BCRP exhibits partial overlap in substrate specificity with P-GP, which suggests that impairment of intestinal P-GP may contribute to greater exposure in diabetic states for orally administered GLB. Other drug transporters, such as multidrug resistance-associated proteins and organic anion transporters, may affect the pharmacokinetic characteristics of GLB under diabetic conditions.

In summary, the present study demonstrated that the increased GLB exposure after oral dosing in diabetic states partly resulted from the suppression of hepatic CYP2C11 and intestinal BCRP activity and expression. Insulin treatment could partly or completely reverse the alterations in the pharmacokinetic features of orally administered GLB induced by diabetes. Additional studies are required to identify the reason for the down-regulation of CYP2C11, as well as the physiological significance of the combined effects of P450s and efflux transporters.
Authorship Contributions

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

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

Performed data analysis: H. Liu, L. Liu, Mei, and Hu.

Wrote or contributed to the writing of the manuscript: H. Liu, L. Liu, and X. Liu.

References


