

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

Gregory J. Anger & Micheline Piquette-Miller

Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto,
Toronto, Ontario, Canada (G.J.A., M.P.-M.)

Running title: Lopinavir exposure in a rat model of gestational diabetes

Corresponding author:

Dr. Micheline Piquette-Miller

Leslie Dan Faculty of Pharmacy

University of Toronto

144 College St.

Toronto, Ontario, Canada, M5S 3M2

Telephone: 1-416-946-3057

Fax: 1-416-978-8511

E-mail: m.piquette.miller@utoronto.ca

Pages: 38

Tables: 3

Figures: 7 (+ 1 Supplemental figure)

References: 45

Abstract word count: 243

Introduction word count: 794

Discussion word count: 1,652

Nonstandard abbreviations: AAG, α 1-acid glycoprotein; AUC, area under concentration versus time curve; BCRP, breast cancer resistance protein; Cyp, cytochrome P450; GDM, gestational diabetes mellitus; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; LC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; LPV, lopinavir; MDR1, multidrug resistance protein 1; MRP2, multidrug resistance-associated protein 2; PXR, pregnane X receptor; RTV, ritonavir.

Abstract

Lopinavir 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 is altered in rodent models of GDM. Because lopinavir 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 minutes after lopinavir injection. In another cohort, fetuses were serially extracted 5-60 minutes after injection. Lopinavir was quantified using LC-MS/MS. Expression of relevant transporters, like Mdr1, and Cyp3a2, which metabolizes lopinavir in rodents, was measured in maternal liver via qRT-PCR and western blot. Expression of relevant transporters was also measured in placenta via qRT-PCR. Protein binding was determined by ultrafiltration. Relative to controls, we observed dramatically reduced maternal and fetal lopinavir exposure in GDM. As compared to controls, maternal hepatic Mdr1 and Cyp3a2 were upregulated and protein binding was reduced in the GDM group. Increased Mdr1 and Cyp3a2-mediated hepatobiliary clearance, coupled with a larger unbound lopinavir fraction, is likely to have facilitated hepatic elimination, thereby decreasing maternal and fetal exposure. Not surprisingly, upregulation of Mdr1 and Cyp3a2's transcriptional regulator, pregnane X receptor, was demonstrated in maternal liver via western blot. Upregulation of Mdr1 in placentas isolated from the GDM group likely also contributed to decreased fetal exposure to lopinavir. This study provides preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction.

Introduction

Antiretroviral drugs are administered to human immunodeficiency virus (HIV)-positive pregnant women in order to prevent both maternal disease progression and vertical HIV transmission. Lopinavir (LPV) co-formulated 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 utilize LPV/RTV as part of their highly active antiretroviral therapy (HAART) 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 (Guiard-Schmid et al., 2003) and is likely 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 extensively metabolized by cytochrome P450 3A4 (CYP3A4) (van Waterschoot et al., 2010). In the liver, MDR1 facilitates LPV's hepatobiliary excretion while CYP3A4 converts it to increasingly hydrophilic metabolites (Fig. 1). Large intersubject differences in the basal expression and activity of CYP3A4 and MDR1 have been reported (Westlind-Johnsson et al., 2003; Meier et al., 2006). In the placenta, MDR1 plays a barrier role by actively transporting substrates back into the systemic circulation. LPV is also highly (>99%) bound to blood proteins, including albumin and α 1-acid glycoprotein (AAG), so even small changes in binding result in large changes in unbound drug fractions. As 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 (Reviewed in Morgan et al., 2008); 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 HAART drugs. In rats with streptozotocin (STZ)-induced GDM, we have recently observed that the plasma protein binding of the protease inhibitor saquinavir decreases because of displacement by elevated lipids while the expression of hepatic Mdr1 and a variety of cytochrome P450 and UDP-glucuronosyltransferase enzymes increases (Anger and Piquette-Miller, 2010). Also, altered disposition has been observed for a variety of drugs in both non-pregnant humans with diabetes (Reviewed in Gwilt et al., 1991) and non-pregnant 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 the 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 like 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 upregulated). 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. Since the quantitative contribution of these processes could change with time, we utilized a novel fetal sampling procedure to determine the time course of fetal exposure. Due to its role in Mdr1 and Cyp3a2 transcription (Lehmann et al., 1998; Tirona, 2011), pregnane X receptor (PXR) expression in maternal liver was also 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.

Methods

Pharmaceutical and chemical products. LPV and RTV were purchased from USP (PubChem Compound IDs 92727 and 392622, Rockville, MD). Lopinavir 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 other chemical products, including streptozotocin (PubChem Compound ID 5300), were purchased from Sigma-Aldrich (Oakville, ON, Canada). For intravenous administration, LPV was dissolved in 4:3:3 (v/v/v) ethanol:propylene glycol: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 Laboratories, Inc. (Senneville, QC, Canada) and housed individually in a temperature controlled facility on a 12:12 hr light-dark cycle. Rats were given free access to water and standard chow (Harlan Teklad Global Diet 2018). 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 randomly assigned to one of three groups: non-treated 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 streptozotocin

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 animals, body weight was measured prior to respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured prior to respective injections (GD6) and on gestational days 7-9, 11 and 20. For animals in the insulin-treated group, blood glucose was measured prior to STZ injection and then daily until sacrifice in order to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a non-fasted 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 employed according to the manufacturer's instructions (Autokit Glucose, Wako Chemicals USA, Inc., Richmond, VA). Mild hyperglycemia was defined as blood glucose concentration at 10 to 14 mM and diabetes was defined as blood glucose concentration at >14 mM. For diabetic rats receiving insulin treatment, treatment was initiated once diabetes was confirmed and consisted of daily subcutaneous injection of insulin glargine as required. On GD20, one cohort of animals (n = 4-5/group) received LPV (10 mg/kg; intravenous) 45 ± 1 min prior to being sacrificed to collect maternal blood (cardiac puncture) and liver as well as up to 10 fetal compartments/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). Amniotic fluid was also 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/group) received LPV (10 mg/kg; intravenous) while anesthetized with continuous inhalation anesthesia (5% isoflurane for induction and 1.5-2% isoflurane for maintenance) and was subsequently subjected to sequential sampling of placentas and fetuses (collectively referred to as fetal compartments) over a period of one hour.

Following LPV administration, a 3-4 cm midline abdominal incision was made so as to provide access to the uterine horns via the intraperitoneal cavity. Five min post-injection, one of the uterine horns was externalized and the most distal fetal unit identified (Supplemental Fig. 1 illustrates fetal positioning within the pregnant rat at approximately GD20). The maternal vasculature supplying this fetal unit's placenta was then blocked via ligation in order 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, were then 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 post-injection. 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). Following the collection of the 60 min fetal unit, maternal blood (cardiac puncture) and liver was collected. Throughout this procedure, maternal body temperatures were kept at approximately 37 °C using heating pads.

Determination of lopinavir and lopinavir metabolite concentrations. For the preparation of samples and standards, a liquid-liquid extraction procedure was adapted from Wang and colleagues (Wang et al., 2006). Briefly, 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 μ l of serum or homogenate was added to tubes containing 20 ng of pre-dried internal standard (internal standard = RTV; final internal standard concentration: 200 ng/ml). For LPV standards, 100 μ l of drug-free serum or homogenate was added to tubes containing both 20 ng of pre-dried internal standard and 1-100 ng of pre-dried LPV (final LPV standard concentrations: 10-1000 ng/ml). LPV metabolites M3/M4 and M1 were only measured in maternal liver because the placenta and fetus are not significant sites of LPV metabolism. For LPV metabolite standards, 100 μ l of drug-free liver homogenate was added to tubes containing internal standard as well as a combination of 1-100 ng of pre-dried LPV, M3/M4 and M1 (final standard concentration for each of LPV, M3/M4 and M1: 10-1000 ng/ml). Fifty μ l of 500 mM Na_2CO_3 was then added to the resulting samples and standards. After mixing by vortex for 30 s, 1.2 ml of 1:1 (v/v) hexane:ethyl acetate was added to each tube. Mixtures were then 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 μ l of the organic supernatant was transferred to a fresh tube and dried under nitrogen gas. Extracts were reconstituted in 200 μ l 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 μ l could be injected into the liquid chromatographic (LC) system. Using concentration

estimates obtained in pilot experiments, serum and homogenates were diluted with volumes of deionized water prior to processing so as to ensure that their concentrations fell within the mid-range 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, Carrboro, 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. Following sample injections, the syringe and injector valve were each washed 3 times with methanol and 3 times with water. MS/MS was performed on an API 4000 triple quadrupole mass spectrometer equipped with a Turbo Ionspray source operating in a positive ion multiple reaction monitoring (MRM) mode (AB Sciex, Concord, ON, Canada). The MRM transitions were m/z 629.3-447.3 (declustering potential 58 volts, collision energy 21 volts) for LPV, m/z 645.3-447.3 (declustering potential 40 volts, collision energy 23 volts) for M3/M4, m/z 643.3-447.2 (declustering potential 80 volts, collision energy 23 volts) for M1 and m/z 721.3-268.0 (declustering potential 60 volts, collision energy 27 volts) 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 (AB 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 then used to calculate sample LPV and/or LPV metabolite concentrations. Across tissues, based on signal-to-noise ratios, the lower limit of detection was < 3 ng/ml and the lower limit of quantification was < 10 ng/ml.

ATPase activation assay. Following the manufacturer's instructions, rat Mdr1b PREDEASY ATPase kits (Solvo Biotechnology, Szeged, Hungary) were used to determine if LPV's M3/M4 and M1 metabolites are transported by rat Mdr1b. Briefly, rat Mdr1b-expressing Sf9 membrane vesicles were incubated in ATPase assay buffer (5 mM DTT, 0.1 mM EGTA, 50 mM KCl, 10 mM MgCl₂, 40 mM MOPS-Tris [pH 7.0] and 4 mM sodium azide) containing 5 mM ATP and M3/M4 or M1, at eight concentrations ranging from 0.05-100 μM for M3/M4 and 0.14-300 μM for M1, for 10 min at 37 °C with and without 1.2 mM sodium orthovanadate. Because it is an established Mdr1 substrate, LPV was also assayed at eight concentrations ranging from 0.14-300 μM. Specific Mdr1-related ATPase activity was determined by calculating the difference of inorganic phosphate liberation measured with and without 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). ABC transporters like 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 (HDL)/low-density lipoprotein (LDL) cholesterol, triglyceride, FFA and albumin concentrations in serum was outsourced to the Banting and Best Diabetes Centre, University of Toronto. Total cholesterol, HDL/LDL cholesterol, triglyceride and albumin concentrations were analyzed using an autoanalyzer and commercially available reagents (Roche Diagnostics, Laval, QC, Canada) while FFA was analyzed using a commercially available ELISA kit (Wako Chemicals USA, Inc.). AAG concentrations were determined in-house using a commercially available, rat-specific ELISA kit (GenWay, San Diego, CA), according to the manufacturer's instructions.

PCR. In this study, *Mdr1a/b*, *Mrp2*, *Bcrp* and *Cyp3a2* mRNA levels in maternal liver ($n = 4-5$ dams/group) and *Mdr1a/b*, *Mrp2* and *Bcrp* mRNA levels in placenta ($n = 3$ placentas/dam; $n = 4-5$ dams/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 Scientific Limited, Nepean, ON) and then reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada), all according to the manufacturer's instructions. qRT-PCR was performed using LightCycler technology with SYBR Green I 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 previously published (Petrovic et al., 2008). Expression levels were normalized to 18S rRNA expression using the efficiency-corrected ΔC_t method and presented as a gene:18S

ratio. The efficiency-corrected ΔC_t method was employed because there were slight differences in the efficiencies of our primer sets.

Western blotting. Mdr1, Cyp3a2 and PXR.1 protein levels were examined in maternal liver via western blotting. Tissue samples were first homogenized in radioimmunoprecipitation (RIPA) assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X 100), containing freshly added DTT (1 mM), PMSF (0.5 mM) and 1X protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates were then 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 to determine total protein concentration. Samples containing 60 μ g (Mdr1), 20 μ g (Cyp3a2) or 50 μ 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 hr and incubated with mouse anti-Mdr1 (C219, 1:500, 1 mg/ml, Abcam, Inc., Cambridge, MA), rabbit anti-Cyp3a2 (ab78279, 1:1000, whole antiserum, 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 hr at room temperature (goat anti-mouse at 1:3,000 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 (Amersham Biosciences, Baie d'Urfé, QC) and scanned using an Alpha Innotech FluorChem imaging system (San Diego, CA). Using 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- β -actin antibody was employed (AC-15, 1:20,000, 2 mg/ml, Sigma-Aldrich). Mdr1, Cyp3a2 and PXR.1 ODs were normalized to β -actin. To correct for variability in gel transfer and other sources of variation that affect western blot quantification, a calibrator sample was randomly selected from the vehicle group and run in each gel. Protein/ β -actin ratios for each blot were converted to percentages of that blot's calibrator sample ratio. For PXR.1 blots, 50 μ g of HeLa cell nuclear extract was also run in each gel to provide a positive control (Santa Cruz Biotechnology, Inc.).

Lopinavir protein binding assay. LPV protein binding in rat serum was determined by ultrafiltration (UF) with Amicon Ultra 0.5 ml centrifugal filter units (MWCO 10,000; Millipore Canada, Ltd., Etobicoke, ON, Canada). Serum samples from 4-5 dams/group, all with known serum LPV concentrations, were first equilibrated for 30 min at 37 °C. Then, 500 μ 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 5,000 *g* for 5 min in a centrifuge maintained at 30 °C. After this initial spin, filtrates were collected and discarded (approximately 200 μ l). Centrifugation was then continued with the remaining sample at 14,000 *g* for 15 min and filtrate samples collected. LPV was

subsequently extracted from aliquots (100 μ l) of filtrate samples and quantified using LC-MS/MS as described. Filtrate sample LPV concentrations were then compared to LPV concentrations in corresponding unfiltered serum samples. Percent unbound (% unbound) was determined using the following equation: % unbound = $(C_{\text{filtered}}/C_{\text{unfiltered}}) \times 100$. Aweeka and colleagues validated the precision and accuracy of this method and found non-specific binding of LPV to centrifugal filter units to be very low (Aweeka et al., 2010).

Statistics. Data were analyzed using Prism 5 for Macintosh (GraphPad Software, Inc., San Diego, CA). Concentration (e.g., lopinavir in serum and tissues, lipids, albumin, etc.), transporter expression (mRNA and protein) and protein binding (% unbound) data were analyzed using one-way ANOVAs followed by Bonferroni post-tests. To assess the effect of uterine horn position on fetal exposure, all fetal concentrations were converted to a percentage of their corresponding litter's average concentration and then subjected to one-way ANOVAs followed by Bonferroni post-tests (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 post-tests were performed. Fetal LPV AUC₀₋₆₀, C_{max} and T_{max} values are presented as geometric group means, derived directly from the concentration-versus-time curve using Prism 5's area under curve analysis feature (trapezoidal method). Linear regression was used to determine correlations (i.e., r^2 values). Levels of significance for all statistical analyses were set at or below $\alpha = 0.05$, indicated as follows: */#, $p < 0.05$, **/##, $p < 0.01$ and ***/###, $p < 0.001$. All results are presented as mean \pm 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 animals in the GDM group were, therefore, diabetic for 13 to 14 days before sacrifice on GD20. Blood glucose concentrations in the GDM group, relative to controls, were higher at GD8, GD11 and GD20 ($p < 0.001$; 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 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 as serum cholesterol (total), triglyceride and FFA concentrations were profoundly elevated ($p < 0.01-0.001$). Polydipsia was observed in the GDM group on GD16. Also, as compared to 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 were also elevated ($p < 0.01$).

A number of fetal characteristics were also 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, GDM fetuses were smaller while GDM placentas 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 GDM as compared to 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 in samples from controls ($p < 0.05$).

Maternal and fetal lopinavir exposure at 45 min. 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 were also determined in 45 min maternal serum samples. Unbound LPV concentrations were 11.7 ng/ml in the GDM group as compared to 12 ng/ml 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, $1.6 \pm 0.8\%$ of the drug was unbound in the GDM group as compared to $0.6 \pm 0.3\%$ and $0.6 \pm 0.4\%$ 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 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. 6). This indicates that maternal and fetal exposure in GDM is lower than in controls even after controlling for changes in protein binding.

Influence of fetal uterine horn position on fetal lopinavir exposure. Position within the uterine horn was noted for all fetal compartments that were sampled 45 min after administration (up to 10 positions: right horn 1-5 and left horn 1-5). After converting fetal concentrations 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 lopinavir exposure in GDM. Pups were surgically removed from dams 5, 10, 15, 20, 30, 45 and 60 min after LPV administration in order to generate a concentration-versus-time curve for fetal exposure within each dam (Fig. 3). While 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 post-injection, 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 post-injection, 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_{\max} for LPV exposure in GDM 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 area under the concentration-versus-time curve (AUC_{0-60}), fetuses in the vehicle group were exposed to more LPV than fetuses in the GDM group (Table 3). The 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 significantly upregulated in the GDM group. Hepatic *Mdr1b/18S* standard ratios were 3.55 ± 2 in GDM compared with 0.33 ± 0.41 in the vehicle group ($p < 0.05$) and 0.26 ± 0.16 in the insulin-treated group ($p < 0.05$). Hepatic *Cyp3a2/18S* standard ratios were 1 ± 0.35 in GDM compared with 0.43 ± 0.04 in the vehicle group ($p < 0.05$) and 0.55 ± 0.22 in the insulin-treated group ($p > 0.05$).

Significant mRNA findings in maternal liver were confirmed at the level of protein (Fig. 5). Hepatic Mdr1/ β -actin standard ratios (% calibrator) were 149 ± 41 in GDM compared with 92 ± 6 in the vehicle group ($p < 0.05$) and 85 ± 6 in the insulin-treated group ($p < 0.05$). Hepatic Cyp3a2/ β -actin standard ratios (% calibrator) were 170 ± 63 in GDM compared with 78 ± 27 in the vehicle group ($p < 0.05$) and 64 ± 14 in the

insulin-treated group ($p < 0.05$). Hepatic *Mdr1a* and *Cyp3a2* protein concentrations correlated well with corresponding *Mdr1a* ($r^2 = 0.9$; $p < 0.001$) and *Cyp3a2* ($r^2 = 0.56$; $p < 0.01$) mRNA concentrations.

In placenta, both *Mdr1b* and *Bcrp* were significantly upregulated in the GDM group. Placental *Mdr1b/18S* standard ratios were 1.9 ± 0.43 in GDM compared with 0.95 ± 0.18 in the vehicle group ($p < 0.01$) and 1.06 ± 0.39 in the insulin-treated group ($p < 0.05$). Placental *Bcrp/18S* standard ratios were 2.49 ± 0.96 in GDM compared with 1.08 ± 0.27 in the vehicle group ($p < 0.05$) and 1.45 ± 0.64 in the insulin-treated group ($p > 0.05$). Significant mRNA findings in placenta were not confirmed at the level of protein due to insufficient sample volume. Intraplacental 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 genes and proteins examined in this study, we did not find a difference between the vehicle and insulin-treated groups ($p > 0.05$).

Lopinavir 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 post-tests; Fig. 6A) and increased M1 ratios ($p < 0.01$ but $p > 0.05$ for Bonferroni post-tests; 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 is 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]) concentrations were 30 ± 13 in GDM compared with 176 ± 77 in the vehicle group ($p < 0.05$) and 182 ± 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 (data not shown). 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 figure 7 illustrates, the expression of hepatic PXR.1 in the GDM group was substantially higher than in control groups. Hepatic PXR.1/ β -actin standard ratios (% calibrator) were 309 ± 55 in GDM compared with 75 ± 33 in the vehicle group ($p < 0.001$) and 96 ± 14 in the insulin-treated group ($p < 0.001$). There was a significant correlation between hepatic PXR.1 protein concentrations and the 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. While unbound serum concentrations were roughly equivalent in all groups, they represented a greater percentage of total serum concentrations in the GDM group. We have previously demonstrated 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 were also significantly reduced, which means there were fewer LPV binding sites. As previously stated, 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 hrs and approximately 86% of this occurred in the first 2 hrs (Kumar et al., 2004). In maternal liver, at 45 and 60 min, LPV concentrations in GDM were less than a third of control values. Moreover, when hepatic LPV concentrations in our study were standardized to unbound serum concentrations, lower values were still observed in GDM. This suggests that more rapid clearance of LPV from liver tissue occurred in the GDM group. Significantly higher hepatic Mdr1 and Cyp3a2 expression in GDM 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 co-formulated with RTV specifically for its

ability to inhibit CYP3A enzymes and thereby increase LPV exposure (Abbott Laboratories Ltd., 2010). It has been proposed by van Waterschoot and colleagues, however, that hepatic Mdr1 and Cyp3a work in a coordinated fashion to eliminate LPV in mice (van Waterschoot et al., 2010). More specifically, it has been proposed that Cyp3a drastically lowers LPV concentrations so as to prevent the saturation of Mdr1-mediated hepatobiliary excretion. Agarwal and colleagues provided evidence supporting the *in vitro* saturability of Mdr1 by LPV when they observed transporter activity at low (0.5 μ M) LPV concentrations but not at high (5-25 μ M) LPV concentrations (Agarwal et al., 2007). Data from our study indicate that LPV metabolites could also 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).

While 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 non-pregnant individuals, approximately $10.4 \pm 2.3\%$ 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). While urinary output was not measured in this study, water intake was nearly 5-

fold higher in the GDM group than in controls (Table 1) and a strong positive correlation exists between urinary output and water intake (unpublished data).

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 GDM. It stands to reason that reduced fetal exposure in GDM was largely the result of reduced maternal exposure. As maternal serum LPV levels were much lower in GDM, 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. This suggests that LPV was also being actively excluded from the fetal compartment. This is likely attributable to placental efflux and this hypothesis is supported by increased *Mdr1b* mRNA expression in GDM placentas.

The time courses of fetal exposure for both the vehicle and insulin-treated groups exhibited an accumulation phase whereas the GDM group's time course did not. Accordingly, fetuses in the vehicle and insulin-treated groups were exposed to more LPV than fetuses in the GDM group. In the absence of placental transporter data, this finding would be surprising given the GDM group's decreased albumin concentrations and decreased LPV protein binding. Studies employing the human cotyledon perfusion model have shown that albumin plays a major role in placental LPV transfer (Gavard et al., 2006; Ceccaldi et al., 2010). For example, Gavard *et al* demonstrated that LPV transfer was $23.6 \pm 6.9\%$ at an albumin concentration of 2 g/l, $20.7 \pm 10\%$ at 10 g/l and only $3.3 \pm 0.5\%$ at 40 g/l (Gavard et al., 2006). Gavard *et al*'s data predicts higher (15%) transfer in our GDM group than in our control groups (6-7%), which is not what we observed.

The role of fetal exposure to antiretrovirals in the prevention of vertical HIV transmission is not fully established but it is generally acknowledged that exposure is desirable. The concentration of protease inhibitors in human cord blood after *in utero* exposure is low but varies greatly by molecule (Mirochnick et al., 2002; Chappuy et al., 2004). While the protease inhibitors RTV, indinavir and saquinavir have low cord blood/maternal blood ratios that range from 0-0.01 (Chappuy et al., 2004), LPV has been found to have a comparatively high ratio of 0.2 ± 0.13 (Stek et al., 2006). Importantly, Gavard *et al* demonstrated in the human cotyledon perfusion model that this ratio leads to fetal compartment concentrations that exceed the 50% HIV inhibitory concentration for LPV under normal physiological conditions (Gavard et al., 2006; Abbott Laboratories Ltd., 2010). The fact that fetal exposure was reduced in STZ-induced GDM raises questions 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. Briefly, it is known that drug transporters and metabolic enzyme transcription is controlled by several nuclear receptors, like PXR, which can themselves be activated by lipids and glucose (Handschin and Meyer, 2005). We have previously demonstrated 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 upregulation 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).

While 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 typically taken 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/AZT and lamivudine/3TC are BCRP substrates and are often 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 upregulation and decreased protein binding are responsible. Enhanced renal elimination in GDM may also contribute but was not directly evaluated. For fetal exposure, our data suggest that while fetal exposure is largely dictated by maternal exposure, increased placental transporter expression in GDM serves to further limit placental transfer. Using 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 employing the clinical formulation of LPV are underway and will determine if the effects described in this study persist in the presence of RTV.

Acknowledgements

We thank Michelle Young and Dr. Matthew Forbes at University of Toronto's Advanced Instrumentation for Molecular Structure (AIMS) Lab (Department of Chemistry) for their invaluable expertise and technical assistance with our LC-MS/MS experiments. We also thank Teresa Feng, Sarabjit Gahir, Ji Zhang and Nick Zhidkov for their input and assistance throughout this study. Teresa Feng was instrumental in validating our approach to determining LPV protein binding in highly turbid serum samples.

Authorship Contributions

Participated in research design: Anger, Piquette-Miller

Conducted experiments: Anger

Contributed new reagents or analytic tools: N/A

Performed data analysis: Anger

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

References

- Abbott Laboratories Ltd. (2010) Kaletra (product monograph).
- Agarwal S, Pal D and Mitra A (2007) Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. *Int J Pharm* **339**:139-147.
- Anger G, Magomedova L and Piquette-Miller M (2009) Impact of acute streptozotocin-induced diabetes on ABC transporter expression in rats. *Chem Biodivers* **6**:1943-1959.
- Anger GJ and Piquette-Miller M (2010) Impact of hyperlipidemia on plasma protein binding and hepatic drug transporter and metabolic enzyme regulation in a rat model of gestational diabetes. *J Pharmacol Exp Ther* **334**:21-32.
- Aweeka FT, Stek A, Best BM, Hu C, Holland D, Hermes A, Burchett SK, Read J, Mirochnick M, Capparelli EV and International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT) P1026s Protocol Team (2010) Lopinavir protein binding in HIV-1-infected pregnant women. *HIV Med* **11**:232-238.
- Boffito M, Arnaudo I, Raiteri R, Bonora S, Sinicco A, Di Garbo A, Reynolds HE, Hoggard PG, Back DJ and Di Perri G (2002) Clinical use of lopinavir/ritonavir in a salvage therapy setting: pharmacokinetics and pharmacodynamics. *AIDS* **16**:2081-2083.
- Ceccaldi P-F, Mandelbrot L, Ferreira C and Farinotti R (2010) Modulations of human placental transfer of lopinavir, ritonavir and enfuvirtide. *Retrovirology* **7**:P164.
- Chandra P and Brouwer KLR (2004) The complexities of hepatic drug transport: current knowledge and emerging concepts. *Pharma Res* **21**:719-735.

- Chappuy H, Tréluyer J-M, Rey E, Dimet J, Fouché M, Firtion G, Pons G and Mandelbrot L (2004) Maternal-fetal transfer and amniotic fluid accumulation of protease inhibitors in pregnant women who are infected with human immunodeficiency virus. *Am J Obstet Gynecol* **191**:558-562.
- Choi JS and Burm JP (2008) Effect of pioglitazone on the pharmacokinetics of verapamil and its major metabolite, norverapamil, in rats. *Arch Pharm Res* **31**:1200-1204.
- Gahir SS and Piquette-Miller M (2011) Gestational and pregnane X receptor-mediated regulation of placental ATP-binding cassette drug transporters in mice. *Drug Metab Dispos* **39**:465-471.
- Gavard L, Gil S, Peytavin G, Ceccaldi P-F, Ferreira C, Farinotti R and Mandelbrot L (2006) Placental transfer of lopinavir/ritonavir in the ex vivo human cotyledon perfusion model. *Am J Obstet Gynecol* **195**:296-301.
- González-Tomé MI, Ramos Amador JT, Guillen S, Solís I, Fernández-Ibieta M, Muñoz E, Almeda J, Rojano X, Rojo P, Nieto O and Spanish Cohort of HIV-Infected Mother-Infant Pairs (2008) Gestational diabetes mellitus in a cohort of HIV-1 infected women. *HIV Med* **9**:868-874.
- Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1-17.
- Guiard-Schmid J-B, Poirier J-M, Meynard J-L, Bonnard P, Gbadoe AH, Amiel C, Calligaris F, Abraham B, Pialoux G, Girard P-M, Jaillon P and Rozenbaum W (2003) High variability of plasma drug concentrations in dual protease inhibitor regimens. *Antimicrob Agents Chemother* **47**:986-990.

- Gwilt PR, Nahhas RR and Tracewell WG (1991) The effects of diabetes mellitus on pharmacokinetics and pharmacodynamics in humans. *Clin Pharmacokinet* **20**:477-490.
- Hanada K, Ikemi Y, Kukita K, Mihara K and Ogata H (2008) Stereoselective first-pass metabolism of verapamil in the small intestine and liver in rats. *Drug Metab Dispos* **36**:2037-2042.
- Handschin C and Meyer UA (2005) Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. *Arch Biochem Biophys* **433**:387-396.
- Hasegawa Y, Kishimoto S, Shibatani N, Inotsume N, Takeuchi Y and Fukushima S (2010) Effects of insulin on CYP3A activity and nifedipine disposition in streptozotocin-induced diabetic rats. *J Pharm Pharmacol* **62**:883-889.
- Kameyama N, Arisawa S, Ueyama J, Kagota S, Shinozuka K, Hattori A, Tatsumi Y, Hayashi H, Takagi K and Wakusawa S (2008) Increase in P-glycoprotein accompanied by activation of protein kinase C α and NF- κ B p65 in the livers of rats with streptozotocin-induced diabetes. *Biochim Biophys Acta* **1782**:355-360.
- Kis O, Robillard K, Chan GNY and Bendayan R (2010) The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters. *Trends Pharmacol Sci* **31**:22-35.
- Kourtis A, Bansil P, McPheeters M, Meikle S, Posner S and Jamieson D (2006) Hospitalizations of pregnant HIV-infected women in the USA prior to and during the era of HAART, 1994-2003. *AIDS* **20**:1823-1831.

- Kumar GN, Jayanti VK, Johnson MK, Uchic J, Thomas S, Lee RD, Grabowski BA, Sham HL, Kempf DJ, Denissen JF, Marsh KC, Sun E and Roberts SA (2004) Metabolism and disposition of the HIV-1 protease inhibitor lopinavir (ABT-378) given in combination with ritonavir in rats, dogs, and humans. *Pharm Res* **21**:1622-1630.
- Lee JH, Yang SH, Oh JM and Lee MG (2010) Pharmacokinetics of drugs in rats with diabetes mellitus induced by alloxan or streptozocin: comparison with those in patients with type I diabetes mellitus. *J Pharm Pharmacol* **62**:1-23.
- Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016-1023.
- Maeng H, Kim M, Jin H, Shin S, Tsuruo T, Kim S, Kim D, Shim C and Chung S (2007) Functional induction of P-glycoprotein in the blood-brain barrier of streptozotocin-induced diabetic rats: Evidence for the involvement of nuclear factor-kappaB, a nitrosative stress-sensitive transcription factor, in the regulation. *Drug Metab Dispos* **35**:1996-2005.
- Martí C, Peña JM, Bates I, Madero R, de José I, Pallardo LF, Arribas JR, Gonzalez-Garcia J, Gonzalez A and Vazquez JJ (2007) Obstetric and perinatal complications in HIV-infected women. Analysis of a cohort of 167 pregnancies between 1997 and 2003. *Acta Obstet Gynecol Scand* **86**:409-415.
- Masquelier B, Breilh D, Neau D, Lawson-Ayayi S, Lavignolle V, Ragnaud J-M, Dupon M, Morlat P, Dabis F, Fleury H and Groupe d' Epidémiologie Clinique du SIDA

- en Aquitaine (2002) Human immunodeficiency virus type 1 genotypic and pharmacokinetic determinants of the virological response to lopinavir-ritonavir-containing therapy in protease inhibitor-experienced patients. *Antimicrob Agents Chemother* **46**:2926-2932.
- Meier Y, Pauli-Magnus C, Zanger UM, Klein K, Schaeffeler E, Nussler AK, Nussler N, Eichelbaum M, Meier PJ and Stieger B (2006) Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. *Hepatology* **44**:62-74.
- Mirochnick M, Dorenbaum A, Holland D, Cunningham-Schrader B, Cunningham C, Gelber R, Mofenson L, Culnane M, Connor J and Sullivan JL (2002) Concentrations of protease inhibitors in cord blood after in utero exposure. *Pediatr Infect Dis J* **21**:835-838.
- Morgan E, Goralski K, Piquette-Miller M, Renton K, Robertson G, Chaluvadi M, Charles K, Clarke S, Kacevska M, Liddle C, Richardson T, Sharma R and Sinal C (2008) Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos* **36**:205-216.
- Mulay S and Varma DR (1984) Influence of streptozotocin-diabetes on the pharmacokinetics, placental transfer and tissue localization of dexamethasone in rats. *Br J Pharmacol* **83**:139-144.
- Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC and Gotoh O (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**:1-51.

- Petrovic V, Wang J and Piquette-Miller M (2008) Effect of endotoxin on the expression of placental drug transporters and glyburide disposition in pregnant rats. *Drug Metab Dispos* **36**:1944-1950.
- Stek AM, Mirochnick M, Capparelli E, Best BM, Hu C, Burchett SK, Elgie C, Holland DT, Smith E, Tuomala R, Cotter A and Read JS (2006) Reduced lopinavir exposure during pregnancy. *AIDS* **20**:1931-1939.
- Tirona RG (2011) Molecular mechanisms of drug transporter regulation. *Handb Exp Pharmacol* **201**:373-402.
- Tracy TS, Korzekwa KR, Gonzalez FJ and Wainer IW (1999) Cytochrome P450 isoforms involved in metabolism of the enantiomers of verapamil and norverapamil. *British Journal of Clinical Pharmacology* **47**:545-552.
- van Waterschoot RAB, ter Heine R, Wagenaar E, van der Kruijssen CMM, Rooswinkel RW, Huitema ADR, Beijnen JH and Schinkel AH (2010) Effects of cytochrome P450 3A (CYP3A) and the drug transporters P-glycoprotein (MDR1/ABCB1) and MRP2 (ABCC2) on the pharmacokinetics of lopinavir. *Br J Pharmacol* **160**:1224-1233.
- Wang J, Scollard D, Teng S, Reilly R and Piquette-Miller M (2005) Detection of P-glycoprotein activity in endotoxemic rats by ^{99m}Tc-sestamibi imaging. *J Nucl Med* **46**:1537-1545.
- Wang PG, Wei JS, Kim G, Chang M and El-Shourbagy T (2006) Validation and application of a high-performance liquid chromatography-tandem mass spectrometric method for simultaneous quantification of lopinavir and ritonavir in

- human plasma using semi-automated 96-well liquid-liquid extraction. *J Chromatogr A* **1130**:302-307.
- Watts DH, Balasubramanian R, Maupin RT, Delke I, Dorenbaum A, Fiore S, Newell M-L, Delfraissy J-F, Gelber RD, Mofenson LM, Culnane M, Cunningham CK and Team PS (2004) Maternal toxicity and pregnancy complications in human immunodeficiency virus-infected women receiving antiretroviral therapy: PACTG 316. *Am J Obstet Gynecol* **190**:506-516.
- Westlind-Johnsson A, Malmebo S, Johansson A, Otter C, Andersson TB, Johansson I, Edwards RJ, Boobis AR and Ingelman-Sundberg M (2003) Comparative analysis of CYP3A expression in human liver suggests only a minor role for CYP3A5 in drug metabolism. *Drug Metab Dispos* **31**:755-761.
- Wilkinson GR and Shand DG (1975) Commentary: a physiological approach to hepatic drug clearance. *Clin Pharmacol Ther* **18**:377-390.
- Wiznitzer A, Mayer A, Novack V, Sheiner E, Gilutz H, Malhotra A and Novack L (2009) Association of lipid levels during gestation with preeclampsia and gestational diabetes mellitus: a population-based study. *Am J Obstet Gynecol* **201**:4821-4828.
- Woodahl EL, Yang Z, Bui T, Shen DD and Ho RJY (2005) MDR1 G1199A polymorphism alters permeability of HIV protease inhibitors across P-glycoprotein-expressing epithelial cells. *AIDS* **19**:1617-1625.

Footnotes

Funding for this study was provided by an operating grant from the Canadian Institutes of Health Research [CIHR Grant #57688]. G.J.A. is the recipient of a Frederick Banting and Charles Best Canada Graduate Scholarship Doctoral Award, which is also from the Canadian Institutes of Health Research.

Legends for figures

Fig. 1. The dominant Cyp3a-mediated metabolic pathway for lopinavir.

LPV (A) is metabolized to M3/M4 (B; 4-hydroxy-LPV) by Cyp3a and M3/M4 is subsequently metabolized 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.

Fig. 2. Tissue/unbound maternal serum lopinavir concentration ratios. LPV

concentrations in maternal livers (A), placentas (B) and fetuses (C) were standardized to unbound concentrations in maternal serum. Resulting ratios are presented as mean \pm S.D. Maternal liver results are from 4-5 dams/group. Placental and fetal results are from 5 randomly selected placentas or fetuses/dam and there were 4-5 dams/group (n = 20-25 data points/group). *, Significantly different from vehicle controls. #, Significantly different from GDM. ***/###, $p < 0.001$.

Fig. 3. Time course of fetal exposure to lopinavir following intravenous maternal

administration. Pups were surgically removed from dams 5, 10, 15, 20, 30, 45 and 60 min after administration in order to generate a concentration-versus-time curve for fetal LPV exposure. All results are presented as mean \pm S.D. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, $p < 0.05$; ***/###, $p < 0.01$; ***/###, $p < 0.001$.

Fig. 4. Drug transporter and Cyp3a2 mRNA expression in GDM. The mRNA expression of Mdr1a/b, Mrp2, Bcrp and Cyp3a2 in maternal liver (left bars) and Mdr1a/b, Mrp2 and Bcrp in placenta (right bars) was determined by qRT-PCR. All results are presented as a percentage of the vehicle group's mean \pm S.D. Maternal liver results are from 4-5 dams/group while placental results represent 4-5 maternal means/group from 3 randomly selected fetuses/dam. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, $p < 0.05$; **/##, $p < 0.01$.

Fig. 5. Hepatic Mdr1 and Cyp3a2 protein expression in GDM. Mdr1 and Cyp3a2 protein concentrations were determined in maternal liver via western blotting. A, Representative blots showing Mdr1, Cyp3a2 and β -actin expression in samples from each group. Quantified Mdr1 (B) and Cyp3a2 (C) results are presented as mean percentages of a calibrator sample's values \pm S.D. and are from 4-5 dams/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, $p < 0.05$.

Fig. 6. Standardized concentration ratios for lopinavir metabolites in maternal liver 45 min after lopinavir administration. Forty-five min after LPV administration, lopinavir and lopinavir 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 were also standardized to LPV concentrations. All results are presented as mean \pm S.D. of 4-5

dams/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. **/###, $p < 0.01$.

Fig. 7. Hepatic PXR.1 protein expression in GDM. Protein concentrations of the PXR.1 PXR isoform were determined in maternal liver via western blotting. A, Representative blots showing PXR.1 and β -actin expression in samples from 3 dams/group. HeLa cell nuclear extract was used as a positive control. B, Quantified results are presented as mean percentages of a calibrator sample's values \pm S.D. and are from 4-5 dams/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. ***/####, $p < 0.001$.

Table 1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics. All results are presented as mean \pm S.D. Maternal results are from 7-8 dams/group while fetal results are from 3-6 randomly selected fetuses/7-8 dams/group (fetal body weight). Amniotic fluid data are from one pooled sample/7-8 dams/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, P < 0.05; **/##, P < 0.01; ***/###, P < 0.001.

Characteristic	Vehicle	GDM	Insulin-treated
Blood glucose:			
GD6 (mM)	6 \pm 0.9	6 \pm 0.4	6.5 \pm 1.3
GD11 (mM)	6.3 \pm 1	23.2 \pm 2.8***	16 \pm 6.6***,##
GD20 (mM)	5.8 \pm 1.1	26.8 \pm 2.9***	9.3 \pm 7.2###
Body weight:			
GD6 (g)	243 \pm 10	242 \pm 21	244 \pm 12
GD11 (% baseline)	116 \pm 1	103 \pm 2**	108 \pm 5
GD20 (% baseline)	154 \pm 6	136 \pm 7***	152 \pm 1###
Blood chemistry (GD20):			
Total cholesterol (mM)	1.9 \pm 0.3	8 \pm 4.7**	2.6 \pm 0.6##
HDL cholesterol (mM)	1.1 \pm 0.5	0.9 \pm 0.3	1.3 \pm 0.7
Triglycerides (mM)	2.9 \pm 2.1	25.9 \pm 16.6***	3.2 \pm 2.2##
Free fatty acids (mEq/l)	1.1 \pm 1.2	7.6 \pm 6.8*	1.1 \pm 0.7#
AAG (μ g/ml)	49 \pm 8	87 \pm 30**	86 \pm 12**
Albumin (g/l)	34 \pm 4	18 \pm 7***	36 \pm 4###

Water intake:

GD16 (ml/kg/day)	160 ± 91	768 ± 205***	168 ± 24###
------------------	----------	--------------	-------------

Fetal characteristics (GD20):

Litter size	16 ± 2	11 ± 2**	13 ± 3
Body weight (g)	2.59 ± 0.14	2.29 ± 0.24*	2.43 ± 0.18
Placenta weight (g)	0.52 ± 0.05	0.65 ± 0.08**	0.5 ± 0.08##
Fetus/placenta weight	5.1 ± 0.6	3.6 ± 0.6**	5 ± 0.4#
Amniotic glucose (mM)	2.6 ± 0.8	32.2 ± 9***	7.5 ± 8.7###

Table 2. Maternal and fetal lopinavir concentrations 45 min after lopinavir

administration. Drug concentrations were determined 45 min after LPV administration via LC-MS/MS. Maternal results are from 4-5 dams/group while placental and fetal results represent 4-5 maternal means/group from 5 randomly selected placentas or fetuses/dam. *, Significantly different from vehicle controls. #, Significantly different from GDM. **/###, $p < 0.01$; ***/####, $p < 0.001$.

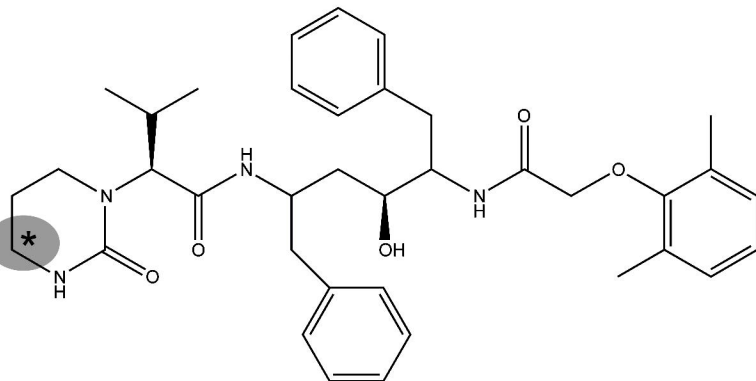
Sample	Vehicle	GDM	Insulin-treated
Maternal serum ($\mu\text{g/ml}$)	2.04 ± 0.37	$0.76 \pm 0.12^{***}$	$2.17 \pm 0.48^{###}$
Maternal liver ($\mu\text{g/g}$)	47.5 ± 16.6	$14.4 \pm 6.02^{**}$	$44.25 \pm 9^{##}$
Placenta ($\mu\text{g/g}$)	2.45 ± 0.93	$0.83 \pm 0.34^{***}$	$2.36 \pm 1.1^{###}$
Fetus ($\mu\text{g/g}$)	0.15 ± 0.05	$0.04 \pm 0.02^{***}$	$0.12 \pm 0.07^{###}$

Table 3. Pharmacokinetic parameters derived from concentration-versus-time curves for fetal lopinavir exposure. AUC₀₋₆₀, C_{max} and T_{max} values as well as maternal serum and liver values are arithmetic groups means. Maternal samples were collected at 60 min. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, $p < 0.05$; **/###, $p < 0.01$; ***/####, $p < 0.001$.

Parameter	Vehicle	GDM	Insulin-treated
Fetal time course:			
AUC ₀₋₆₀ (µg.min/g)	15.63 ± 1.21	4.76 ± 0.77***	9.03 ± 1.29***,###
C _{max} (µg/g)	0.37 ± 0.06	0.16 ± 0.05**	0.21 ± 0.02*
T _{max} (min)	27 ± 6	8 ± 6*	13 ± 8
Maternal samples:			
Serum [LPV] (µg/ml)	2.17 ± 0.32	0.62 ± 0.32**	1.44 ± 0.38
Liver [LPV] (µg/g)	47.39 ± 15.83	9.13 ± 7.12*	42.76 ± 15.37#

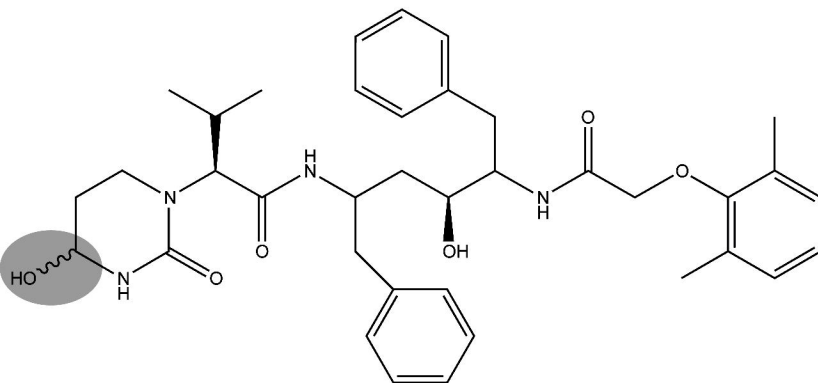
Figure 1

A: LPV (Parent)



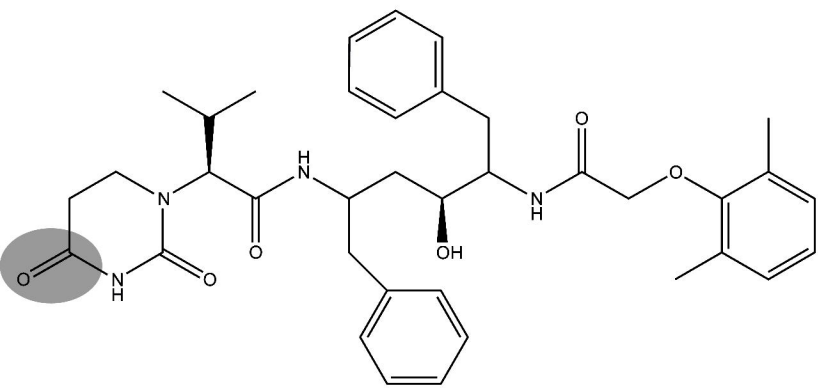
→ MDR1 efflux

B: M3/M4 (Product of LPV metabolism)



→ MDR1 efflux?

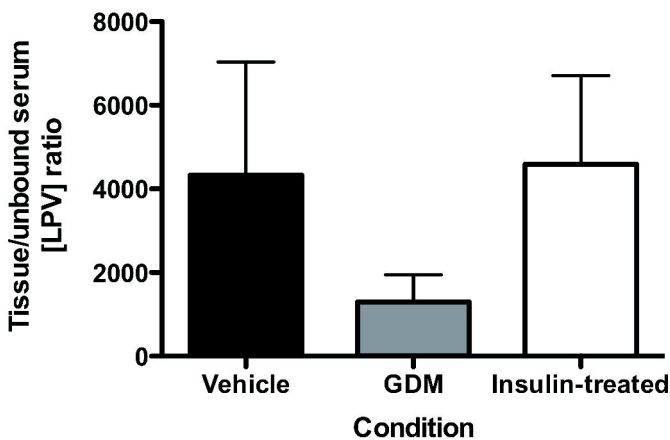
C: M1 (Product of M3/M4 metabolism)



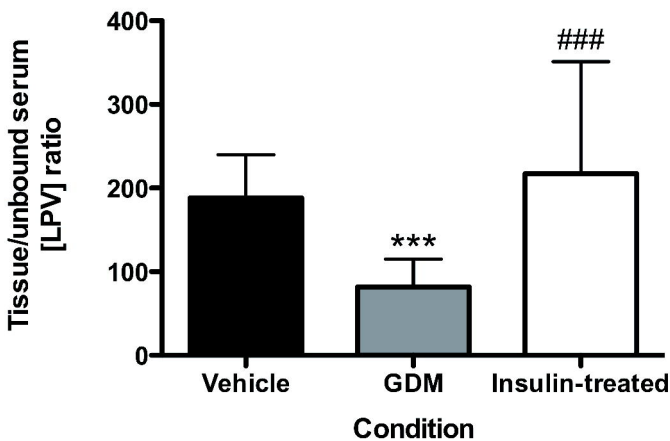
→ MDR1 efflux?

Figure 2

A: Maternal liver



B: Placenta



C: Fetus

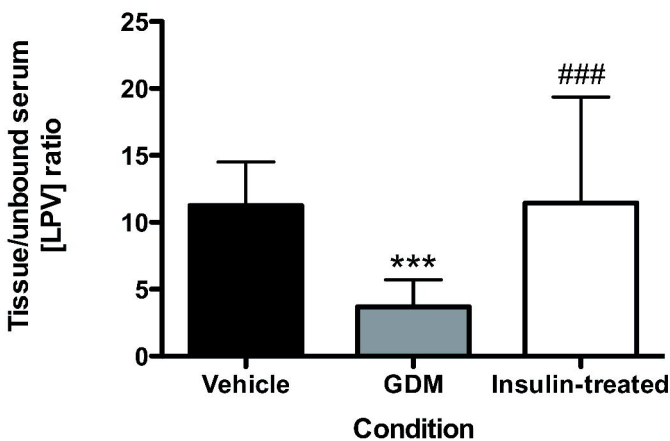


Figure 3

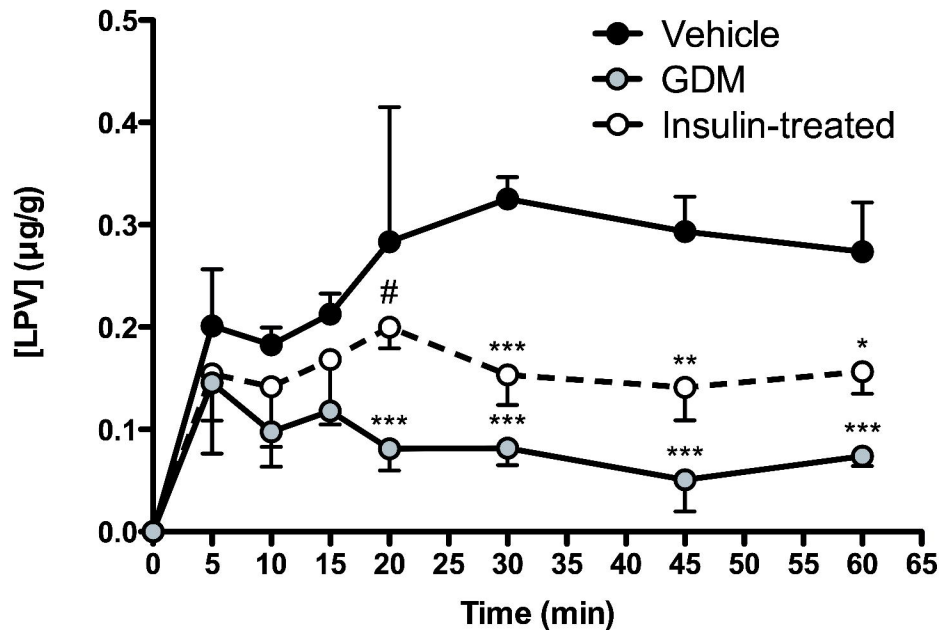


Figure 4

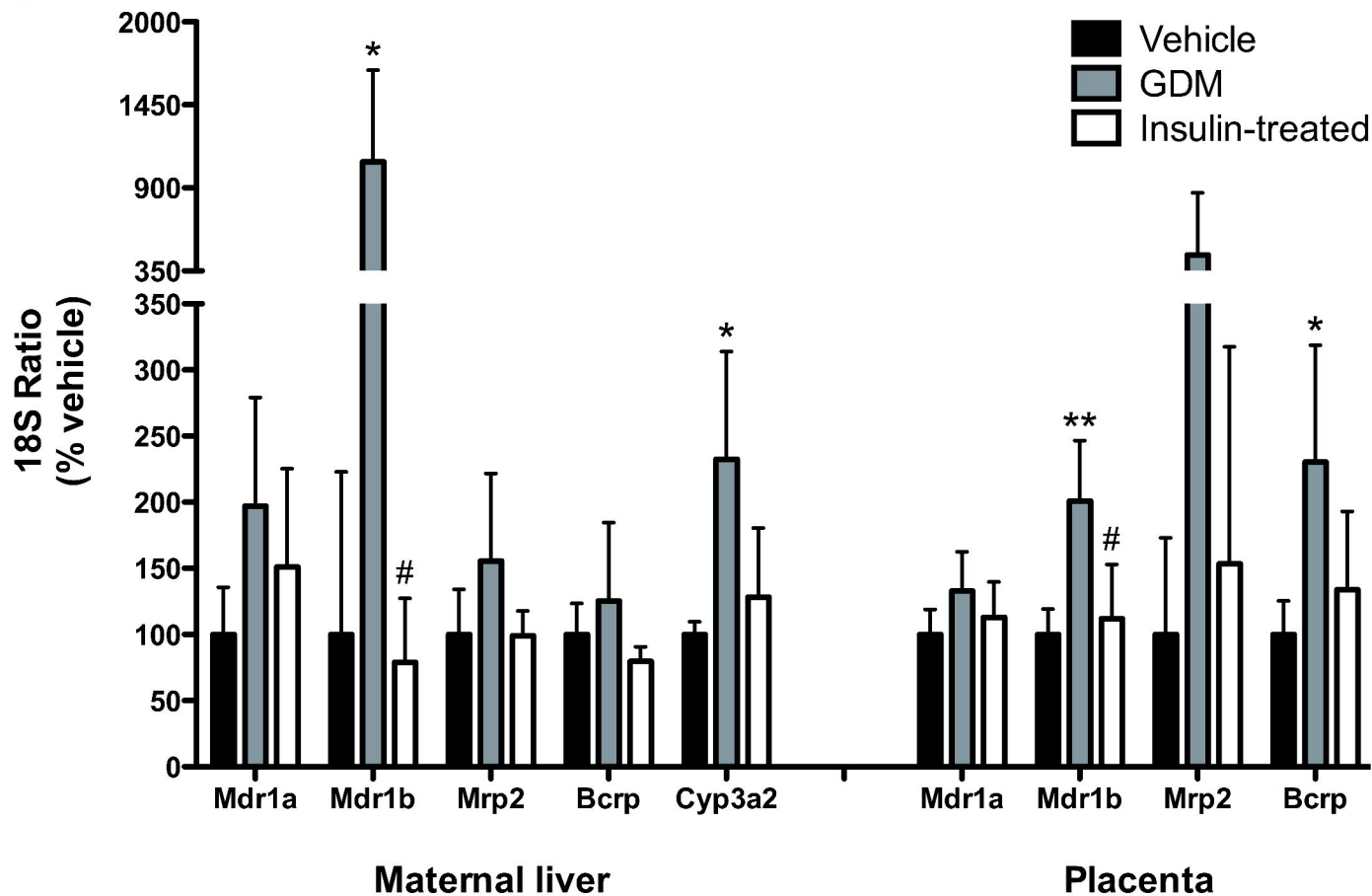
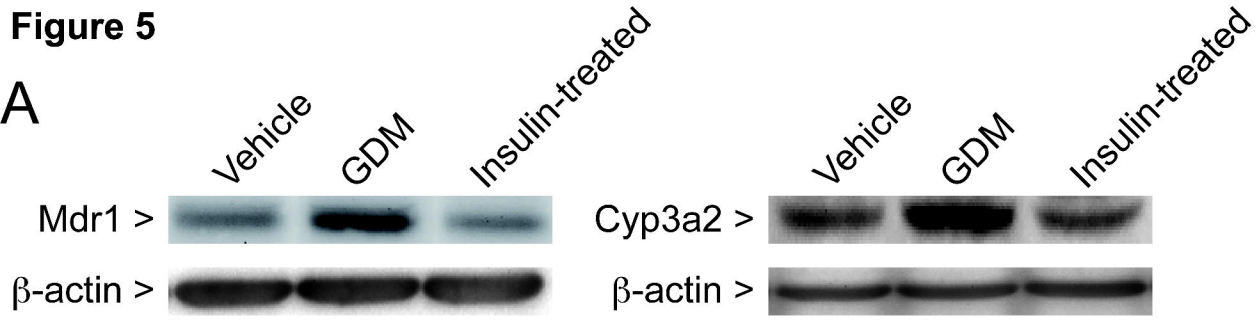
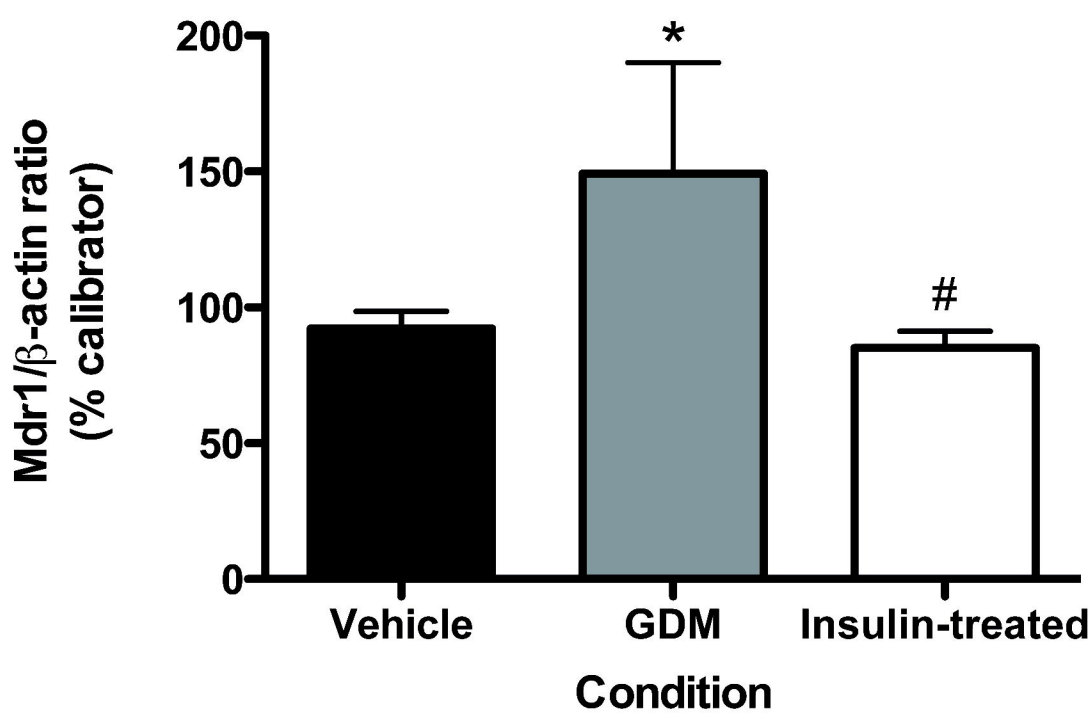


Figure 5

A



B



C

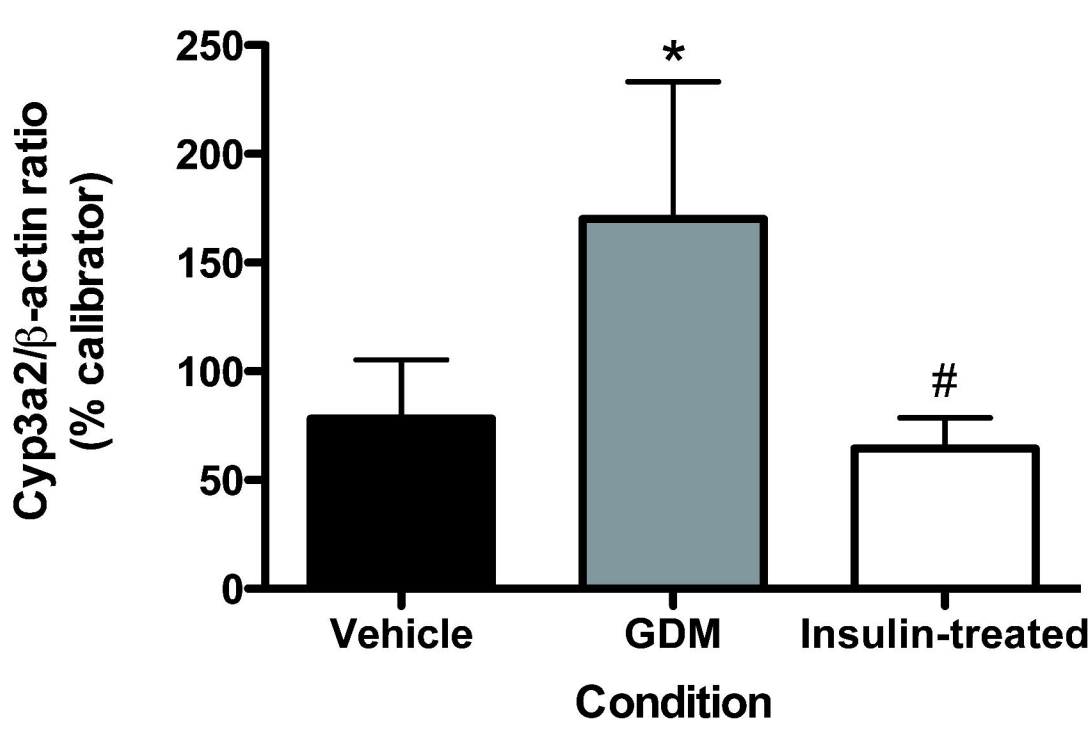
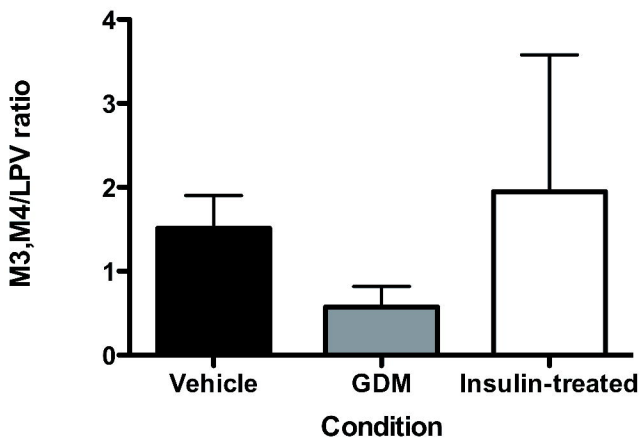
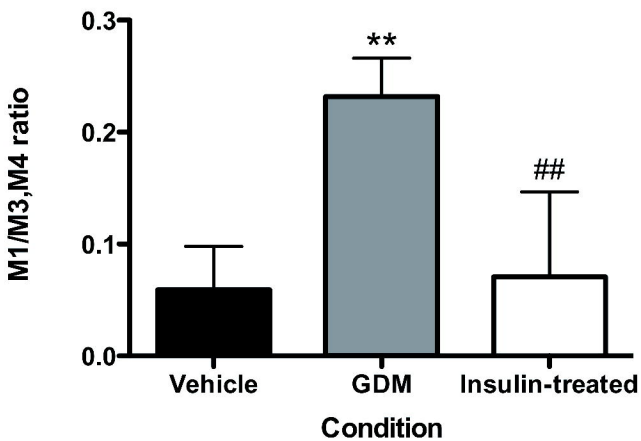


Figure 6

A: M3/M4 to LPV



B: M1 to M3/M4



C: M1 to LPV

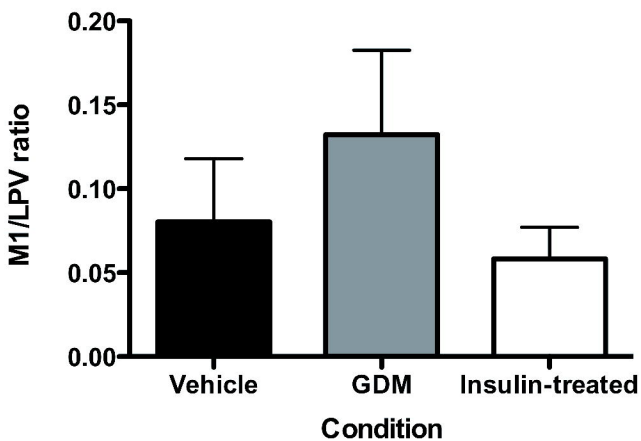
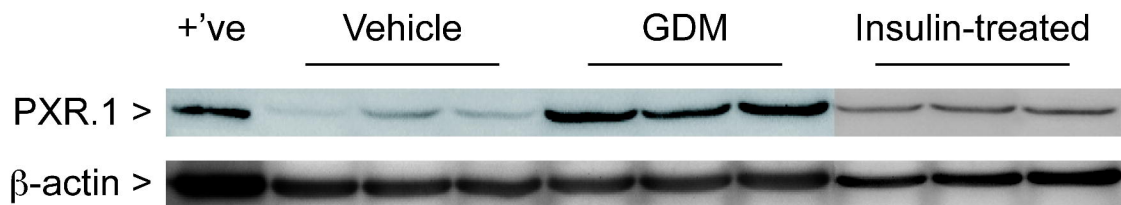


Figure 7

A



B

