

**Polyinosinic/Polycytidylic Acid-Mediated Changes in Maternal and
Fetal Disposition of Lopinavir in Rats**

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Running title: LPV exposure in a pregnant rat model of viral infection

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List of non-standard abbreviations:

ABC: ATP-binding cassette
AAG: α 1-acid glycoprotein
ARV: antiretroviral
AUC: area under the concentration-versus-time curve
BCRP: breast cancer resistance protein (ABCG2)
CYP: cytochrome P-450
ELISA: enzyme-linked immunosorbent assay
IFN: interferon
LC-MS/MS: liquid chromatography-tandem mass spectrometry
LPV: lopinavir
MRP: multidrug resistance-associated protein (ABCC)
MTCT: mother-to-child transmission
P-gp: P-glycoprotein or multidrug resistance protein 1 (MDR1/ABCB1)
Poly(I:C): polyinosinic/polycytidylic acid
qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction
RTV: ritonavir

ABSTRACT

Maintenance of optimal lopinavir (LPV) concentration is essential for effective antiretroviral therapy and prevention of mother-to-child transmission of HIV. However little is known about the effects of inflammation on the pharmacokinetics of this protease inhibitor and drug transporter substrate, particularly during gestation. Our objective was to study the effect of polyinosinic/polycytidylic acid [poly(I:C)], a viral mimetic, on key maternal drug transporters and to examine the effect on maternal and fetal disposition of LPV in rats. Poly(I:C) (5.0 mg/kg i.p.) or saline vehicle was administered to pregnant Sprague-Dawley rats on gestational day 17-18. At 24 hours post-injection, all rats were administered LPV (10 mg/kg i.v.) and plasma and tissues collected at 5-120 min post-administration. Plasma IFN- γ levels were measured by ELISA and transporter expression was measured via real-time polymerase chain reaction. Maternal plasma, hepatic, placental and fetal LPV concentrations were determined by LC-MS/MS. Administration of poly(I:C) induced IFN- γ plasma levels and downregulated the expression of several important ABC drug efflux transporters in placenta and liver of pregnant rats, compared to controls ($p < 0.05$). Maternal LPV plasma concentration and AUC were significantly increased in poly(I:C) group. Plasma protein binding was also significantly higher in poly(I:C)-treated rats. Pronounced increases in hepatic, placental, and fetal LPV tissue:unbound plasma concentrations were seen in the poly(I:C) group, however absolute tissue concentrations were not changed. Since the majority of commonly used and clinically important antiretroviral drugs are known to be ABC transporter substrates, inflammation-mediated changes in transporter expression could affect their maternal disposition and fetal exposure.

INTRODUCTION

Prescription or over-the-counter medications are a necessity for as many as 80% of pregnant women (Anger and Piquette-Miller, 2008). Human immunodeficiency virus (HIV) infections are prevalent in 20 million women of child-bearing age, world-wide. Clinically, antiretroviral (ARV) therapy is crucial for the prevention of mother-to-child-transmission (MTCT) of HIV, but information on the disposition of ARVs in pregnancy or the effect of concomitant infections is still largely lacking. Being the front line barrier against xenobiotics and blood-borne toxins between the mother and the fetus, the integrity of the placenta is of utmost importance. Evidence from the literature demonstrates that the placenta expresses a number of transporters, some of which function in the removal of endogenous compounds and xenobiotics. Specifically, several of the ATP-binding cassette (ABC) drug efflux transporters, including P-glycoprotein (P-gp/ MDR1/ ABCB1), the multidrug resistance-associated proteins (MRPs/ ABCCs), and the breast cancer resistance protein (BCRP/ ABCG2) are highly expressed in placental tissues and have been found to profoundly limit the *in vivo* passage of potentially toxic xenobiotics into the fetus (Petropoulos *et al.*, 2007; Zhou *et al.*, 2008).

While the role of ABC proteins in drug transport has been examined under normal physiological conditions, the impact of maternal disease on drug transporter regulation and activity is still poorly understood. Infection and inflammation occur frequently in pregnancy and induction of pro-inflammatory cytokines is seen with many prevalent obstetric complications including preeclampsia, gestational diabetes and various infections (Romero *et al.*, 2007). Previous studies from our group and others have reported inflammation-mediated alterations in the expression and activity of rodent ABC

drug transporters in the biologically protective barriers of the liver, intestine and brain, as well as the placenta (reviewed in (Petrovic *et al.*, 2007)). Moreover, altered drug disposition due to the inflammation-mediated suppression of P-glycoprotein was previously observed *in vivo*, in male mice (Hartmann *et al.*, 2005).

Currently, the protease inhibitor lopinavir (LPV) is widely used in clinical regimens for pregnant HIV-positive women. LPV is a Biopharmaceutics Classification System class II antiretroviral drug with a therapeutic dose range of approximately 5-7 µg/ml (mean steady state trough concentration) and peak plasma concentration in the range of 10 µg/ml. Maintenance of optimal LPV concentration is essential for effective therapy and MTCT prevention, but little is known about the effects of inflammatory mediators on the pharmacokinetics of this protease inhibitor. Previous *in vitro* studies have established LPV is a substrate for P-gp and MRP2 (Agarwal *et al.*, 2007; Janneh *et al.*, 2007). As HIV-positive pregnant women are susceptible to opportunistic infections (Watts *et al.*, 2004), it is plausible that changes in drug transporters may occur and in turn mediate changes in LPV disposition. Indeed, we have previously shown that the inflammation-mediated downregulation of placental Bcrp led to the increased accumulation of the Bcrp substrate, glyburide in rat fetus (Petrovic *et al.*, 2008), therefore changes in P-gp could alter fetal accumulation of LPV. Furthermore, LPV is extensively metabolized by cytochrome P450 (CYP) 3A, a metabolizing enzyme that is sensitive to inflammation-mediated changes in expression and functionality (Renton, 2001; Kumar *et al.*, 2004). LPV is also highly bound (≥99%) to plasma proteins that can fluctuate during infection, mainly α1-acid glycoprotein (AAG) and, to a lesser extent, albumin, thus having the

potential to affect the concentration of free drug available for intracellular and transplacental passage (Kumar *et al.*, 2004; Chandwani and Shuter, 2008).

Under the MTCT-prevention premise, the benefits of ARV therapy far outweigh the risks of fetal exposure to ARV drugs. However, the fetal safety of most ARV drugs has not been fully established and long-term effects of increased exposure are still unknown (Panel on Treatment of HIV-Infected Pregnant Women and Prevention of Perinatal Transmission). On the other hand, underexposure may result in compromised prevention of virus transmission. Understanding disease-induced changes in the maternal and fetal disposition of the antiretroviral agents commonly prescribed in pregnancy is imperative. Therefore we examined the effect of a viral-like infection, modelled by polyinosinic/polycytidylic acid [poly(I:C), a synthetic viral mimetic double-stranded RNA], on *in vivo* maternal and fetal disposition of lopinavir.

MATERIALS AND METHODS

Animals and experimental design

Pregnant near-term Sprague-Dawley rats (gestational day (GD) 17-18; Charles River Laboratories, Senneville, QC) were injected i.p. with a single 5.0 mg/kg dose of poly(I:C) (Amersham Biosciences, Piscataway, NJ) dissolved in phosphate buffered saline. Control pregnant rats (GD 17-18) were injected with sterile phosphate buffered saline. Twenty-four hours after the initial injection, all animals received LPV (USP, Rockville, MD) through a tail vein injection (10 mg/kg, i.v.). LPV was dissolved (10 mg/ml) in 4:3:3 (v/v/v) ethanol/ propylene glycol/ 5% dextrose in water. At various times (5, 25, 45, 60 and 120 min) after LPV administration, dams (n = 4 per time point) were sacrificed under anesthesia by exsanguination. Maternal blood was collected and plasma obtained via centrifugation (3000 g, 15 min, 4 °C) and preserved at -80 °C. Maternal livers, placentas, and fetuses were immediately harvested, frozen in liquid nitrogen and preserved at -80 °C for further analyses. All animal studies were approved by the Office of Research Ethics at the University of Toronto and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Cytokine measurements

Maternal plasma concentrations of interferon-gamma (IFN- γ) were determined via commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The sensitivity of this assay is 10 pg/ml. Each sample was examined in duplicate and results within the

standard curve range reported. Results were pooled from all 5 time points in each study group (n = 20 / group).

qRT-PCR and mRNA expression

Methods for RNA isolation, cDNA synthesis and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) have been previously described (Wang *et al.*, 2005; Petrovic and Piquette-Miller, 2010). Briefly, RNA was extracted from tissues (50-100 mg) using TRIzol reagent (Invitrogen, Burlington, ON). RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and then reverse-transcribed to cDNA by use of the First Strand cDNA synthesis kit (Fermentas, Burlington, ON) according to the manufacturer's protocol. Placental and hepatic mRNA expression levels of relevant genes were measured by qRT-PCR using the Roche LightCycler™ with the LC FastStart DNA Master SYBR Green I® kit (Roche, Laval, QC). Oligonucleotides for previously reported primer sequences were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON) (Petrovic and Piquette-Miller, 2010). All mRNA levels were normalized to *18s* rRNA and the ratios are presented as the percentage of control values.

Lopinavir LC-MS/MS Assay

Samples and standards were prepared as previously described (Anger and Piquette-Miller, 2011) using a liquid-liquid extraction adapted from Wang *et al.* (Wang *et al.*, 2006). In brief, fetuses, placentas and maternal livers were homogenized in deionized water. 100 µl of thawed plasma or tissue homogenates comprised the samples. The same

volume of drug-free plasma or tissue homogenate was used for constructing LPV standards by adding to tubes containing pre-dried LPV. For plasma and hepatic samples, the final LPV standard concentrations ranged from 62.5–2000 ng/ml, whereas for fetal, and placental samples, the standards measured 6.25–200 and 25–1000 ng/ml, respectively. Ritonavir (RTV) was used as the internal standard (final concentration, 200 ng/ml). For extraction, 50 μ l of 500mM NaCO₃ was mixed with the samples followed by adding 1.2 ml of hexane/ethyl acetate (1:1 v/v). The mixture was then vortexed (1 min) and centrifuged (21000 g, 5 min, 4 °C). The organic layer (700 μ l) was transferred to a clean vial, evaporated under nitrogen gas, and the extract reconstituted in 200 μ l of 80% methanol. The final extracts were aliquoted in autosampler vials and 10 μ l were used for sampling injection.

Plasma and tissue LPV concentrations were measured using high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (Anger and Piquette-Miller, 2011). Briefly, for liquid chromatography we used a CTC PAL autosampler (LEAP Technologies, Carrboro, NC) and an Agilent Technologies (Santa Clara, CA) 1100 series pump with a 50 x 4.6 mm, 5 micron, LiChrosorb RP-8 column (Phenomenex, Torrance, CA), while MS/MS was performed on an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems/MDS Sciex, Foster City, CA). Analyst software, version 1.4.2 was used (Applied Biosystems/MDS Sciex) for analysis of LPV and RTV peak areas and LPV quantification. The lower limit of detection was < 3 ng/ml and the lower limit of quantification was < 10 ng/ml.

Pharmacokinetic Analysis

At each time point, plasma and hepatic tissue were obtained from 4 dams, while 3 fetuses and placentas were collected from each dam for a pooled analysis (per dam) of fetal and placental concentrations. Concentration-time curves, $C(t)$ were obtained by plotting the average of data points. Since this study design entailed one-point sampling data (one blood or tissue sample from each rat), a non-compartmental analysis program for sparse sampling (Phoenix WinNonlin 6.3, Certara, St. Louis, MO) was utilized to calculate key pharmacokinetic parameters derived from maternal plasma concentrations. Additionally, the Bailer method for destructive measurement sampling (Bailer, 1988; Hartmann *et al.*, 2005; Zhou *et al.*, 2008) was used to estimate the mean and S.E. of the maternal plasma AUC and the fetal, placental and hepatic tissue AUCs, from 5-120 min. The Bailer method was previously adapted by Zhou *et. al.* to the following mathematical form (Zhou *et al.*, 2008):

$$E(AUC) = \sum_{i=2}^m (E(C_i) + E(C_{i-1})) \times (t_i - t_{i-1})/2$$

$$V(AUC) = \sum_{i=2}^m (V(C_i) + V(C_{i-1})) \times ((t_i - t_{i-1})/2)^2$$

where $E(AUC)$ and $V(AUC)$ are the mean and variance of AUC, m is the number of time points of the time course experiment and C_i and t_i are concentration and time, respectively. The time course was integrated by the trapezoidal formula without extrapolation. $E(C_i)$ and $V(C_i)$ are the mean and variance of concentration at time t_i , defined as

$$E(C_i) = \frac{1}{n_i} \sum_{j=1}^{n_i} C_{ij}$$

$$V(C_i) = \frac{1}{n_i - 1} \sum_{j=1}^{n_i} (C_{ij} - E(C_i))^2$$

where n_i is the number of the concentration data points at time t_i . The variance of mean AUC is defined as

$$V(\overline{AUC}) = \sum_{i=2}^m (V(C_i)/n_i + V(C_{i-1})/n_{i-1}) \times ((t_i - t_{i-1})/2)^2$$

The difference between groups is considered statistically significant if $Z_0 > 1.96$ (95% confidence interval, $p < 0.05$), where

$$Z_0 = \frac{|\overline{\phi}_1 - \overline{\phi}_2|}{\sqrt{SE_1^2 + SE_2^2}}$$

$\overline{\phi}_1$ and $\overline{\phi}_2$ are the means of AUC, and SE_1 and SE_2 are the standard errors of AUC for the two groups. SE of the AUC is defined as the square root of the variance of mean AUC.

Protein Binding Assay

LPV plasma protein binding was measured by ultrafiltration using Amicon Ultra-0.5 Centrifugal Filter Devices with nominal molecular weight limit of 10,000 (Millipore, Billerica, MA). Plasma samples taken at 5 min were thawed and incubated at 37 °C for 30 min, then transferred to filter devices and equilibrated at 30 °C for 30 min. The samples were then centrifuged (14000 g, 15 min, 30 °C) and the ultrafiltrate collected. Filtrate LPV concentrations were quantified using LC-MS/MS as described for plasma and tissues. For standard curve calibration, filtrate was collected from drug-free plasma samples. LPV concentrations in the filtrate samples were compared against the LPV

concentrations in corresponding unfiltered plasma samples and the percentage unbound (% unbound) was calculated as follows: % unbound = $(C_{\text{filtered}} / C_{\text{unfiltered}}) \times 100$.

Statistical analysis

Data were analyzed using Prism 6 (GraphPad Software, San Diego, CA). All results are expressed as means \pm standard error (SE). With the exception of the pharmacokinetic analysis described above, for comparison of the effects between poly(I:C) and control, a two-tailed independent-samples *t*-test was used. Significance was assigned at $\alpha = 0.05$, and is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

RESULTS

Interferon response to poly(I:C)

Interferon-gamma levels were examined in maternal plasma samples from poly(I:C)-treated and vehicle control rats. Viruses or administration of double stranded RNA such as poly(I:C) are known to induce Type I interons and trigger an acute phase response. Indeed, our ELISA results demonstrated a significant induction of IFN- γ , 24 hours after administration of 5.0 mg/kg poly(I:C), as compared to control (Figure 1). Measurements of IFN- γ were below detection limit in many control samples (11 out of 20) and were thus considered to be <10 pg/ml.

Effect of poly(I:C) on maternal drug transporter and Cyp3a2 expression

The impact of poly(I:C) on the expression of transporters in placental and hepatic tissues was examined by qRT-PCR ($n=7-10$ /group) in order to confirm previously observed poly(I:C)-mediated changes in the expression of transporters that are reportedly implicated in LPV transport, namely the two P-gp isoforms *Abcb1a* and *Abcb1b*, as well as *Abcc2* (Mrp2) (Agarwal *et al.*, 2007; Petrovic and Piquette-Miller, 2010).

In line with our previous observations, administration of poly(I:C) imposed significant changes in the expression of these transporters in the liver (Figure 2). After administration of 5.0 mg/kg poly(I:C), *Abcb1a* was significantly reduced to $61 \pm 9\%$ of control values, while *Abcb1b* was drastically increased ($p < 0.05$). Expression of hepatic *Abcc2* (Mrp2) was significantly reduced to $61 \pm 6\%$ of control levels ($p < 0.05$). Additionally, expression levels of the metabolizing enzyme *Cyp3a2* were examined as downregulation has been reported in numerous models of acute and chronic

inflammation. We found a significant downregulation of hepatic *Cyp3a2* to $70 \pm 8\%$ of control mRNA value ($p < 0.05$).

In placenta, we also observed significant changes in the mRNA expression of examined transporters (Figure 2). Placental *Abcb1a* and *Abcb1b* were significantly downregulated in poly(I:C)-treated dams, to $70 \pm 9\%$ and $66 \pm 8\%$ of control mRNA levels, respectively ($p < 0.05$). *Abcc2* mRNA levels were decreased in the poly(I:C)-treated group to $44 \pm 22\%$ of control value, but this did not reach significance.

Influence of poly(I:C) on maternal and fetal LPV exposure

Maternal plasma, hepatic, placental and fetal LPV concentrations were determined by a validated LC-MS/MS assay, as described under *Materials and Methods*. Overall, our data showed that the fetal and placental LPV tissue concentrations were much lower than the maternal total plasma LPV concentrations indicating limited fetal distribution. Plasma levels in the pregnant rats were in line with the typical clinical therapeutic range of LPV in HIV-infected patients. A significant increase in maternal plasma concentrations of LPV was seen in rats administered poly(I:C), as compared to vehicle control, with the most pronounced differences seen at 25-60 minutes after LPV administration (Figure 3). Table 1 summarizes the pharmacokinetic parameters calculated for both groups of pregnant rats. We obtained a terminal half-life of approximately 1.5 h, which is in line with previous reports (Kumar *et al.*, 2004). Terminal half-life was unchanged in the poly(I:C)-treated group. However, large differences in time-zero concentration, clearance and volume of distribution are evident between the treatment groups. As expected, LPV was highly bound to plasma proteins (>

99%) and significantly different between treatment groups. The unbound fraction in the control rats was $0.85 \pm 0.33\%$, compared to $0.1 \pm 0.01\%$ in poly(I:C) group ($p < 0.05$).

Plasma AUC between 5 and 120 min was significantly higher in the poly(I:C) group, (126 ± 13 in controls vs. $283 \pm 28 \mu\text{g} \cdot \text{min}/\text{ml}$ in poly(I:C)), as calculated by Bailer's method (Table 2). The AUC_{5-120} for fetal, placental and hepatic tissue concentrations did not differ significantly between the two groups (Table 2), reflecting similar absolute tissue concentrations (Supplemental Figure 1). However, when normalized to unbound LPV plasma concentrations, higher levels of accumulation were seen in these tissues at all time points, with significantly higher levels at 1-2 hrs ($p < 0.05$) (Figure 4).

DISCUSSION

Lopinavir is commonly used in HIV-infected pregnant women as part of highly active antiretroviral therapy, however the impact of co-existing conditions such as infection or inflammation on disposition is largely unknown (Watts, 2006; Lambert *et al.*, 2011). Maintaining adequate drug concentrations is necessary as subtherapeutic concentrations can increase the risk of MTCT, while supratherapeutic concentrations can increase risk of drug toxicities (Ofotokun *et al.*, 2011).

In the present study, we examined the impact of an acute inflammatory response elicited by poly(I:C) on the disposition of LPV in pregnant rats. Consistent with induction of inflammation following a viral infection, we saw a dramatic increase in plasma concentrations of IFN- γ in the poly (I:C) group (Fortier *et al.*, 2004; Petrovic and Piquette-Miller, 2010). Likewise, the maternal plasma AUC_{5-120min} of LPV was significantly increased by more than 2-fold in the poly(I:C) group, suggesting that inflammation imposes changes in the pharmacokinetics of LPV. It is well recognized that induction of inflammation by bacteria or virus can cause changes in drug disposition (Morgan *et al.*, 2008).

Increased LPV levels in pregnant poly(I:C)-treated rats could occur through a number of mechanisms such as altered plasma protein binding, metabolism and distribution. LPV is highly bound ($\geq 99\%$) to plasma proteins (Kumar *et al.*, 2004; Chandwani and Shuter, 2008). An unbound fraction of 0.89% has been reported for LPV in serum from healthy pregnant women (Gulati *et al.*, 2009), which is comparable to the 0.85% we saw in the

control pregnant rats. A significant correlation of LPV protein binding with AAG concentrations has been reported in pregnant and post-partum HIV-infected women (Aweeka *et al.*, 2010). It is well known that inflammation can cause an induction of AAG (Schreiber *et al.*, 1989). In humans, the serum concentration of AAG rises two to five times during an acute phase response (Hochepped *et al.*, 2003). Various models of acute inflammation in rats have also demonstrated an increase in AAG levels resulting in altered pharmacokinetics of highly bound drugs (Murai-Kushiya *et al.*, 1993; Laethem *et al.*, 1994). Consistent with reported increases in AAG, we found a significant increase in plasma protein binding of LPV in poly(I:C)-treated rats, as compared to control group. Generally, increased protein binding of a drug is associated with a decrease in the steady state volume of distribution (V_d or V_{ss}). The calculated values for control and poly(I:C)-treated dams suggest a relative 2.5-fold difference in V_{ss} between the groups. Therefore the reduction in V_{ss} likely contributes to higher plasma concentrations of LPV in poly(I:C)-treated dams. Of note, it was not surprising that k_e values were not different between the groups, since half-life is a function of clearance and volume of distribution and these were both decreased after treatment with poly(I:C).

It is well established that inflammation can lead to changes in drug disposition through downregulation of drug metabolizing enzymes (Morgan, 1997; Renton, 2001). For example, impaired theophylline clearance during confirmed viral infection episodes is attributed to an IFN-mediated downregulation of CYP1A2-mediated metabolism. In humans, LPV is primarily cleared via CYP3A-mediated metabolism (Kumar *et al.*, 2004; Chandwani and Shuter, 2008). A dramatic increase in LPV systemic exposure is seen in

Cyp3a^{-/-} mice when compared with wild-type mice, indicating its importance in determination of LPV pharmacokinetics (Waterschoot *et al.*, 2010). We measured the hepatic mRNA expression levels of Cyp3a2, the rat equivalent of CYP3A4 in humans, and we observed a significant downregulation in poly(I:C)-treated rats. This poly (I:C)-mediated downregulation is consistent with previous reports (Knickle *et al.*, 1992; Petrovic and Piquette-Miller, 2010). As plasma levels of metabolites were not measured in this study, the resultant effect of poly(I:C) on the hepatic clearance of LPV is not immediately obvious; however, previous studies have demonstrated that LPV metabolite concentrations in rats are reflective of Cyp3a2 mRNA levels (Anger and Piquette-Miller, 2011). LPV is a highly extracted drug and is administered clinically with low-dose RTV in order to inhibit CYP3A enzymes and increase oral bioavailability. In this study we administered LPV intravenously in order to minimize variability due to intestinal transit times and circumvent the potential effects of poly(I:C) on intestinal and hepatic first pass extraction. Hepatic metabolism of highly extracted drugs such as LPV is more sensitive to changes in hepatic flow than to changes in plasma protein binding and intrinsic metabolism. Nevertheless it is still plausible that the decreased expression of Cyp3a along with increased plasma protein binding of LPV, could result in decreased hepatic metabolism, thereby contributing to altered LPV levels in the poly(I:C)-treated dams.

The co-ordinated activity of CYP3A with drug transporters can also impact systemic drug exposure (Benet and Cummins, 2001; Morgan *et al.*, 2008). LPV is transported by the apical drug efflux transporter P-gp. Several *in vitro* transport studies have demonstrated evidence that LPV is a substrate of P-gp, a finding that is also supported by

studies in an *Abcb1* knockout mouse model (Agarwal *et al.*, 2007; Janneh *et al.*, 2007; Waterschoot *et al.*, 2010). We saw a decrease in mRNA expression of *Abcb1a* in the liver and placenta of poly(I:C)-treated rats. Interestingly, levels of *Abcb1b* were increased in maternal liver but decreased in placenta. We have previously shown that P-gp protein expression is decreased in the livers of poly(I:C) treated rats despite increased mRNA levels of *Abcb1b* (Petrovic and Piquette-Miller, 2010). A decreased P-gp expression in liver could therefore contribute to a decreased hepatobiliary clearance of LPV. Indeed we saw increased hepatic levels of LPV (relative to unbound concentration) in the pregnant rats that were administered poly(I:C), compared to controls. This could be due to decreased P-gp-mediated secretion of LPV into bile. Previous studies demonstrated that poly(I:C)-mediated changes in the expression of endogenous bile acid transporters were associated with elevated levels of bile acids in serum (Petrovic and Piquette-Miller, 2010). It is likely that similar mechanisms are involved in the biliary transport of exogenous substrates, such as LPV. There is still speculation whether CYP3A is a greater determinant of LPV plasma concentrations in comparison with P-gp (Wyen *et al.*, 2008; Waterschoot *et al.*, 2010), however our findings suggest that it is the combined action of these pathways that impact the overall LPV exposure, with evidence of the impact of P-gp on LPV tissue distribution. While we also observed a significant downregulation of *Abcc2* in livers of poly(I:C)-treated rats, which could further contribute to altered hepatobiliary clearance of LPV, the role of Mrp2 in LPV disposition is controversial. Although studies in MRP2 transfected cells demonstrate LPV transport, studies in Mrp2 knockout mice models failed to reveal a role of this transporter in *the*

vivo disposition of LPV (Waterschoot *et al.*, 2010). Hence the role of this transporter on LPV disposition in pregnancy remains to be delineated.

It has been shown that placental transfer of LPV is highly variable, as evidenced by intersubject differences in the ratio of cord-to-maternal blood ranging from 5-25% (Gingelmaier *et al.*, 2006). Likewise cord-to-maternal ratios ranging from 7-43% are reported for nelfinavir (van Heeswijk *et al.*, 2004). As both P-gp expression and high plasma protein binding play important roles in restricting placental transfer of these agents, it is plausible that disease or inflammation-mediated changes could contribute to clinical variability. The observed decrease in placental expression of P-gp in the poly(I:C)-treated rats could lead to increased accumulation of drug substrates in fetal tissues. On the other hand, the observed inflammation-mediated increase in LPV plasma protein binding might lead to decreased transplacental transfer, as only free drug is able to cross cell membranes. Overall, we observed significant increases in LPV accumulation in placenta and fetal tissues of poly(I:C)-treated rats, when normalized to unbound plasma concentrations. This is in line with other studies that have shown an increase in accumulation of P-gp substrates attributed to decreased P-gp expression and activity. For instance, a markedly higher accumulation of the P-gp substrate ^{99m}Tc-sestamibi was seen in fetal tissues isolated from endotoxin-treated dams (Wang *et al.*, 2005). Decreased P-gp expression and increased protein binding are opposing factors that attenuate the impact of inflammatory response on absolute tissue accumulation. To this point, when we examined the total (free and bound) concentrations of LPV in fetal tissues, the levels in poly(I:C) and control groups were not significantly different,

suggesting there is likely no appreciable difference to pharmacodynamics. However, the fact that increased levels of tissue accumulation are seen when normalized to unbound LPV plasma concentrations indicates that transfer of free drug is greater in the poly(I:C) group. This suggests that, in addition to altered plasma protein binding, decreased expression of the efflux transporters plays a role in LPV disposition. While LPV is clinically administered orally as a co-formulation with low-dose RTV in order to increase bioavailability, it has been reported that LPV binding to AAG is not affected by RTV at therapeutically relevant concentrations (Gulati *et al.*, 2009).

In conclusion, our study in a rat model of viral infection demonstrated that inflammation-mediated changes in plasma protein binding, drug metabolism and drug transport significantly alter maternal and fetal disposition of LPV. It remains to be elucidated whether a similar trend would be seen in clinical cases of co-infection in pregnant women. As LPV metabolism and P-gp mediated transport appear highly comparable between rodents and humans (Kumar *et al.*, 1999), further clinical investigation of inflammation-mediated effects on LPV is warranted. Moreover as LPV is generally administered in combination with other antiretrovirals that are substrates of P-gp, MRP2, BCRP, and CYP3A (Kis *et al.*, 2010), the effects of inflammation on combination ARV therapies remain to be elucidated.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: V.P. and M.P.M.

Conducted experiments: V.P.

Performed data analysis: V.P.

Wrote or contributed to the writing of the manuscript: V.P. and M.P.M.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: *IFN- γ concentration in maternal plasma.* Pregnant rats were injected with poly(I:C) and sacrificed 24 h later. IFN- γ plasma concentrations were determined via ELISA and are presented as pg/ml of plasma. Data represent the mean \pm S.E. ($n=20$ /group) and statistics calculated by *t*-test. ***, $p < 0.001$, as compared to saline control.

Figure 2: *Effect of poly(I:C) on the hepatic and placental mRNA expression.* Hepatic and placental tissues were collected from near term pregnant rats 24–26 h after poly(I:C) administration (5mg/kg, i.p). Analysis of mRNA expression was performed via qRT-PCR and gene expression normalized to *18s*, as described under *Materials and Methods*. Data represent the mean \pm S.E. as a percentage of control value ($n = 7-10$ /group) with statistics calculated by *t*-test. *, $p < 0.05$, as compared to saline control.

Figure 3: *Time course of LPV concentration in maternal circulation.* Pregnant rats were administered LPV (10 mg/kg, i.v.) 24 h after exposure to poly(I:C) (5 mg/kg, i.p.). Maternal blood samples were collected at 5, 25, 45, 60 and 120 min post-LPV injection ($n = 4$ dams/ group/ time point). Total drug concentrations in maternal plasma were determined via LC-MS/MS, as described under *Materials and Methods*. Results are presented as mean \pm S.D. Statistical differences between the two groups at each time point were calculated by *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Figure 4: *Fetal and tissue accumulation of LPV.* Pregnant rats were administered LPV (10 mg/kg, i.v.) 24 h after exposure to poly(I:C) (5 mg/kg, i.p.). Maternal blood, hepatic and placental tissue and fetuses were collected at 5, 25, 45, 60 and 120 min post-LPV injection. Drug concentrations in maternal plasma unbound fraction, tissue and fetal homogenates were determined via LC-MS/MS, as described under *Materials and Methods*. Results are presented as mean \pm S.E. ($n = 4$ dams/group/time point; for fetal and placental concentrations $n = 3$ / dam). Statistical differences between the two groups at each time point were calculated by *t*-test. *, $p < 0.05$.

Table 1

Pharmacokinetic parameters of lopinavir in plasma of control versus poly(I:C)-treated pregnant rats following a 10 mg/kg i.v dose

		control	poly(I:C)
λ_z^a	(1/min)	0.0076	0.0080
C_o^a	(ng/ml)	6199	17329
AUC_{0-1NF}^a	(min*ng/ml)	220085	482860
CL^a	(ml/min/kg)	45	21
V_{ss}^a	(ml/kg)	4567	1803
$f_{u, plasma}^b$	(%)	0.85 ± 0.33	0.10 ± 0.01*

λ_z : first-order elimination rate constant, k_e ; C_o : concentration at time zero; CL: clearance;

V_{ss} : volume of distribution; f_u : fraction unbound

^a Predicted values calculated by non-compartmental analysis (Phoenix WinNonlin)

^b percentage unbound LPV (mean ± SE) calculated as described under *Materials and*

Methods.

* Significantly different from controls, $p < 0.05$

Table 2

Lopinavir plasma and tissue AUC_{5-120 min}

Maternal plasma and fetal, placental and hepatic AUCs (5–120 min) were calculated using Bailer’s approach as described under *Materials and Methods*. Data represent mean \pm SD ($n = 4$ dams per time point, 3 fetus or placenta per dam). Z_o values were calculated to assess the statistical differences of AUCs (shown as p -values) between the control and poly(I:C)-treated pregnant dams.

AUC _{5-120 min}	control	poly(I:C)	Z_o	p
Maternal plasma (ng*min/ml plasma)	126060 \pm 13530	282550 \pm 27720	5.07	<0.001
Fetus (ng*min/g fetus)	2900 \pm 400	2050 \pm 420	1.45	>0.05
Placenta (ng*min/g placenta)	26830 \pm 2240	30200 \pm 3470	0.82	>0.05
Liver (ng*min/g liver)	82660 \pm 11820	83330 \pm 8510	0.05	>0.05

Figure 1

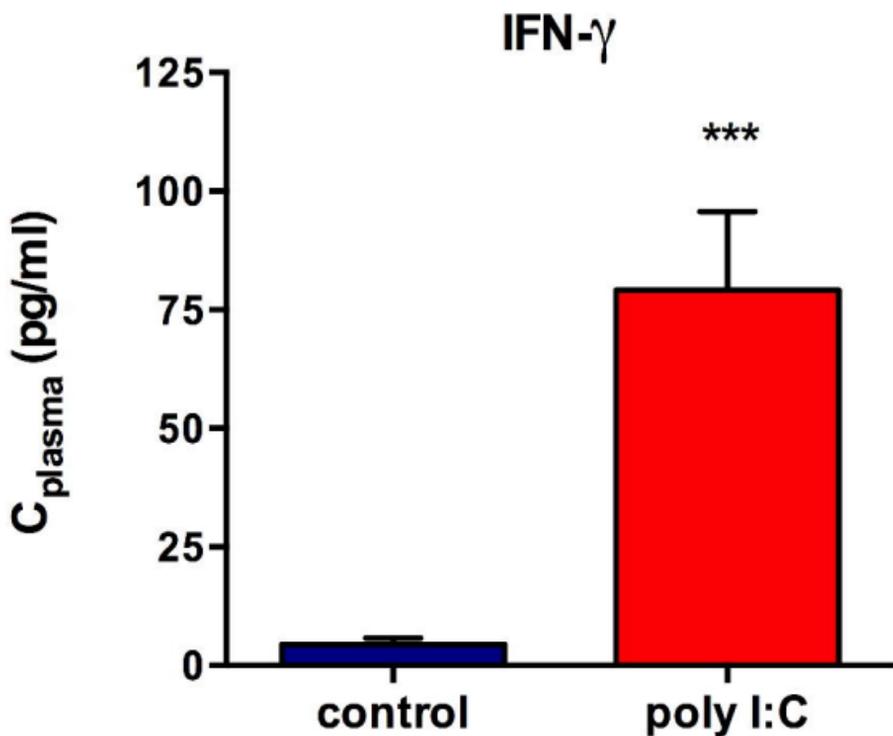


Figure 2

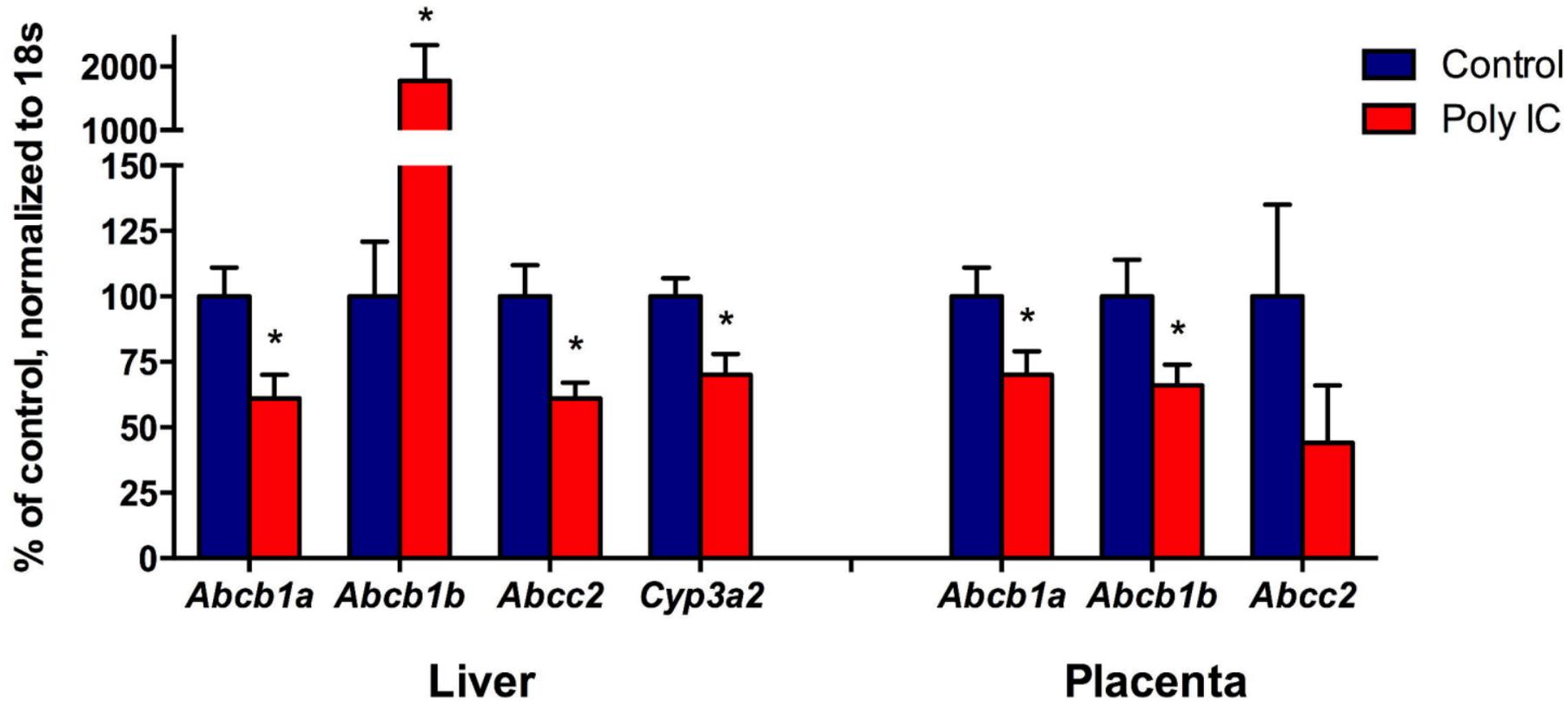


Figure 3

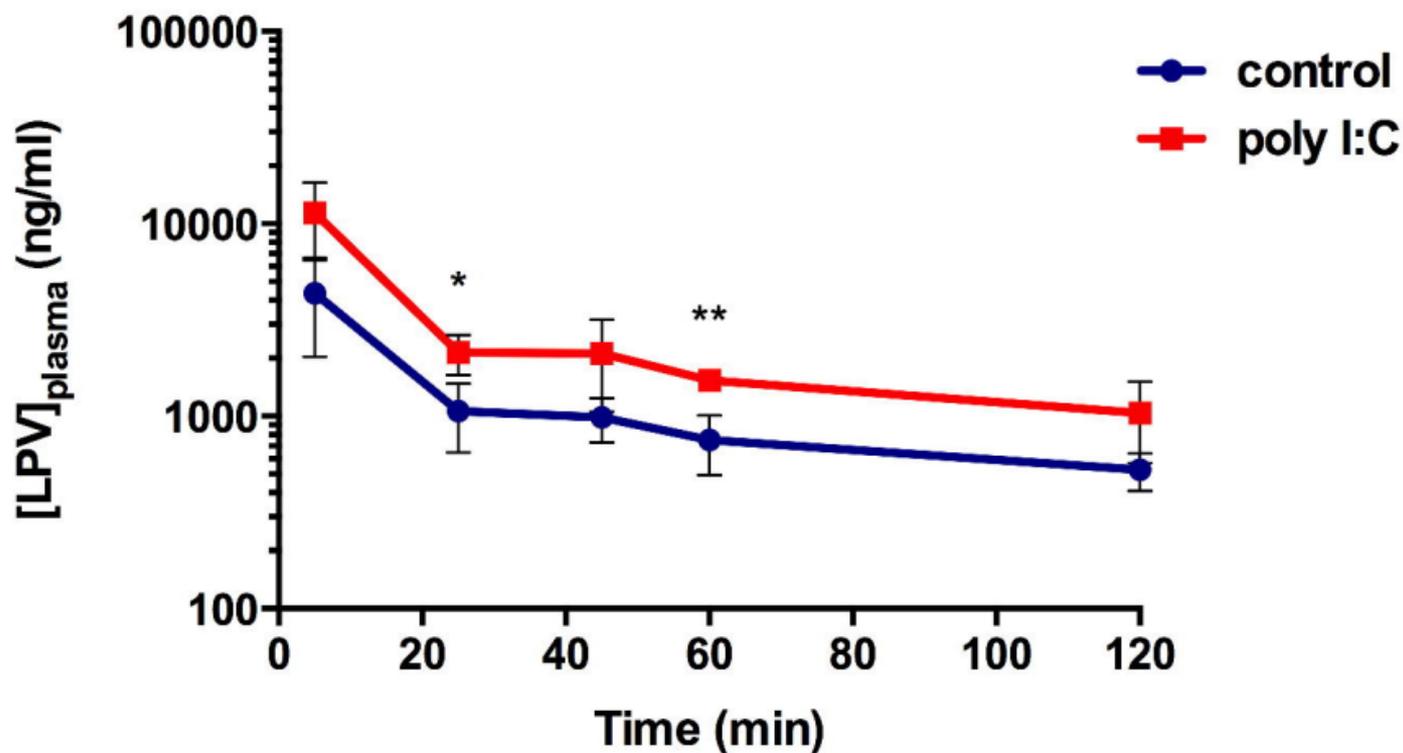
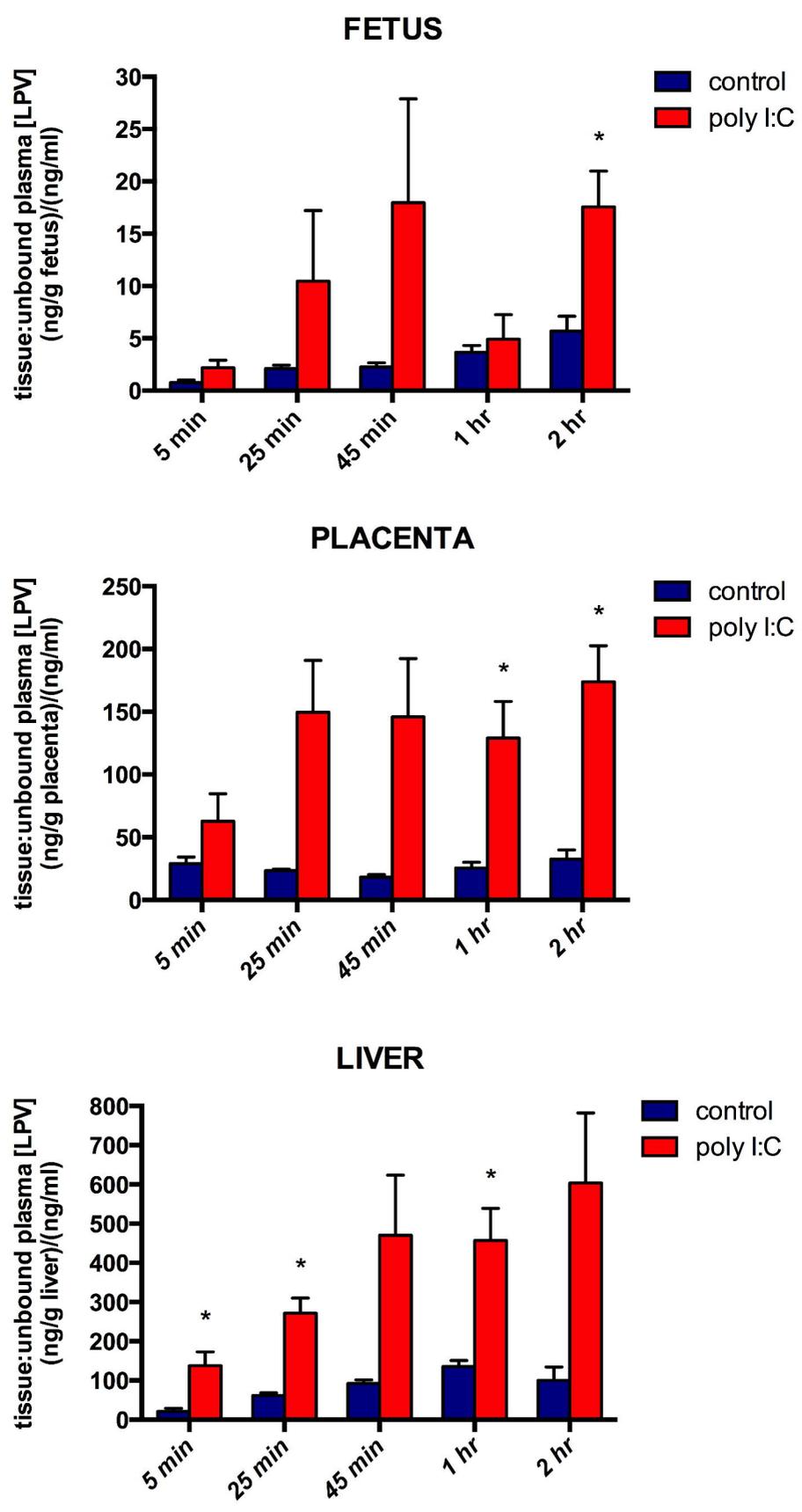


Figure 4



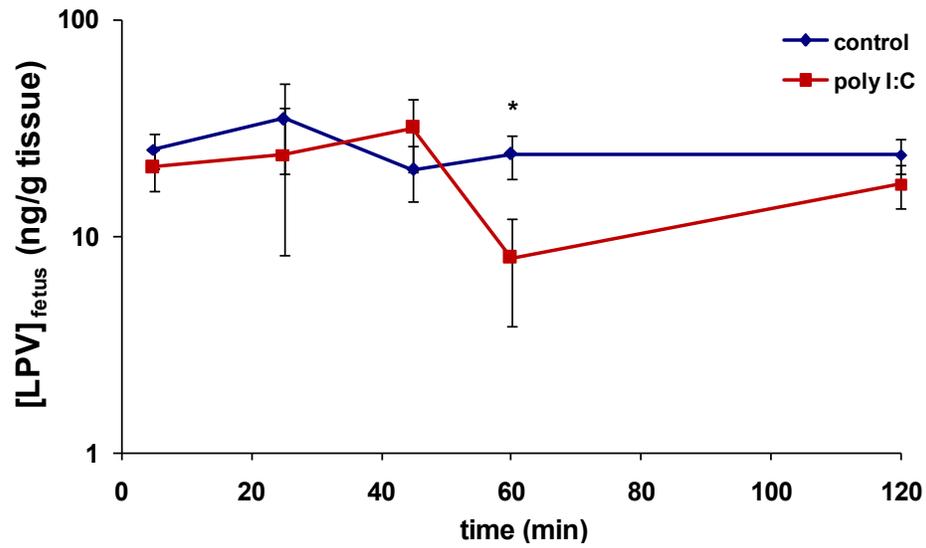
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Authors: Vanja Petrovic and Micheline Piquette-Miller

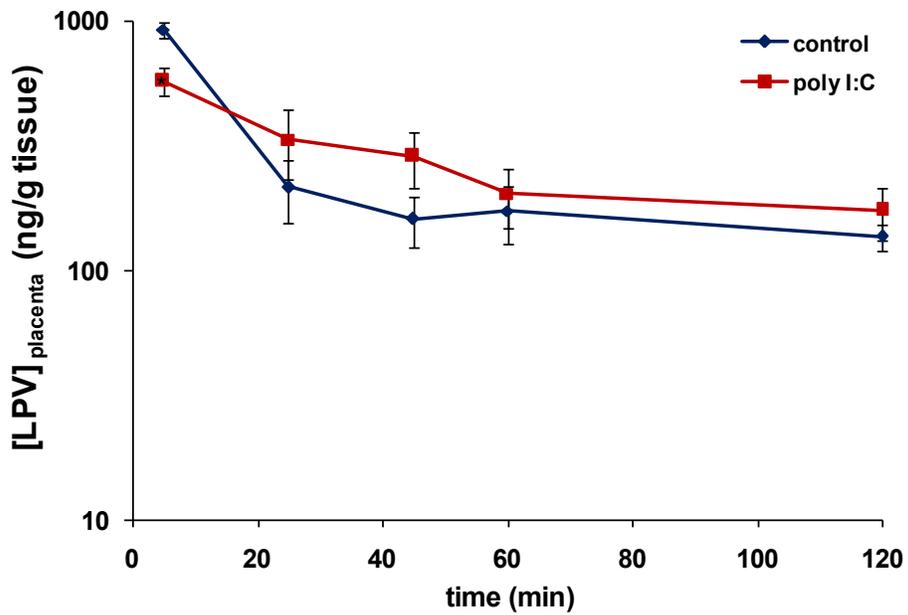
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Supplemental Figure 1

(A)

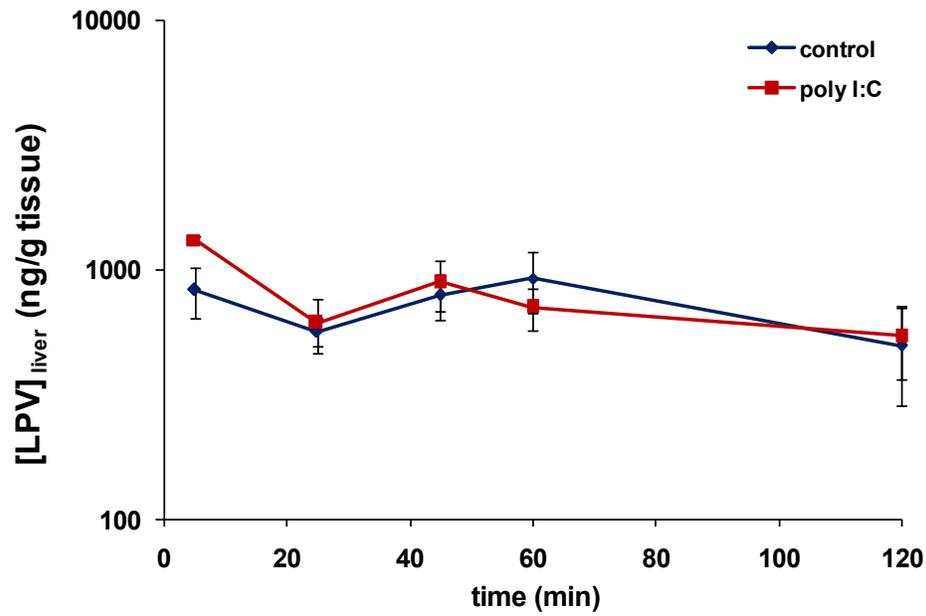


(B)



Article title: Polyinosinic/Polycytidylic Acid-Mediated Changes in Maternal and Fetal Disposition of Lopinavir in Rats
Authors: Vanja Petrovic and Micheline Piquette-Miller
Journal title: Drug Metabolism and Disposition

(C)



Supplemental Figure 1: Time course of LPV concentration in tissues: (A) Fetus, (B) Placenta, (C) Liver. Pregnant rats were administered LPV (10 mg/kg, i.v.) 24 h after exposure to poly(I:C) (5 mg/kg, i.p.). Tissue samples were collected at 5, 25, 45, 60 and 120 min post-LPV injection ($n = 4$ dams/ group/ time point). Total drug concentrations in tissues were determined via LC-MS/MS, as described under *Materials and Methods*. Results are presented as mean \pm S.E. Statistical differences between the two groups at each time point were calculated by *t*-test. *, $p < 0.05$.