Mechanisms of Reduced Maternal and Fetal Lopinavir Exposure in a Rat Model of Gestational Diabetes

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ABSTRACT:

Lopinavir (LPV) is the preferred HIV protease inhibitor in pregnancy, but it is unknown if gestational diabetes mellitus (GDM) affects its disposition. Hepatic protein expression and plasma protein binding are altered in rodent models of GDM. Because LPV is influenced by hepatic transporters and metabolic enzymes and is highly protein bound, it was hypothesized that streptozotocin-induced GDM would alter its disposition. Maternal and fetal tissues were collected from GDM rats and controls 45 min after LPV injection. In another cohort, fetuses were serially extracted 5 to 60 min after injection. LPV was quantified using liquid chromatography tandem mass spectrometry. Expression of relevant transporters, such as Multidrug resistance protein 1 (Mdr1), and cytochrome P450 3A2 (Cyp3a2), which metabolizes LPV in rodents, was measured in maternal liver via quantitative reverse transcriptase polymerase chain reaction and Western blot analysis. Expression of relevant transporters also was measured in placenta via quantitative reverse transcriptase polymerase chain reaction. Protein binding was determined by ultrafiltration. Relative to controls, we observed dramatically reduced maternal and fetal LPV exposure in GDM. Compared with controls, maternal hepatic Mdr1 and Cyp3a2 were up-regulated, and protein binding was reduced in the GDM group. Increased Mdr1- and Cyp3a2-mediated hepatobiliary clearance, coupled with a larger unbound LPV fraction, is likely to have facilitated hepatic elimination, thereby decreasing maternal and fetal exposure. Not surprisingly, up-regulation of Mdr1 and Cyp3a2’s transcriptional regulator, pregnane X receptor, was demonstrated in maternal liver via Western blot analysis. Up-regulation of Mdr1 in placentas isolated from the GDM group likely also contributed to decreased fetal exposure to LPV. This study provides preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction.

Introduction

Antiretroviral drugs are administered to HIV-positive pregnant women to prevent both maternal disease progression and vertical HIV transmission. Lopinavir (LPV) coformulated with low-dose ritonavir (RTV) is currently the preferred HIV protease inhibitor combination for pregnant women because of superior efficacy and extensive patient experience in pregnancy (http://aidsinfo.nih.gov). As such, the majority of HIV-positive pregnant women in North America now use LPV/RTV as part of their highly active antiretroviral therapy regimens. For LPV/RTV to be effective, optimal LPV plasma concentrations must be maintained throughout gestation (Boffito et al., 2002; Masquelier et al., 2002).

Variation in LPV exposure has been observed with standard dosing (Giard-Schmid et al., 2003) and likely is caused by individual variability in protein binding and drug transporter and metabolic enzyme expression. LPV is a substrate for the multidrug resistance protein 1 (MDR1) drug efflux transporter (Woodahl et al., 2005; Agarwal et al., 2007) and is metabolized extensively by cytochrome P450 3A4 (CYP3A4) (van Waterschoot et al., 2010). In the liver, MDR1 facilitates LPV’s hepatobiliary excretion, whereas CYP3A4 converts it to increasingly hydrophilic metabolites (Fig. 1). Large intersubject differences in the basal expression and activity of MDR1 and CYP3A4 have been reported (Westlund-Johnsson et al., 2003; Meier et al., 2006). In the placenta, MDR1 plays a barrier role by actively transporting substrates back into systemic circulation. LPV is also highly bound (>99%) to blood proteins, including albumin and α1-acid glycoprotein (AAG), so even small changes in binding result in large changes in unbound drug fractions. Because only unbound drugs traverse cell membranes, variation in LPV protein binding translates into deviations in the efficiency with which LPV passes into maternal and fetal tissues.

Individual variability in these mechanisms can be introduced by a variety of factors, including disease. Reports of drug disposition being sensitive to disease have appeared in the literature for nearly 40 years.

REFERENCES:

Lopinavir, ritonavir, MDR1, multidrug resistance protein 1; AAG, α1-acid glycoprotein; GDM, gestational diabetes mellitus; STZ, streptozotocin; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; PXR, pregnane X receptor; GD, gestational day; LC-MS/MS, liquid chromatography tandem mass spectrometry; FFA, free fatty acid; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; Ct, crossover threshold; OD, optical density; ANOVA, analysis of variance; AUC, area under the concentration-versus-time curve.

Abbreviations:

LPV, lopinavir; RTV, ritonavir; MDR1, multidrug resistance protein 1; AAG, α1-acid glycoprotein; GDM, gestational diabetes mellitus; STZ, streptozotocin; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; PXR, pregnane X receptor; GD, gestational day; LC-MS/MS, liquid chromatography tandem mass spectrometry; FFA, free fatty acid; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; Ct, crossover threshold; OD, optical density; ANOVA, analysis of variance; AUC, area under the concentration-versus-time curve.
However, only in the last decade have the effects of disease during pregnancy, including gestational diabetes mellitus (GDM), been investigated (Wang et al., 2005; Petrovic et al., 2008; Anger and Piquette-Miller, 2010). Studies indicate that the prevalence of GDM in HIV-positive pregnant women far exceeds that of HIV-negative pregnant women (Watts et al., 2004; Kourtis et al., 2006; Martí et al., 2007; González-Tomé et al., 2008), but to the best of our knowledge, it is unknown if this has an effect on the disposition of highly active antiretroviral therapy drugs. In rats with streptozotocin (STZ)-induced GDM, we have observed recently that the plasma protein binding of the protease inhibitor saquinavir decreases because of displacement by elevated lipids, whereas the expression of hepatic Mdr1 and a variety of cytochrome P450 and UDP-glucuronosyltransferase enzymes increases (Anger and Piquette-Miller, 2010). In addition, altered disposition has been observed for a variety of drugs in both nonpregnant humans with diabetes (reviewed in Gwilt et al., 1991) and nonpregnant rats with STZ-induced diabetes (reviewed in Lee et al., 2010).

In this study, maternal and fetal LPV exposure was examined in rats with STZ-induced GDM and related to protein binding and hepatic and placental drug transporter/metabolic enzyme expression data. In terms of transporters, the expression of Mdr1 (encoded in rodents by the Mdr1a and Mdr1b genes), multidrug resistance-associated protein 2 (Mrp2), and breast cancer resistance protein (Bcrp) was determined in maternal liver and placenta. Mrp2 and Bcrp were included because of documented interactions with LPV and/or other antiretroviral drugs. In terms of metabolic enzymes, only Cyp3a2 was examined. Whereas CYP3A4 is the dominant constitutive CYP3A subfamily isoform in humans, Cyp3a2 is the dominant constitutive Cyp3a subfamily isoform in rats (Nelson et al., 1993) and has been shown to metabolize prototypical human CYP3A4 substrates such as verapamil (Tracy et al., 1999; Choi and Burm, 2008; Hanada et al., 2008). Given our previous findings with this model (Anger and Piquette-Miller, 2010), we hypothesized that maternal LPV exposure would be reduced. Our hypothesis regarding fetal LPV exposure was dependent on whether protein binding or active feto-maternal transport dominated LPV transfer (and whether placental Mdr1 was up-regulated). Decreased protein binding could increase fetal LPV exposure by facilitating materno-fetal placental transfer, but placental Mdr1 could limit fetal exposure by actively transporting LPV in a feto-maternal direction. Because the quantitative contribution of these processes could change with time, we used a novel fetal sampling procedure to

**Fig. 1.** The dominant Cyp3a-mediated metabolic pathway for LPV. LPV (A) is metabolized to M3/M4 (B) (4-hydroxy-LPV) by Cyp3a, and M3/M4 is metabolized subsequently to M1 (C) (4-oxo-LPV), also by Cyp3a. Carbon-4 is the location for these metabolic reactions, which is indicated by an asterisk in A. LPV is a substrate for MDR1. The M3/M4 and M1 metabolites are likely substrates for MDR1.
determine the time course of fetal exposure. Due to its role in Mdr1 and Cyp3a2 transcription (Lehmann et al., 1998; Tirona, 2011), pregnant X receptor (PXR) expression in maternal liver also was determined.

Our findings further advance our understanding of the impact that GDM can have on drug disposition mechanisms and provide preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction. Effects associated with GDM would compound the drug disposition effects that have been associated with pregnancy itself.

Materials and Methods

Pharmaceutical and Chemical Products. LPV and RTV were purchased from USP (Rockville, MD) (PubChem Compound IDs: 92727 and 392622). LPV metabolites M1 and M3/M4 were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Insulin glargine was purchased from a commercial pharmacy in Toronto, ON (Sanofi-Aventis Canada, Inc., Laval, QC, Canada). Ethanol and methanol were purchased from Commercial Alcohols (Brampton, ON, Canada) and Caledon (Georgetown, ON, Canada), respectively. Unless otherwise noted, all of the other chemical products, including STZ (PubChem Compound ID 5300), were purchased from Sigma-Aldrich (St. Louis, MO). For intraovarian administration, LPV was dissolved in 4:3:3 (v/v/v) ethanol/propanediol/5% dextrose in water (10 mg/ml).

Animals. Timed pregnant Sprague-Dawley rats (208–265 g on gestational day 6) were purchased directly from Charles River Canada (Montreal, QC, Canada) and housed individually in a temperature-controlled facility on a 12:12 h light/dark cycle. Rats were given free access to water and standard chow (Harlan Teklad Global Diet 2018; Harlan Teklad, Madison, WI). Experiments were approved by the Office of Research Ethics at the University of Toronto and performed in accordance with Canadian Council on Animal Care guidelines.

Animal Treatments/Monitoring and Tissue Collection. Rats were assigned randomly to one of three groups: nontreated diabetics (the GDM group), vehicle controls (the vehicle group), and insulin-treated diabetics (the insulin-treated group). On gestational day 6 (GD6), diabetes was induced by way of a single subcutaneous injection of STZ in 0.1 M citrate buffer (pH 4.5) at a dosage of 45 mg/kg. Vehicle control animals received a single subcutaneous injection of citrate buffer on GD6. For all of the animals, body weight was measured before respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured before respective injections (GD6) and on GD7 to GD9, GD11, and GD20. For animals in the insulin-treated group, blood glucose was measured before STZ injection and then daily until sacrifice to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a nonfasted state, using a typical glucometer with blood obtained via tail prick. When values exceeded the range of this glucometer, a commercially available enzymatic glucose assay was used according to the manufacturer’s instructions (Autokit Glucose; Wako BioProducts, Richmond, VA). Mild hyperglycemia was defined as a concentration, a 3- to 4-cm midline abdominal incision was made so as to provide access to the uterine horns via the intraperitoneal cavity. Five minutes postinjection, one of the uterine horns was externalized, and the most distal fetal unit was identified. (Supplemental Fig. 1 illustrates fetal positioning within the pregnant rat at approximately GD20.) The maternal vasculature supplying this fetal unit’s placenta then was blocked via ligature to minimize both maternal and fetal blood loss. Once the placental blood supply was blocked, the fetal unit was removed through a small incision made in the uterine wall. The remaining pups, still situated within the uterine horn, then were returned to the maternal intraperitoneal cavity to await future time points. Additional fetal compartments were collected from more proximal uterine horn positions and from the unused uterine horn at 10, 15, 20, 30, 45, and 60 min postinjection.

Sampling of Fetal Compartments for Time Course Determination. On GD20, a second cohort of animals (n = 4–5 per group) received LPV (10 mg/kg i.v.) 45 ± 1 min before being sacrificed to collect maternal blood (cardiac puncture) and liver as well as up to 10 fetal compartments per dam. The location of each fetal compartment within the uterine horn was noted (e.g., left versus right horn and distance, in fetus number, away from the cervical stump). Anemic fluid also was collected from this first subset of animals. Aliquots of whole blood were transferred to thrombin-coated BD Vacutainer tubes (BD Canada, Mississauga, ON, Canada) and spun to collect serum. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use.

Sampling of Fetal Compartments for Time Course Determination. On GD20, a second cohort of animals (n = 3 per group) received LPV (10 mg/kg i.v.) while anesthetized with continuous inhalation anesthesia (5% isoflurane for induction and 1.5–2% isoflurane for maintenance) and was subjected subsequently to sequential sampling of placentas and fetuses (collectively referred to as fetal compartments) over a period of 1 h. After LPV administration, a 3- to 4-cm midline abdominal incision was made so as to provide access to the uterine horns via the intraperitoneal cavity. Five minutes postinjection, one of the uterine horns was externalized, and the most distal fetal unit was identified. (Supplemental Fig. 1 illustrates fetal positioning within the pregnant rat at approximately GD20.) The maternal vasculature supplying this fetal unit’s placenta then was blocked via ligature to minimize both maternal and fetal blood loss. Once the placental blood supply was blocked, the fetal unit was removed through a small incision made in the uterine wall. The remaining pups, still situated within the uterine horn, then were returned to the maternal intraperitoneal cavity to await future time points. Additional fetal compartments were collected from more proximal uterine horn positions and from the unused uterine horn at 10, 15, 20, 30, 45, and 60 min postinjection. Fetal compartments were not collected if their placental blood supply was compromised during the collection of another fetal unit (e.g., if they shifted position in such a way that their blood vessels sheared). After the collection of the 60-min fetal unit, maternal blood (cardiac puncture) and liver were collected. Throughout this procedure, maternal body temperatures were kept at approximately 37°C using heating pads.

Determination of LPV and LPV Metabolite Concentrations. For the preparation of samples and standards, a liquid-liquid extraction procedure was adapted from Wang et al. (2006). In brief, serum and tissue samples were thawed to room temperature. Tissues (maternal livers, placentas, and fetuses) were homogenized in deionized water in glass tubes. For samples, 100 μl of serum or homogenate was added to tubes containing 20 ng of predried internal standard (internal standard, RTV; final internal standard concentration, 200 ng/ml). For LPV standards, 100 μl of drug-free serum or homogenate was added to tubes containing both 20 ng of predried internal standard and 1 to 100 ng of predried LPV (final LPV standard concentrations, 10–1000 ng/ml). LPV metabolites M1 and M3/M4 only were measured in maternal liver because the placenta and fetuses are not significant sites of LPV metabolism. For LPV metabolite standards, 100 μl of drug-free liver homogenate was added to tubes containing internal standard as well as a combination of 1 to 100 ng of predried LPV, M1, and M3/M4 (final standard concentration for each of LPV, M1, and M3/M4, 10–1000 ng/ml). Fifty microliters of 500 mM Na2CO3 then was added to the resulting samples and standards. After each tube was mixed by vortex for 30 s, 1.2 ml of 1:1 (v/v) hexane/ethyl acetate was added. Mixtures then were vortexed for 2 min to ensure complete extraction. The organic layer was separated from the aqueous layer by centrifugation at 21,000 g for 10 min at 4°C. Seven-hundred microliters of the organic supernatant was transferred to a fresh tube and dried under nitrogen gas. Extracts were reconstituted in 200 μl of 80% methanol, vortexed for 30 s, and then centrifuged at 21,000 g for 5 min at 4°C. Aliquots of the resulting extracts were transferred to autosampler vials and stored at 4°C until 10 μl could be injected into the liquid chromatographic system. Through the use of concentration estimates obtained in pilot experiments, serum and homogenates were diluted with volumes of deionized water before processing so as to ensure that their concentrations fell within the midrange of calibration curves.

LPV concentrations were determined using high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS). The liquid chromatographic system consisted of a CTC PAL autosampler (LEAP Technologies, Carbboro, NC) and an Agilent Technologies (Santa Clara, CA) 1100 series pump with a 50 mm × 4.6 mm, 5 μm, LiChrosorb RP-8 column (Phenomenex, Torrance, CA), controlled at room temperature with a flow rate of 700 μl/min. Separation of LPV and internal standard was accomplished using isocratic elution with 20:80 parts of 0.1% formic acid in water and 80% methanol. After peak elution, the mobile phase was changed to 100% methanol to wash the column. After sample injections, the syringe and injector valve each were washed three times with methanol and three times with water. MS/MS was performed on an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray source operating in a positive ion multiple reaction monitoring mode (Applied Biosystems/MSD Sciex, Foster City, CA). The multiple reaction monitoring transitions were m/z 629.3 to 447.3 (declustering potential, 55 V; collision energy, 21 V) for LPV, m/z 645.3 to 447.3 (declustering potential, 40 V; collision energy, 23 V) for M3/M4, m/z 645.3 to 447.2 (declustering potential, 80 V; collision energy, 23 V) for M1, and m/z 721.3 to 268.0 (declustering potential, 60 V; collision energy, 27 V) for RTV (the internal standard). Source temperature was set to 500°C. Peak areas of LPV, LPV metabolites, and the internal standard were determined using Analyst software, version 1.4.2 (Applied Biosystems/MSD Sciex). For quantification,
calibration curves were derived for each sample type by plotting the peak area ratio (LPV or LPV metabolite peak area/internal standard peak area) of standards against their concentrations. Linear regression equations with 1/\(x\) weighting were used to calculate sample LPV and/or LPV metabolite concentrations. Across tissues, based on signal-to-noise ratios, the lower limit of detection was <0.3 ng/ml, and the lower limit of quantification was <0.05 ng/ml.

ATPase Activation Assay. According to the manufacturer’s instructions, rat Mdr1b PREDEASY ATPase kits (Solvo Biotechnology, Szeged, Hungary) were used to determine whether LPV’s Mdr1a/b, Mrp2, and Bcrp metabolites are transported by rat Mdr1b. In brief, rat Mdr1b-expressing S9 membrane vesicles were incubated in ATPase assay buffer [5 mM diithiothreitol, 0.1 mM EGTA, 50 mM KCl, 10 mM MgCl2, 40 mM 3-(N-morpholino)propanesulfonic acid-Tris (pH 7.0), and 4 mM sodium azide] containing 5 mM ATP and M1 or M3/M4, at eight concentrations ranging from 0.14 to 300 \(\mu M\) for M1 and 0.05 to 0.01 \(\mu M\) for M3/M4 for 10 min at 37°C with and without 1.2 mM sodium orthovanadate. Because it is an established Mdr1 substrate, LPV also was assayed at eight concentrations ranging from 0.14 to 300 \(\mu M\). Specific Mdr1-related ATPase activity was determined by calculating the difference in inorganic phosphate liberation measured with and without 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). ATP-binding cassette transporters such as Mdr1b are inhibited by sodium orthovanadate, so determining vanadate-sensitive ATPase activity distinguishes Mdr1-related ATPase activity from the activity of other ATPases that are found in membrane preparations.

Blood Chemistry. Determination of total cholesterol, high-density lipoprotein/low-density lipoprotein cholesterol, triglyceride, free fatty acid (FFA), and albumin concentrations in serum was outsourced to the Battening and Best Diabetes Centre, University of Toronto. Total cholesterol, high-density lipoprotein/low-density lipoprotein cholesterol, Mrp2, and albumin concentrations were analyzed using an AutoAnalyzer and commercially available reagents (Roche Diagnostics, Indianapolis, IN), whereas FFA was analyzed using a commercially available ELISA kit (Wako Bioproducts). AAG concentrations were determined in-house using a commercially available, rat-specific ELISA kit (GenWay, San Diego, CA), according to the manufacturer’s instructions.

Polymerase Chain Reaction. In this study, Mdr1a/b, Mrp2, Bcrp, and Cyp3a2 mRNA levels in maternal liver (\(n = 4-5\) dams per group) and Mdr1a/b, Mrp2, and Bcrp mRNA levels in placenta (\(n = 3\) placentas per dam; \(n = 4-5\) dams per group) were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissue using the TRIzol method (Invitrogen, Carlsbad, CA), subjected to quality and quantity measurements using a NanoDrop (Thermo Fisher Scientific, Waltham, MA), and then reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, MD), all according to the manufacturer’s instructions. qRT-PCR was performed using LightCycler technology with SYBR Green fluorescence detection (Roche Diagnostics). PCR oligonucleotides were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON, Canada), and their sequences have been published previously (Petrovic et al., 2008). Expression levels were normalized to 18S RNA expression using the efficiency-corrected \(\Delta Ct\) method and presented as a gene-to-18S ratio. The efficiency-corrected \(\Delta Ct\) method was used because there were slight differences in the efficiencies of our primer sets.

Western Blot Analysis. Mdr1, Cyp3a2, and PXR.1 protein levels were examined in maternal liver via Western blot analysis. Tissue samples first were homogenized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100), containing freshly added diithiothreitol (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and 1 \(\times\) protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates then were incubated on ice for 20 min and subsequently centrifuged at 18,000 \(g\) for 15 min. For each sample, the supernatant was isolated and subjected to a Bradford assay (Bradford, 1976) to determine total protein concentration. Samples containing 60 \(\mu g\) (Mdr1), 20 \(\mu g\) (Cyp3a2), or 50 \(\mu g\) (PXR.1) of protein in Laemmli sample buffer were heated at 36°C for 20 min (Mdr1) or 95°C for 5 min (Cyp3a2 and PXR.1) and then separated via 8% (Mdr1) or 10% (Cyp3a2 and PXR.1) SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON, Canada). Membranes were blocked in 5% fat-free milk powder at room temperature for 1 h and incubated with mouse anti-Mdr1 (C219, 1:500, 1 mg/ml; Abcam, Inc., Cambridge, MA), rabbit anti-Cyp3a2 (ab87287, 1:1000, whole antisem; Abcam, Inc.), or goat anti-PXR.1 (A-20, 1:100, 0.2 mg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies at 4°C overnight on a rocker. After a series of washes, membranes were incubated with a peroxidase-labeled secondary antibody for 2 h at room temperature (goat anti-mouse at 1:3000 for Mdr1, goat anti-rabbit at 1:10,000 for Cyp3a2, and donkey anti-goat at 1:10,000 for PXR.1) all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive proteins were detected in membranes using ECL Plus (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and scanned using a FluorChem imaging system (Alpha Innotech, San Leandro, CA). Through the use of AlphaEaseFC (version 6.0) software, also from Alpha Innotech, the optical density (OD) of each band was determined. To confirm equivalent protein loading after determining Mdr1, Cyp3a2, and PXR.1 band ODs, a peroxidase-labeled anti-\(\beta\)-actin antibody was used (AC-15, 1:20,000, 2 mg/ml; Sigma-Aldrich). Mdr1, Cyp3a2, and PXR.1 ODs were normalized to that of \(\beta\)-actin. To correct for variability in gel transfer and other sources of variation that affect Western blot quantification, a calibrator sample was selected randomly from the vehicle group and run in each gel. Protein-to-\(\beta\)-actin ratios for each blot were converted to percentages of that blot’s calibrator sample ratio. For PXR.1 blots, 50 \(\mu g\) of HeLa cell nuclear extract also was run in each gel to provide a positive control (Santa Cruz Biotechnology, Inc.).

LPV Protein Binding Assay. LPV protein binding in rat serum was determined by ultrafiltration with Amicon Ultra 0.5-ml centrifugal filter units (molecular weight cutoff, 10,000; Millipore Canada, Ltd., Etobicoke, ON, Canada). Serum samples from 4 to 5 dams per group, all with known serum LPV concentrations, first were equilibrated for 30 min at 37°C. Then, 500 \(\mu l\) of each individual sample was transferred to centrifugal filter devices and equilibrated for another 30 min at 30°C. After equilibration, samples were centrifuged at 5000 \(g\) for 5 min in a centrifuge maintained at 30°C. After this initial spin, filtrates were collected and discarded (approximately 200 \(\mu l\)). Centrifugation then was continued with the remaining sample at 14,000 \(g\) for 15 min, and filtrate samples were collected. LPV was extracted subsequently from aliquots (100 \(\mu l\)) of filtrate samples and quantified using LC-MS/MS as described. Filtrate sample LPV concentrations then were compared with LPV concentrations in corresponding unfiltered serum samples. Percentage unbound (% unbound) was determined using the following equation: \% unbound = \((C_{filtered}/C_{unfiltered}) \times 100\). Awecka et al. (2010) validated the precision and accuracy of this method and found nonspecific binding of LPV to centrifugal filter units to be very low.

Statistical Analysis. Data were analyzed using Prism 5 for Macintosh (GraphPad Software, Inc., San Diego, CA). Concentration (e.g., LPV in serum and tissues, lipids, albumin, etc.), transporter expression (mRNA and protein), and protein binding (% unbound) data were analyzed using one-way analyses of variance (ANOVARs) followed by Bonferroni posttests. To assess the effect of uterine horn position on fetal exposure, all of the fetal concentrations were converted to a percentage of their corresponding litter’s average concentration and then subjected to one-way ANOVAs followed by Bonferroni posttests (this was done within and across groups). To analyze blood glucose, body weight, and some aspects of fetal exposure time courses, two-way ANOVAs followed by Bonferroni posttests were performed. Fetal LPV area under the concentration-versus-time curve (AUC0–\(\infty\), \(C_{\text{max}}\), and \(T_{\text{max}}\) values are presented as geometric mean values, derived directly from the concentration-versus-time curve using Prism 5’s area under curve analysis feature (trapazoidal method). Linear regression was used to determine correlations (i.e., \(r^2\) values). Levels of significance for all of the statistical analyses were set at or below \(\alpha = 0.05\), indicated as follows: \(*p < 0.05; **p < 0.01; and ***p < 0.001. All of the results are presented as mean ± S.D.

Results

Characterization of STZ-Induced GDM. All of the STZ-injected rats developed at least mild hyperglycemia within 24 h and then developed GDM (>14 mM) by GD8. All of the animals in the GDM group therefore were diabetic for 13 to 14 days before sacrifice on GD20. Blood glucose concentrations in the GDM group, relative to those of controls, were higher at GD8, GD11, and GD20 (\(p < 0.001;\))
Maternal and Fetal LPV Exposure at 45 Minutes. Relative to controls, LPV concentrations were lower in the GDM group’s serum and tissue samples 45 min after administration (Table 2). Concentrations in serum and tissue samples from the vehicle and insulin-treated groups were not statistically different from each other ($p > 0.05$). Unbound LPV concentrations also were determined in 45-min maternal serum samples. Unbound LPV concentrations were 11.7 ng/ml in the GDM group compared with 12 and 12.8 ng/ml in the vehicle and insulin-treated groups, respectively ($p > 0.05$). When expressed as percentages of total serum LPV concentrations, 1.6 ± 0.8% of the drug was unbound in the GDM group compared with 0.6 ± 0.3 and 0.6 ± 0.4% in the vehicle and insulin-treated groups, respectively ($p < 0.05$). LPV plasma protein binding in the rat has been shown to be constant across a range of total LPV concentrations that encompasses all of the values observed in our study (Kumar et al., 2004).

When LPV concentrations in tissues were divided by corresponding unbound LPV concentrations in maternal serum, significantly decreased ratios were observed in GDM maternal livers, placentas, and fetuses ($p < 0.05$; Fig. 2). This indicates that maternal and fetal exposure in the GDM group is lower than that in controls even after controlling for changes in protein binding.

Influence of Fetal Uterine Horn Position on Fetal LPV Exposure. Position within the uterine horn was noted for all of the fetal compartments that were sampled 45 min after administration (up to 10 positions, right horn 1–5 and left horn 1–5). After fetal concentrations were converted to a percentage of their corresponding litter’s average concentration, which corrects for variation in exposure between dams, one-way ANOVA failed to detect a significant overall difference between the position means ($p > 0.05$). This was true across and within this study’s three groups.

Time Course of Fetal LPV Exposure in GDM. Pups were removed surgically from dams 5, 10, 15, 20, 30, 45, and 60 min after LPV administration to generate a concentration-versus-time curve for fetal exposure within each dam (Fig. 3). Although LPV concentrations did not differ significantly until the 20-min time point, the overall time course of fetal LPV exposure for each group did. From 5 to 30 min postinjection, fetal concentrations in the vehicle group rose in a manner consistent with the distribution of LPV from maternal circulation (site of administration) to the fetal compartment. From 30 to 60 min postinjection, fetal concentrations in the vehicle group began to fall in a manner consistent with the clearance of LPV from the fetal compartment. In the GDM group, there was only one apparent phase after the 5-min time point: a phase dominated by clearance. The $C_{\text{max}}$ for LPV exposure in the GDM group occurred at the first time point that was examined (5 min), and fetal concentrations continued to fall thereafter. The insulin-treated time course exhibited both a distribution and a clearance phase, but the distribution phase lasted only 20 min. With respect to $\text{AUC}_{0-\infty}$ fetuses in the vehicle group were exposed to more LPV than fetuses in the GDM group (Table 3).

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-Treated</th>
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<td>Blood glucose (mM)</td>
<td>6 ± 0.9</td>
<td>6 ± 0.4</td>
<td>6.5 ± 1.3</td>
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<td>GD11 (mM)</td>
<td>6.3 ± 1</td>
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<td>16 ± 6.6**##</td>
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<td>GD20 (mM)</td>
<td>5.8 ± 1</td>
<td>26.8 ± 2.9***</td>
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<td>Body weight (g)</td>
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<td>244 ± 12</td>
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<td>GD11 (% baseline)</td>
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<td>GD20 (% baseline)</td>
<td>154 ± 6</td>
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<tr>
<td>Total cholesterol (mM)</td>
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<td>2.6 ± 0.6**</td>
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<td>High-density cholesterol (mM)</td>
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<td>1.3 ± 0.7</td>
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<td>Triglycerides (mM)</td>
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<td>3.2 ± 2.2**</td>
</tr>
<tr>
<td>FFAs (mEq/l)</td>
<td>1.1 ± 1.2</td>
<td>7.6 ± 6.8*</td>
<td>1.1 ± 0.7*</td>
</tr>
<tr>
<td>AAG (μg/ml)</td>
<td>49 ± 8</td>
<td>87 ± 30**</td>
<td>86 ± 12**</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>34 ± 4</td>
<td>18 ± 7***</td>
<td>36 ± 4##</td>
</tr>
<tr>
<td>Water intake (GD16[ml/kg/day])</td>
<td>160 ± 91</td>
<td>768 ± 205**</td>
<td>168 ± 24**</td>
</tr>
<tr>
<td>Fetal characteristics (GD20)</td>
<td>16 ± 2</td>
<td>11 ± 2*</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Litter size</td>
<td>16 ± 2</td>
<td>22.9 ± 0.24*</td>
<td>2.43 ± 0.18</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>5.25 ± 0.14</td>
<td>0.65 ± 0.08**</td>
<td>0.5 ± 0.08**</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>5.1 ± 0.6</td>
<td>3.6 ± 0.6**</td>
<td>5 ± 0.4*</td>
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<tr>
<td>Amniotic glucose (mM)</td>
<td>2.6 ± 0.8</td>
<td>32.2 ± 9.9**</td>
<td>7.5 ± 8.7###</td>
</tr>
</tbody>
</table>

Table 1. For the insulin-treated group, insulin treatment was initiated when blood glucose concentrations first exceeded 14 mM and was efficacious within hours. Blood glucose concentrations in the insulin-treated group fluctuated but were significantly lower than values obtained in the GDM group on GD11 and GD20 ($p < 0.01$–0.001; Table 1).

As expected during gestation, all of the animals gained weight across the study duration; however, animals with GDM were lighter than controls from GD8 until sacrifice ($p < 0.01$–0.001; see Table 1 for GD11 and GD20 values). Animals in the vehicle group and insulin-treated group did not differ in weight across the study duration ($p > 0.05$).

Blood chemistry data for animals with GDM revealed additional characteristics that were consistent with unmanaged, or poorly managed, GDM (Table 1). Hyperlipidemia was evident in the GDM group because serum cholesterol (total), triglyceride, and FFAs concentrations were profoundly elevated ($p < 0.01$–0.001). Polydipsia was observed in the GDM group on GD16. In addition, compared with the vehicle group, serum from animals in the GDM group contained higher levels of AAG ($p < 0.01$) and lower levels of albumin ($p < 0.001$). AAG concentrations in the insulin-treated group also were elevated ($p < 0.01$).

A number of fetal characteristics also were altered in the GDM group (Table 1). First, litters were smaller in the GDM group at an average of 11 pups per dam versus 16 in the vehicle group and 13 in the insulin-treated group. Second, relative to controls, GD fetuses were smaller, whereas GD placenta were larger. Consistent with this, when the weight of each fetus was divided by the weight of its placenta, the resulting ratios were found to be lower in the GDM group compared with those in the vehicle ($p < 0.01$) and insulin-treated ($p < 0.05$) groups. Finally, glucose concentrations in amniotic fluid samples obtained from the GDM group were much higher than those in samples from controls ($p < 0.05$).
insulin-treated group had an AUC_{0–60} that fell between values observed in the vehicle and GDM groups (Table 3). Maternal serum and liver concentrations were evaluated in this cohort at the 60-min time point. Serum and liver LPV concentrations in the GDM condition were lower than those of the vehicle and insulin-treated groups, which did not differ from each other (Table 3).

**Drug Transporter and Cyp3a2 Expression in GDM.** The expression of Mdr1a/b, Mrp2, Bcrp, and Cyp3a2 mRNA was determined in maternal liver, and the expression of Mdr1a/b, Mrp2, and Bcrp mRNA was determined in placenta (Fig. 4). In maternal liver, both Mdr1b and Cyp3a2 were up-regulated significantly in the GDM group. Hepatic

### TABLE 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal time course</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0–60} (μg · min/g)</td>
<td>15.63 ± 1.21</td>
<td>4.76 ± 0.77***</td>
<td>9.03 ± 1.29***</td>
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<tr>
<td>C_{max} (μg/g)</td>
<td>0.37 ± 0.06</td>
<td>0.16 ± 0.05**</td>
<td>0.21 ± 0.02*</td>
</tr>
<tr>
<td>T_{max} (min)</td>
<td>27 ± 6</td>
<td>8 ± 6*</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>Maternal samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum [LPV] (μg/ml)</td>
<td>2.17 ± 0.32</td>
<td>0.62 ± 0.32**</td>
<td>1.44 ± 0.38</td>
</tr>
<tr>
<td>Liver [LPV] (μg/g)</td>
<td>47.39 ± 15.83</td>
<td>9.13 ± 7.12*</td>
<td>42.76 ± 15.37*</td>
</tr>
</tbody>
</table>

Asterisks represent significant differences from vehicle controls, and pound signs represent significant differences from GDM. *p < 0.05; **p < 0.01; ***p < 0.001.
correlations were observed between Mdr1a and Mdr1b, Mdr1b and Mrp2, Mdr1b and Bcrp, and Mrp2 and Bcrp ($p < 0.0001$).

With respect to the expression of all of the genes and proteins examined in this study, we did not find a difference between the vehicle and the insulin-treated groups ($p > 0.05$).

**LPV Metabolite Formation in Maternal Liver at 45 min.** When metabolite concentrations were divided by corresponding LPV concentrations, there was a trend toward decreased M3/M4 ratios ($p < 0.05$ but $p > 0.05$ for Bonferroni posttests; Fig. 6A) and increased M1 ratios ($p < 0.01$ but $p > 0.05$ for Bonferroni posttests; Fig. 6C). However, when M1, a secondary metabolite, was divided by corresponding M3/M4 concentrations, the metabolite from which it is formed, a significant increase in M1 ratios was observed in the GDM group ($p < 0.001$; Fig. 6B). Decreased M3/M4-to-LPV ratios with increased M1-to-M3/M4 ratios are indicative of increased M3/M4 metabolism to M1, a process mediated by Cyp3a2. Absolute combined LPV and metabolite concentrations (i.e., [LPV] + [M3/M4] + [M1]) were $30 \pm 13$ in the GDM group compared with $176 \pm 77$ in the vehicle group ($p < 0.05$) and $182 \pm 122$ in the insulin-treated group ($p < 0.05$). ATPase activation assays were performed with the M1 and M3/M4 metabolites, but the results were somewhat inconclusive. In these assays, ATPase activity briefly rose but then fell below baseline (G. J. Anger, unpublished observations). Mdr1 has basal ATPase activity, and depression of this activity is observed with slowly transported substrates. In support of this, LPV produced a similar pattern of ATPase activity, and it is an established Mdr1 substrate.

**Hepatic PXR.1 Protein Expression in GDM.** Hepatic PXR.1 protein levels were examined due to its integral role in the regulation of both Mdr1 and Cyp3a2. As Fig. 7 illustrates, the expression of hepatic PXR.1 in the GDM group was substantially higher than that in control groups. Hepatic PXR.1/β-actin standard ratios (% calibrator) were $309 \pm 55$ in the GDM group compared with $75 \pm 33$ in the vehicle group ($p < 0.001$) and $96 \pm 14$ in the insulin-treated group ($p < 0.001$). There was a significant correlation between hepatic PXR.1 protein concentrations and hepatic concentrations of both Mdr1 ($r^2 = 0.64; p < 0.001$) and Cyp3a2 ($r^2 = 0.43; p < 0.01$).

**Discussion**

Experimental GDM had a significant impact on maternal and fetal LPV exposure in this study. Total LPV concentrations in serum from dams with GDM were less than half of control values. Although unbound serum concentrations were roughly equivalent in all of the groups, they represented a greater percentage of total serum concentrations in the GDM group. We have demonstrated previously that GDM-induced hyperlipidemia in rats, which was observed in this study (Table 1) and is observed clinically in GDM (Wiznitzer et al., 2009), leads to drug displacement (Anger and Piquette-Miller, 2010). Albumin concentrations in GDM also were reduced significantly, which means there were fewer LPV binding sites. As stated previously, decreased protein binding facilitates the passage of LPV into eliminating organs and the fetal compartment.

LPV is eliminated from the body primarily by hepatobiliary excretion. In a study of LPV metabolism and disposition in the rat, 69.5% of an intravenous LPV dose was recovered in bile after 24 h and approximately 86% of this occurred in the first 2 h (Kumar et al., 2004). In maternal liver, at 45 and 60 min, LPV concentrations in GDM were less than one third of control values. Moreover, when hepatic LPV concentrations in our study were standardized to unbound serum concentrations, lower values still were observed in the GDM group. This suggests that more rapid clearance of LPV from liver tissue occurred in the GDM group. Significantly higher hepatic Mdr1 and Cyp3a2 expression in the GDM group supports this. Functionally, data supporting enhanced hepatic metabolism of the M3/M4 primary metabolites to the M1 secondary metabolite were obtained in GDM livers. The human equivalent of Cyp3a2, CYP3A4, is the principle determinant of LPV pharmacokinetics in humans. In fact, LPV is coformulated with RTV specifically for its ability to inhibit CYP3A enzymes and thereby increase LPV exposure (Abbott Laboratories Ltd., 2010). However, it has been proposed by van Waterschoot et al. (2010) that hepatic Mdr1 and Cyp3a work in a coordinated fashion to eliminate LPV in mice. More specifically, it has been proposed that Cyp3a dramatically lowers LPV concentrations so as to prevent the saturation of Mdr1-mediated hepatobiliary excretion. Agarwal et al. (2007) provided evidence supporting the in vitro saturability of Mdr1 by LPV when they observed transporter activity at low LPV concentrations (0.5 μM) but not at high LPV concentrations (5–25 μM). Data from our study indicate that LPV metabolites also could contribute to Mdr1 saturation. By monitoring ATPase activity, we demonstrated that the M3/M4 and M1 metabolites interact with rat Mdr1 in a manner similar to LPV. That said, the fact that absolute combined LPV and metabolite concentrations were much lower in GDM livers suggests that both Mdr1-mediated efflux and Cyp3a2-mediated metabolism contributed to enhanced hepatobiliary
clearance of LPV and its metabolites in GDM (Abbott Laboratories Ltd., 2010).

Although the specific reason for the observed pharmacokinetic findings in the GDM group dams has not been fully established, it is clear that important mechanisms have been altered. In addition to the above speculation regarding hepatic elimination, it is plausible that there was a contribution from increased renal elimination. In nonpregnant individuals, approximately 10.4 ± 2.3% of a LPV dose is recovered in urine after 8 days (Abbott Laboratories Ltd., 2010). Urinary output dramatically increases in STZ-induced diabetes as a result of osmotic diuresis (Anger et al., 2009; Anger and Piquette-Miller, 2010). Although urinary output was not measured in this study, water intake was nearly 5-fold higher in the GDM group than that in controls (Table 1), and a strong positive correlation exists between urinary output and water intake (G. J. Anger, unpublished observations).

To better assess fetal exposure, LPV concentrations were determined in placentas and fetuses at 45 min and in fetuses from a second cohort at multiple time points. At 45 min, LPV concentrations were significantly lower in the GDM group. It stands to reason that reduced fetal exposure in the GDM group was largely the result of reduced maternal exposure. Because maternal serum LPV levels were much lower in the GDM group, less LPV would have been available for placental transfer; however, when placental and fetal LPV concentrations were standardized to unbound LPV concentrations in maternal serum, ratios in the GDM group were still lower than control ratios.

**Fig. 5.** Hepatic Mdr1 and Cyp3a2 protein expression in GDM. Mdr1 and Cyp3a2 protein concentrations were determined in maternal liver via Western blot analysis. A, representative blots showing Mdr1, Cyp3a2, and β-actin expression in samples from each group. B and C, quantified Mdr1 (B) and Cyp3a2 (C) results are presented as mean percentages of a calibrator sample’s values ± S.D. and are from four to five dams per group. All of the results are presented as mean ± S.D. Asterisks represent significant differences from vehicle controls, and pound signs represent significant differences from GDM.

**Fig. 6.** Standardized concentration ratios for LPV metabolites in maternal liver 45 min after LPV administration. Forty-five minutes after LPV administration, LPV and LPV metabolite concentrations were determined in maternal liver samples via LC-MS/MS. A, because M3/M4 is a product of LPV (i.e., a primary metabolite), concentrations of M3/M4 were standardized to LPV concentrations. B, because M1 is a product of M3/M4 (i.e., a secondary metabolite), concentrations of M1 were standardized to M3/M4 concentrations. C, M1 concentrations also were standardized to LPV concentrations. All of the results are presented as mean ± S.D. of four to five dams per group. All of the results are presented as mean ± S.D. Asterisks represent significant differences from vehicle controls, and pound signs represent significant differences from GDM.
that fetal exposure was reduced in STZ-induced GDM raises ques-
tions about GDM’s potential impact on vertical HIV transmission risk and could warrant clinical investigation.

Induction of Mdr1 and Cyp3a has been observed in tissues from male rats with STZ-induced diabetes (Maeng et al., 2007; Kameyama et al., 2008; Hasegawa et al., 2010), but reports in female rats with STZ-induced diabetes or STZ-induced GDM are limited (Mulay and Varma, 1984; Anger et al., 2009; Anger and Piquette-Miller, 2010). We posit that disruptions to lipid and glucose homeostasis underlie the alterations to drug transporter and Cyp3a2 expression that were observed in STZ-induced GDM. In brief, it is known that drug transporters and metabolic enzyme transcription is controlled by several nuclear receptors, such as PXR, which can be activated themselves by lipids and glucose (Handschin and Meyer, 2005). We have demonstrated previously that nuclear receptor networks with roles in the regulation of hepatic drug transporters and metabolic enzymes are activated in STZ-induced GDM (Anger and Piquette-Miller, 2010). In this study, we observed a significant up-regulation in the PXR.1 isoform in GDM livers. PXR is Mdr1 and Cyp3a2’s primary transcriptional regulator, so not surprisingly, its hepatic protein concentrations positively correlated with hepatic Mdr1 and Cyp3a2 concentrations. It is conceivable that a similar regulatory effect may be at work in the placenta, although recent work with PXR knockout mice has drawn the role of placental PXR in placental transporter regulation into question (Gahir and Piquette-Miller, 2011).

Although this study focused on LPV, the disposition of other drugs would likely be altered in experimental GDM. Protein binding aside, many drugs that are used today are substrates for MDR1 and/or BCRP (Chandra and Brouwer, 2004), and CYP3A4 is believed to be involved in the metabolism of approximately half of all drugs (Guengerich, 1999). Relating this to anti-HIV therapy, LPV is taken typically with other anti-HIV drugs, and most of these drugs are substrates for at least one of MDR1, BCRP, and CYP3A4 (reviewed in Kis et al., 2010). For example, zidovudine/azidothymidine and lamivudine/3'-dideoxy-3'-thiacytidine are BCRP substrates and often are taken alongside LPV/RTV in pregnancy (Kis et al., 2010).

In conclusion, our findings demonstrate that maternal and fetal LPV exposure is reduced in a rat model of GDM. For maternal exposure, our data suggest that PXR-mediated hepatic Mdr1 and Cyp3a2 up-regulation and decreased protein binding are responsible. Enhanced renal elimination in GDM also may contribute but was not evaluated directly. For fetal exposure, our data suggest that although fetal exposure is dictated largely by maternal exposure, increased placental transporter expression in GDM serves to further limit placental transfer. Through the use of a novel sampling procedure to characterize fetal exposure, it was determined that GDM fetuses accumulated very little LPV. This study further advances our understanding of the impact that GDM can have on drug disposition mechanisms and provides preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction. If confirmed in humans, the effects reported in this study would need to be considered when exposure targets are not met in GDM pregnancies. Studies using the clinical formulation of LPV are underway and will determine whether the effects described in this study persist in the presence of RTV.

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LOPINAVIR EXPOSURE IN A RAT MODEL OF GESTATIONAL DIABETES

1859

Authorship Contributions

Participated in research design: Anger and Piquette-Miller.

Conducted experiments: Anger.

Performed data analysis: Anger.

Wrote or contributed to the writing of the manuscript: Anger and Piquette-Miller.

References


Mechanisms of reduced maternal and fetal lopinavir exposure in a rat model of gestational diabetes

Gregory J. Anger & Micheline Piquette-Miller

*Drug Metabolism & Disposition*

**Supplemental figure 1.** Cartoon illustration of fetal positioning within the pregnant rat at approximately GD20. **A,** The rat uterus consists of two uterine horns, a left horn (LH) and a right horn (RH), that extend into the abdominal cavity. In my experiments, fetuses were identified first by the uterine horn in which they implanted (LH or RH) and then by their relative order, with fetus one being the fetus closest to the cervical stump/vagina (LH1-n or RH1-n). **B,** Rat fetuses reside inside isolated compartments with independent access to maternal circulation (shown in red). Since rat placentation is hemochorial, fetal trophoblasts are in direct contact with maternal blood.