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Selective modulation of placental and fetal MDR transporters by chronic *in utero* exposure to NRTIs in Sprague-Dawley rats: Importance for fetoprotection

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ABSTRACT

Multidrug resistance (MDR) transporters present in placenta and fetal tissues reduce intracellular accumulation of their substrates. Consequently, induction of protein expression may further reduce toxic effects of specific xenobiotics. This work aimed to study whether sustained drug treatments *in utero* could modulate MDR transporters P-gp, BCRP, and MRP2 and thus impact their fetoprotective action. Pregnant Sprague-Dawley rats were daily treated by gavage with zidovudine (AZT, 60 mg/kg) or lamivudine (3TC, 30 mg/kg) from gestation day (GD) 11 to 20. On GD 21, DNA damage and MDR protein abundance were assessed by comet assay and western blotting, respectively. Moreover, a single IV dose of AZT or 3TC was administered on GD 21 and drug concentrations were measured in maternal blood and fetal liver by HPLC-UV. Chronic exposure to 3TC caused significantly higher DNA damage than AZT in fetal liver cells, whereas no differences were observed in maternal blood cells. Increased levels of BCRP protein were found in the placenta and fetal liver after AZT, but not 3TC, chronic *in utero* exposure. Contrarily, no modifications in the protein abundance of P-gp or MRP2 were found after sustained exposure to these drugs. The area under the curve of AZT in fetal liver was significantly lower in the AZT-pretreated rats than in the VEH or 3TC groups. Moreover, pre-administration of the BCRP inhibitor gefitinib (20 mg/kg, IP) increased AZT levels to the values observed in the VEH-treated group in this tissue. On the other hand, the disposition of 3TC in maternal blood or fetal liver was not modified after chronic treatment in either group. In conclusion, chronic exposure to AZT selectively induces BCRP expression in the placenta and fetal liver decreasing its own accumulation which may account for the lower DNA damage observed for AZT compared to 3TC in fetal liver cells.

1. Introduction

ABC transporters are a large and ubiquitous superfamily of membrane transporter proteins that pump their substrates out of cells at the expense of ATP hydrolysis (Choi, 2005). They transport various amphiphilic molecules including endogenous metabolites, nutrients, toxic compounds, and drugs (Pavek and Smutny, 2014; Tocchetti et al., 2016). At the placenta, ABC transporters are localized in the apical and

Abbreviations: 3TC, Lamivudine; T-DNA %, Percentage of DNA in the comet tail; ABC, ATP-binding Cassette; AUC, Area Under the Curve; AZT, Zidovudine; BCRP, Breast cancer resistance protein, also known as Abcg2; BW, Body Weight; cART, Combination Antiretroviral Therapy; GD, Gestation Day; GFT, Gefitinib; HBBS, Hank's Balanced Salt Solution; HIV, Human Immunodeficiency Virus; IP, Intraperitoneal; IV, Intravenous; MDR, Multidrug Resistance; MRP2, Multidrug Resistance-associated Protein 2, also known as Abcc2; NRTI, Nucleoside Reverse Transcriptase Inhibitor; PBS, Phospate Saline Buffer; P-gp, P-glycoprotein, also known as Abcb1; VEH, Vehicle.

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basolateral membranes of the syncytiotrophoblast as well as in endothelial cells (for a detailed review see Joshi et al., 2016). Apical ABC transporters protect the developing fetus from endogenous toxic compounds and xenobiotics (Bloise et al., 2016). Several ABCs confer a multidrug resistance phenotype (MDR) to humans against drugs used in pharmacological treatments. P-gp (P-glycoprotein, ABCB1) and BCRP (breast cancer resistance protein, ABCG2) are especially important from a toxicological point of view. These transporters are expressed on the apical membrane of the syncytiotrophoblast in humans and in layer II of the syncytiotrophoblast in rats (Han et al., 2018). P-gp expression in rat placenta is higher at term than in early gestation whereas BCRP peaks at mid-gestation and declines towards term. In humans, P-gp decreases along gestation while reports on BCRP expression over gestation are conflicting (Han et al., 2018). The inhibition of P-gp and BCRP at the placental level increases the access of xenobiotics to the fetus supporting its fetoprotective role (Huisman et al., 2001; Jonker et al., 2000; Lankas et al., 1998; Wang et al., 2003). Thus, they are critical gatekeepers expelling toxic compounds away from the fetal compartment to the maternal circulation (Ceckova-Novotna et al., 2006; Hahnova-Cygalova et al., 2011).

The fetal liver acquires the capacity for biotransformation and elimination of drugs in late gestation (Han et al., 2018). P-gp and BCRP are localized in the bile canalicular membrane of fetal hepatocytes in rats and humans (Han et al., 2018). Even though there are no studies on the role of P-gp and BCRP in the fetal liver, based on their localization they could facilitate the biliary elimination of drugs from the fetus.

Five Multidrug Resistance-associated Proteins (MRPs/ABCCs) are expressed in human placenta. MRP1 and MRP2 show prominent levels, whereas MRP3, 4, and 5 are poorly expressed (Slot et al., 2011). MRP1 is found on the basal membrane of syncytiotrophoblast and in endothelial cells and its primary role seems to be nutrient rather than xenobiotics transport (Atkinson et al., 2003). MRP2 is apically expressed in the syncytiotrophoblast in humans and in the second layer of syncytiotrophoblast in rats (Meyer Zu Schwabedissen et al., 2005; St-Pierre et al., 2004) transporting substances in the fetal-to-maternal direction (Aye and Keelan, 2013). MRP2 was found to be expressed in the placenta from week 32 and from day 12 of gestation in humans and rats, respectively. In both species, the expression of placental MRP2 increases towards the end of gestation (Joshi et al., 2016; Meyer Zu Schwabedissen et al., 2005; St-Pierre et al., 2004). In the rat fetal liver, MRP2 appears in the canalicular membrane on GD 12 reaching levels at term markedly higher than those in the adult liver (Meyer Zu Schwabedissen et al., 2005; St-Pierre et al., 2004). Like P-gp and BCRP, the polarized localization of MRP2 in fetal liver suggests its importance in fetal detoxification (Gao et al., 2004).

Nucleoside reverse transcriptase inhibitors (NRTIs) are prescribed to pregnant women as part of the combination antiretroviral therapy (cART). This therapy keeps HIV infections under control and prevents mother-to-child transmission (Council, 2021). Lamivudine (3TC) and zidovudine (AZT) are the dual-NRTI backbone of cART used in developing countries, achieving a vertical transmission rate below 1% (Townsend et al., 2014). Nonetheless, NRTIs are genotoxic lipophilic compounds able to cross the placenta which may cause damage to the developing fetus (Gilmore et al., 2021; Poirier et al., 2015).

AZT is a substrate of BCRP in human lymphocytes (Wang et al., 2003). Moreover, AZT is transported and induces BCRP in the placenta and the fetal brain barrier of rats (Filia et al., 2017; Neumanova et al., 2016). Also, the literature reports that AZT would be a substrate and inducer of P-gp in rat adipocytes, placenta and intestine (Janneh et al., 2010; Neumanova et al., 2016; Quevedo et al., 2011). In contrast, the interaction between AZT and P-gp in human cells has been disregarded (Anderson et al., 2006; Eilers et al., 2008; Lucia et al., 1995; Weiss et al., 2008). On the other hand, AZT is not transported by MRPs in the rat placenta (Neumanova et al., 2016). In turn, limited literature exists about whether 3TC is a substrate of MDR proteins. A study shows that 3TC is not a substrate of P-gp, BCRP or MRPs nor does it modulate these transporters (Ceckova et al., 2016). However, another study found that 3TC is transported by BCRP *in vitro* in human lymphocytes (Wang et al., 2003).

In summary, P-gp, BCRP and MRP2 might restrict the passage of NRTIs to the fetus at the placental level and facilitate their excretion back to maternal blood in the fetal liver. Understanding how sustained drug treatments modulate their function *in vivo* will provide valuable information for optimizing drug therapy during pregnancy. To accomplish this objective, we generated a model of chronic oral administration of xenobiotics to pregnant rats. The pregnant rat model is appropriate to study drug passage *in vivo* since the gestation time is short and the MDR transporters have the same localization as in humans. In addition, each fetus is contained in an individual sac with the placenta and amniotic fluid which allows for serial samples to be obtained at different time intervals.

Here, we analyze the effects of sustained *in utero* exposure to AZT or 3TC on the abundance of P-gp, BCRP and MRP2 in the placenta and fetal liver. In addition, we evaluated whether changes in MDR transporters impact drug levels and DNA toxicity in maternal blood and fetal liver. This study aims to shed light on the relevance of iatrogenic modulation of MDR proteins in drug delivery to the fetal tissues. Our hypothesis is that sustained *in utero* exposure to a substrate drug, selectively induces MDR transporters in the placenta and fetal liver as a dynamic response to toxic injury.

2. Materials and methods

2.1. Materials

Zidovudine (1-[(2*R*,4*S*,5*S*)-4-azido-5-(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dione[1] and lamivudine (4-amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one) were provided by Laboratorios Richmond (Buenos Aires, Argentina). Gefitinib (*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine; hydrochloride) was provided by AstraZeneca, Chesire, UK). Protease inhibitor cocktail (Complete mini, Roche Applied Science, Mannheim, Germany) and phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO), and all the other chemicals not listed here were of the highest purity.

2.2. Methods

2.2.1. Animals

Female Sprague-Dawley rats (8 weeks, 150–200 g body weight) were housed under a 12:12-h light-dark cycle, at controlled room temperature with food and water *ad libitum*. All procedures involving animals were conducted in accordance with guidelines provided by the National Research Council (Guide for the Care and Use of Laboratory Animals, 8th ed., 2011) and approved by the Institutional Animal Care and Use Committee (CICUAL-FFyB). The 3Rs principle (Reduction, Refinement, and Replacement) was considered to design the animal protocols. Pregnancy was confirmed by the presence of sperm in the vaginal smear the morning after matting and was considered post gestational day 0 (GD 0). Litter sex composition was not determined and fetuses for experiments were chosen on a random basis.

2.2.2. Treatments

Pregnant rats were randomized into three groups to be administered with AZT (60 mg/kg), 3TC (30 mg/kg) or the corresponding vehicle (VEH, 0.02% ethanol) from GD 11 to GD 20 by esophageal gavage every 24 h ($n = 6$ animals per group). Dose of AZT was selected based on reported evidence showing quantifiable values in placenta, amniotic fluid, and whole fetal tissue homogenates in rats (Brown et al., 2003). Regarding 3TC, the dose was selected from preliminary studies conducted on GD 11 in order to reach similar levels than with AZT in both maternal blood and fetal liver (data not shown).

2.2.3. Pharmacokinetic analysis

2.2.3.1. Sample collection. Pharmacokinetics studies were performed in rats on GD 21 after daily oral pretreatment with either AZT, 3TC or VEH from GD 11 to GD 20. Anesthesia was conducted with a single dose of urethane 20 mg/kg IP, and surgery started only when extinction of corneal reflex was confirmed. Rats were cannulated in the femoral vein for intravenous (IV) administration of AZT (60 mg/kg) or 3TC (30 mg/kg) and the carotid artery for blood sampling. A laparotomy was performed and a small incision was made in the uterine wall to random sampling of the pups. Serial samples of maternal blood and fetal sacs were obtained at 0, 1, 2, 3 and every 5 min for 30 min from each dam (6–8 dams per treatment group). Maternal blood samples (100 μl) were collected in heparinized tubes. The fetus was dissected under the binocular loupe and the fetal liver was separated from the Glisson's capsule and homogenized in two volumes of deionized water. Blood and liver samples were subjected to protein precipitation by the addition of 15% trichloroacetic acid (final concentration) on ice for 15 min and then centrifuged at 5000 xg for 10 min. Supernatants were stored at −80 °C until analysis. To evaluate the effect of BCRP inhibition, the selective BCRP inhibitor gefitinib (GFT) was administered (20 mg/kg IP) 2 h before the IV administration of drugs as in Roma et al., 2015.

2.2.3.2. Quantification of AZT and 3TC in biological samples. AZT and 3TC in maternal blood and fetal liver were quantified by HPLC-UV, as previously described by Fan and Stewart, 2002. This method was validated for the quantification of AZT y 3TC in human plasma with an intra-day and inter-day variability lower than 10%. Instrumentation: The HPLC system consisted of a Spectra series P100 pump with a 20 μl injection loop (Thermo separation products, Virginia, USA). Separation was performed using a Luna C8 column (250 × 4.6 mm, 5 μm particle size, Phenomenex, CA, USA) for AZT samples and a Gemini-NX C18 column (15 × 4.6 mm, 5 μm particle size, Phenomenex, CA, USA) for 3TC samples. Chromatographic conditions: Isocratic elution at room temperature; flow rate: 1.2 ml/min. UV Detection: 266 nm for AZT, 280 nm for 3TC; Mobile phase: 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt)–acetonitrile (86:14, v/ v) with pH adjusted to 3.2 with phosphoric acid (Fan and Stewart, 2002). Internal standard: In the AZT trials, 3TC was used as the internal standard and *vice versa*. When it was required to measure both analytes, nevirapine was used as the internal standard. Recovery: Analytes from plasma and fetal liver were higher than 85%. Quantification limit: 0.05 μg/ml for AZT and 0.08 μg/ml for 3TC. Samples were injected in duplicate into the HPLC column.

2.2.4. Alkaline comet assay (single cell gel electrophoresis)

The extent of DNA strand breaks in the fetal liver tissue and maternal blood cells on GD 21 from the three groups ($n = 6$ per group) were assessed using the alkaline comet assay, as previously described by Tice et al., 2000. Samples were obtained at time zero. Briefly, after electrophoresis, DNA was stained with ethidium bromide. Scoring: Two different observers performed blind measurements to ensure objectivity. Single cells (comets) were observed at 200× magnification in an Eclipse 50i NIKON light microscopbinoe equipped with a fluorescent lamp (excitation filter of 515–560 nm) coupled to a Nikon DS-SM camera. Fifty comets photographed from different areas of two slides per tissue sample were scored using the free access software CometScore (TriTek CometScore Freeware 1.6.1.13). Misshaped, overlapped, or debris-covered comets were excluded from the analysis. The percentage of DNA in the comet tail (T-DNA %) was chosen as the parameter to evaluate DNA damage. The mean T-DNA % for each animal was calculated for both maternal blood and fetal liver cells. In addition, the distribution of comets according to three levels of T-DNA % (low: 0–33%, medium: 34–66% and high: 67–100%) was analyzed for fetal liver samples.

2.2.5. Western blot analysis

Fetal livers and placentas from rats on GD 21 were obtained at time zero, as detailed in pharmacokinetic analysis were processed as a pool of three according to Filia et al., 2017. Briefly, 50 μg of protein per well were loaded, separated on a 7.5% SDS-polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane (PVDF, Amersham Hybond GE Healthcare Life Sciences, UK). Membranes were blocked with 5% non-fat milk prepared in TBS-T (1 h, at room temperature). Blots were incubated with mouse monoclonal anti P-gp (MDR1; D-11; 1:500; Santa Cruz Biotechnologies; USA), mouse monoclonal anti BCRP (BXP-21 1:800; Santa Cruz Biotechnologies; USA) and mouse monoclonal anti MRP2 (M2III-5; 1:150; Abcam; UK), diluted in 1% serum albumin in TBS-Tween 20. Rabbit polyclonal anti-actin (1:1000; Sigma-Aldrich) was used as loading control. The immune complexes were detected by incubation for 90 min with goat anti-rabbit IgG horseradish peroxidase-conjugated (sc-2004; 1:2000; Santa Cruz Biotechnologies; USA) or goat anti-mouse IgG horseradish peroxidase-conjugated (sc-2005; 1:2000; Santa Cruz Biotechnologies; USA). The bands were detected by chemiluminescence (Amersham ECL Biosciences, Amersham, UK) and quantified by densitometric analysis using ImageJ software (1.34S, US National Institutes of Health, Bethesda, MD).

2.3. Data analysis

All experiments were performed by triplicate using tissues from 6 to 8 dams per treatment group. Statistical analysis was performed using GraphPad Software. Analyzed data fit a normal distribution according to D'Agostino & Pearson as well as Shapiro-Wilk normality tests. We did not detected outliers by Rout method under a Q value specified at 1%. Statistical significance between two groups was assessed by a two-tailed Student's *t*-test for unpaired experimental values. Comparison between multiple groups was performed using one-way analysis of variance (ANOVA) or two-way ANOVA when corresponding followed by Bonferroni post-hoc test. Results are presented as mean ± s.e.m.

3. Results

3.1. Effect of chronic treatment with NRTIs on the weight of pups and fetal resorptions

Daily oral administration with 60 mg/kg AZT and 30 mg/kg 3TC to pregnant rats from GD 11 to GD 20 did not modify weight gain in pregnant rats (Fig. 1A) but significantly decreased the average fetal weight per litter compared to VEH-treated animals (Fig. 1B). Furthermore, the average fetal weight after chronic *in utero* exposure to 3TC was significantly lower compared to AZT exposure (Fig. 1B). In turn, the average weight of the placentas was significantly lower after chronic *in utero* exposure to 3TC, but not AZT, when compared to VEH-treated animals (Fig. 1C). Moreover, there was a significant increase in the number of fetal resorptions per litter in the 3TC group compared to VEH- and AZT-treated rats while no differences were found in fetal resorptions between VEH- and AZT-treated rats (Fig. 1D). These results showed higher developmental toxicity in fetuses exposed *in utero* to 3TC than in those exposed to AZT.

3.2. Effect of chronic treatment with NRTIs on DNA damage in maternal blood and fetal liver

Since AZT and 3TC are proven genotoxic compounds, maternal blood cells and fetal liver cells were analyzed *via* the alkaline single cell electrophoresis assay (comet assay). Images of cells with different DNA damage level obtained during the analysis of maternal blood (Fig. 2A–C) and fetal liver (Fig. 3A–C) are shown for all treatment groups. The genotoxicity, evaluated as the percentage of DNA in the comet tail (T-DNA %), was significantly greater in maternal blood cells isolated from pregnant rats chronically treated with either AZT or 3TC compared to

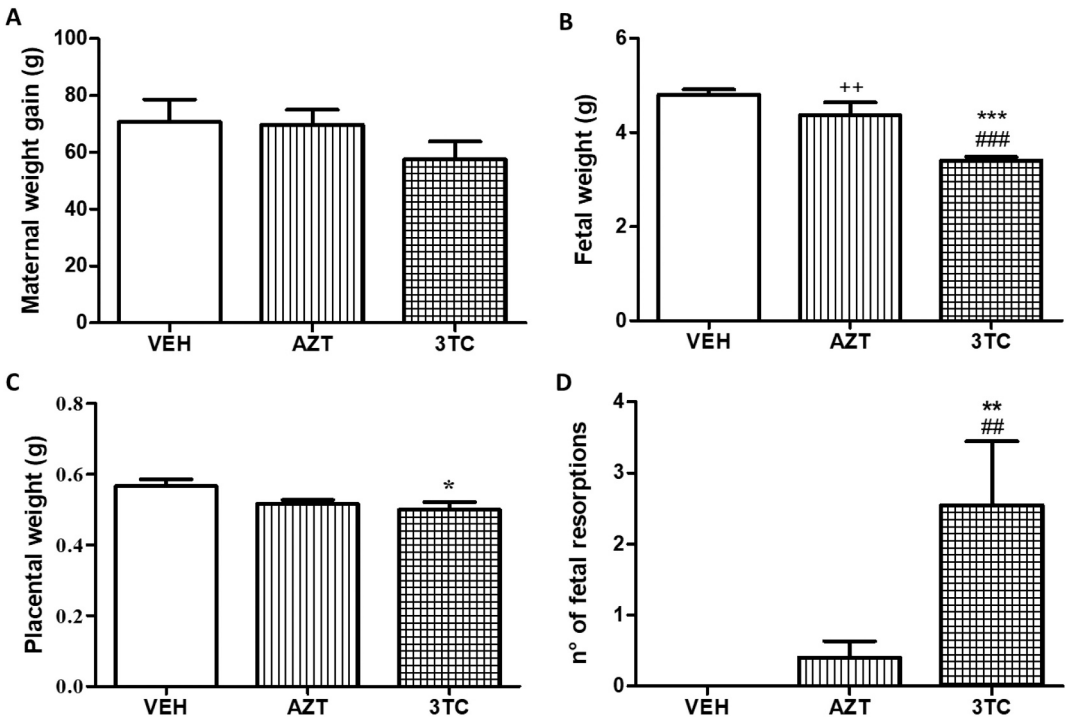

Fig. 1. Effect of chronic treatment with NRTIs on maternal and developmental parameters of pups. Effect of chronic treatment with VEH (white bars), AZT (striped bars) and 3TC (squared bars) on maternal weight gain (A), fetal weight (B), placental weight (C), and number of fetal resorptions (D) found during laparotomy for extraction of pups. Data ($n = 6$ per group) are presented as media ± s.e.m. and was analyzed by one-way ANOVA and Bonferroni's post-hoc test. ***: $p <$ 0.001 between 3TC and AZT. #: $p < 0.05$ and ###: p < 0.001 between 3TC and VEH. ++: $p < 0.01$ between AZT and VEH.

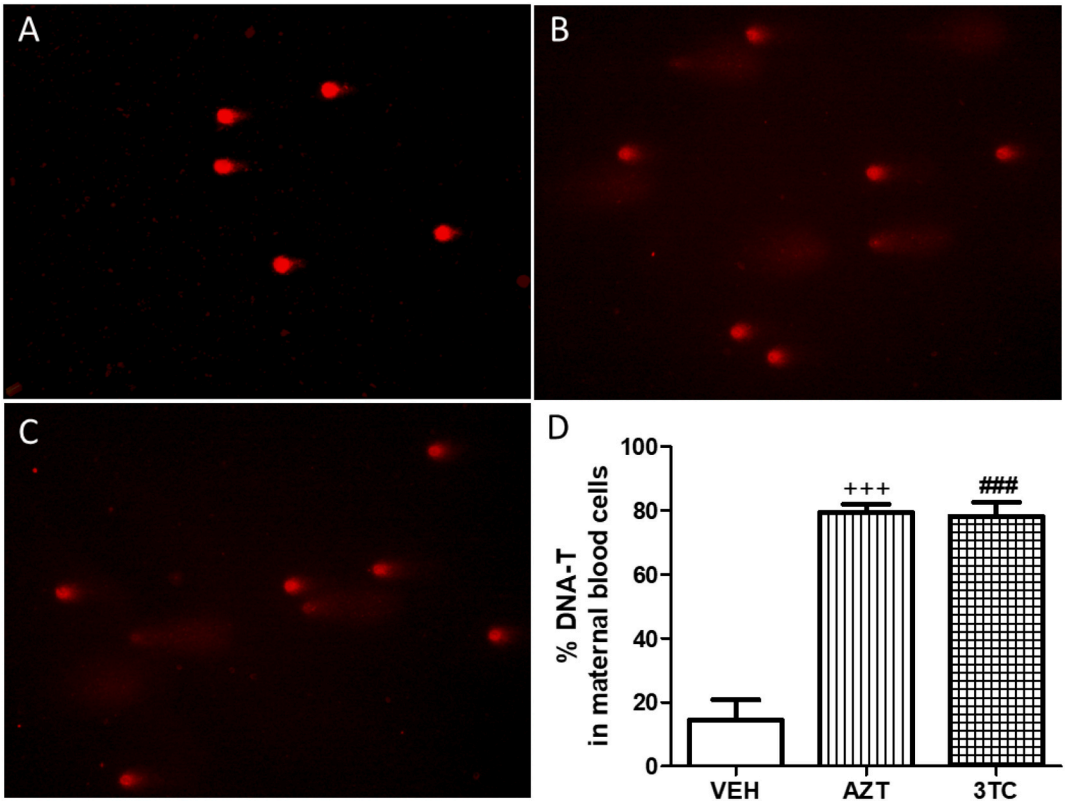

Fig. 2. DNA damage of maternal whole blood cells determined by *in vivo* alkaline comet assay. Representative photomicrographs of blood cells comets isolated from pregnant rats (GD 21) exposed to VEH (A), 60 mg/kg AZT (B) or 30 mg/kg 3TC (C) daily by gavage from GD 11 to GD 20. Images taken a 200× magnification (scale bar represents 50 μm). (D) DNA damage measured *via* the percentage of DNA in the comet tail (T-DNA %) of 100 comets per slide by duplicated. Results are expressed as mean ± s.e.m. of n = 6 per group. Statistics were calculated by one-way ANOVA with Bonferroni's post-hoc test. ###: p < 0.001 between 3TC and VEH. +++: p < 0.001 between AZT and VEH.

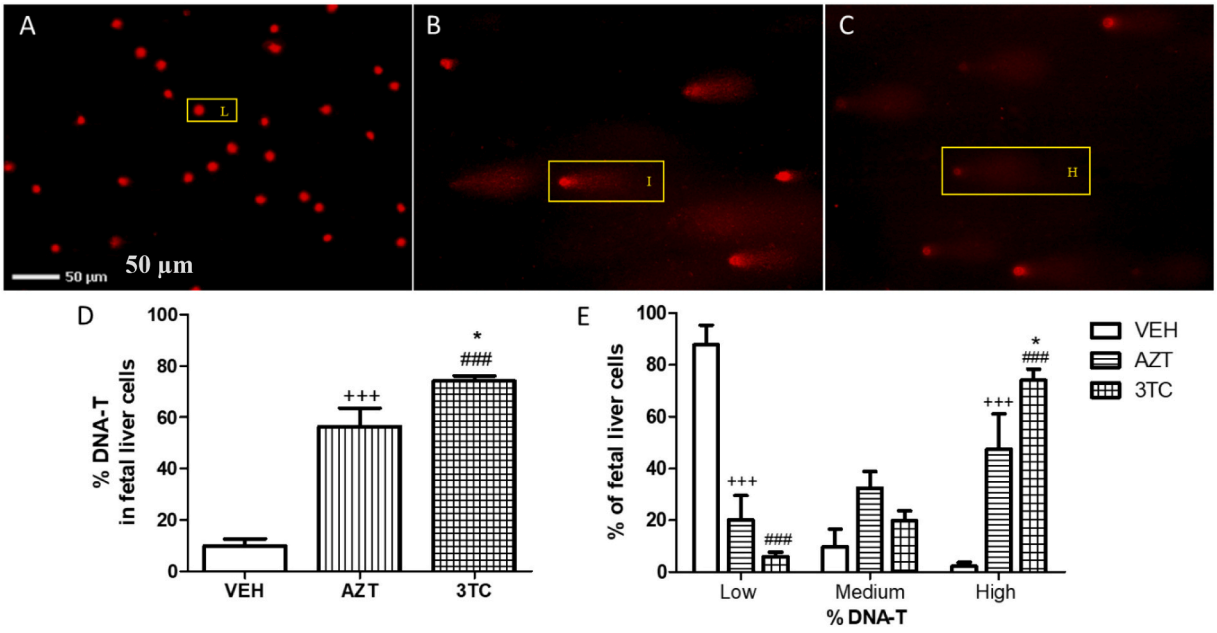

Fig. 3. DNA damage of fetal liver cells determined by *in vivo* alkaline comet assay. Representative photomicrographs of fetal liver comets isolated from pregnant rats (GD 21) exposed to VEH (A), 60 mg/kg AZT (B) or 30 mg/kg 3TC (C) daily by gavage from GD 11 to GD 20. Images taken with a 200× magnification (scale bar represents 50 µm). (D) DNA damage measured *via* the percentage of DNA in the comet tail (T-DNA %) of 100 comets per slide by duplicated. (E) DNA damage distribution in liver cells portraying low (0–33 T-DNA %, L), medium (34–66 T-DNA %, I) and high (67–100 T-DNA %, H). Results are expressed as mean ± s.e.m. of n = 6 per group. Statistics were calculated by one-way ANOVA (D) or two-way ANOVA (E) with Bonferroni's post-hoc test. *: $p < 0.05$ between 3TC and AZT, ###: $p < 0.001$ between 3TC and VEH +++: $p < 0.001$ between AZT and VEH.

the VEH-treated group (Fig. 2D). Moreover, T-DNA % in maternal blood cells was similar after exposure to AZT or 3TC. In turn, in fetal liver, DNA damage for each of the treatment groups was firstly evaluated also by comparing the means of T-DNA %. As observed for maternal blood cells, T-DNA % in fetal liver cells caused by AZT and 3TC was significantly higher than in VEH. In addition, it was found that T-DNA % in fetal liver after *in utero* exposure to 3TC was significantly higher than after exposure to AZT (Fig. 3D). Besides, for fetal liver cells, a genotoxic damage stratification was performed at three levels (T-DNA % low: 0–33%, medium: 34–67%, and high: 77–100%; Fig. 3E). As expected, a percentage close to 100% of fetal liver cells with low T-DNA % was found in VEH group. Compared with VEH group, the percentage of cells with low DNA damage level was significantly reduced by *in utero* treatment with AZT and 3TC treatment, the latter being even significantly lower. In contrast, no significant differences were found in the percentage of cells with a medium level of DNA damage. Finally, in the VEH group, the percentage of cells with high level of damage was negligible and significantly lower than under both NRTI treatments. Furthermore, in the group chronically treated with 3TC, it was observed that the percentage of fetal liver cells with high T-DNA % level was close to 80% and significantly higher than in the AZT group (Fig. 3E).

3.3. Modulation of membrane transporters in the placenta and fetal liver by chronic AZT and 3TC administration

Fig. 5A shows representative immunoblots fo P-gp, MRP2 and BCRP according to the treatment group and tissue. The chronic oral administration of AZT to pregnant rats from GD 11 to GD 20 did not statistically modify the expression level of MRP2 and P-gp neither in the placenta nor in the fetal liver (Fig. 4B–E). However, AZT treatment selectively increased the protein abundance of BCRP in the placenta as well as in the fetal liver (Fig. 4F and G).

Concerning animals treated chronically with 3TC, no modifications in the expression levels of ABC transporters proteins were found in the placenta or fetal liver (Fig. 4B–F). The present results suggest that the lower toxicity of AZT in fetal livers isolated from pups chronically exposed *in utero* to AZT could be related to the overexpression of BCRP in the placenta as well as in the fetal liver.

3.4. Effect of chronic oral administration of AZT and 3TC on their accumulation in maternal blood and fetal liver

To analyze the disposition of AZT and 3TC, the levels of these NRTIs in maternal blood and fetal liver were quantified by HPLC-UV. An IV dose of 30 mg/kg BW of 3TC or 60 mg/kg BW of AZT was administered to pregnant rats on GD 21 that had been chronically exposed to each of the drugs or the VEH. Drug levels were quantified in maternal blood and fetal liver during the first 30 min post-administration. No residual concentrations were found at time 0, which coincided with a period of 24 h after the last oral administration. In VEH treated animals, Cmax of 70,065 ± 12,543 µg/ml and 72,8225 ± 12,679 µg/ml in maternal blood and 604,125 ± 2,359,853 µg/ml and 9887 ± 3738 µg/ml in fetal liver were achieved for AZT and 3TC, respectively. The concentration-time profile of 3TC in maternal blood (Fig. 5A) and fetal liver (Fig. 5B) did not show significant changes between groups. Therefore, no difference in the ratio of AUCs between fetal liver and maternal blood was found for 3TC (Fig. 5C). Regarding AZT, although a trend to higher concentrations in maternal blood was observed in the AZT chronically pretreated group, these differences were not significant. The AUC values in maternal blood for each group were 958,5 ± 268,3 µg.ml-1.min-1 for VEH, 1176 ± 381,8 µg.ml-1.min-1 for AZT and 1034 ± 465,7 µg.ml-1.min-1 for 3TC group (Fig. 5D). On the other hand, a marked decrease in the concentrations of AZT at times 15, 20, 25 and 30 min in the fetal liver was observed for the litter of dams exposed to AZT in comparison with those from mothers exposed to the VEH (Fig. 5E). Also, lower concentrations of AZT were found at 20, 25 and 30 min in fetal livers of AZT group compared to 3TC (Fig. 5E). These differences led to a significant decrease in the accumulation of AZT in the fetal tissue of the pups exposed to AZT compared to VEH and 3TC as observed for the decreased ratio between fetal liver and maternal blood AUC (Fig. 5F).

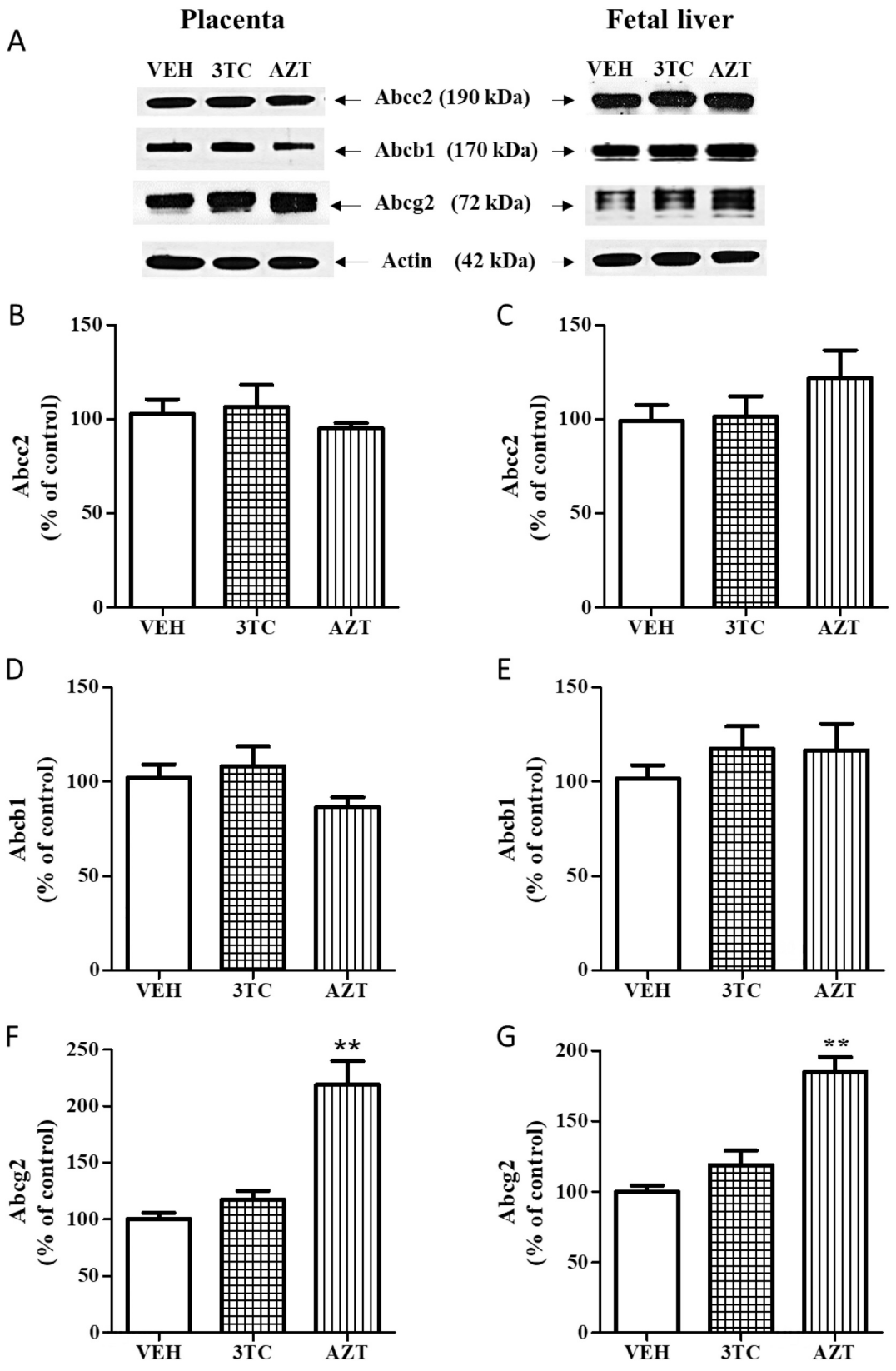

Fig. 4. Effect of chronic 3TC and AZT on the expression of MRP2, P-gp and BCRP in placenta and fetal liver.
(A) Representative immunoblots of MRP2, P-gp, BCRP and actin. Semiquantitative protein expression of MRP2 (B–C), P-gp (D-E) and BCRP (F-G) in placenta (left panel) and fetal liver (right panel) on GD 21 after daily treatment with VEH (white bars), 30 mg/kg 3TC (squared bars) or 60 mg/kg AZT (striped bars) by gavage from GD 11 to GD 20. Densitometric units were calculated as the ratio between optical density of the corresponding ABC transporter band and the actin band (loading control) obtained from the immunoblots. Bar graphs represent the expression of each transporter as a percentage value referred to the VEH group. Results are expressed as the media ± s.e.m. of six animals per group and were analyzed by one-way ANOVA plus Bonferroni post-hoc test. **: $p < 0.01$ between AZT and VEH.

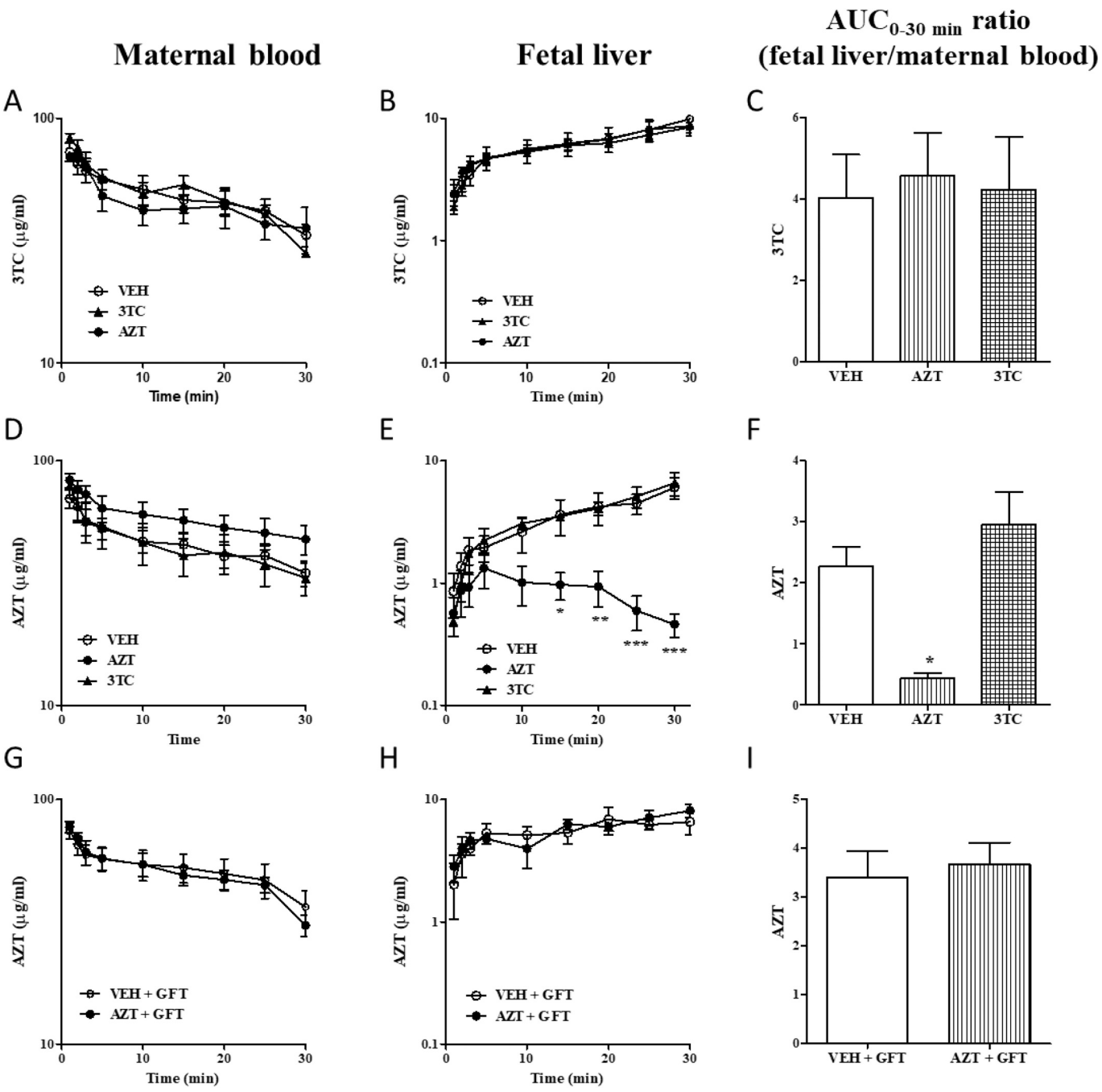

Fig. 5. Effect of chronic 3TC and AZT on the disposition of drugs in maternal blood and fetal liver.
After chronic treatment with AZT (black circles and striped columns), 3TC (black triangles and squared columns) or VEH (white circles and white columns), pregnant rats were administered on GD 21 with an IV dose of 30 mg/kg 3TC (A-C), 60 mg/kg AZT (D–F) or 60 mg/kg AZT plus an IP dose of 20 mg/kg GFT (VEH-GFT and AZT-GFT groups in G-I). Drugs were quantified in maternal blood (A, D, and G) and fetal liver (B, E, and H) at minutes 1, 2, 3, 5, 10, 15, 20, 25 and 30. Ratio between fetal liver AUC0-30min and maternal blood AUC0-30min of 3TC (C), AZT (F) and AZT + GFT (I). Data are presented as the mean ± s.e.m. of six rats per group. +: $p < 0.05$; ++: $p < 0.01$ and +++: $p < 0.001$ between VEH and AZT. **: $p < 0.01$ and ***: $p < 0.001$ between 3TC and AZT.

3.5. Effect of BCRP inhibition in the accumulation of AZT in fetal liver

In the presence of the selective BCRP inhibitor gefitinib 20 mg/kg, the concentration-time profiles of AZT in maternal blood (Fig. 5G) and in the fetal liver (Fig. 5H) showed no differences in pregnant rats chronically exposed to the drug with respect to VEH. Consequently, similar ratios of AUC between fetal liver and maternal blood for the AZT and VEH groups were obtained (Fig. 5I). This result suggests that the decrease in the accumulation of AZT in the liver of fetuses isolated from mothers chronically exposed to the drug (Fig. 5F) was due to the overexpression of BCRP observed for this group.

4. Discussion

We demonstrated that chronic exposure to AZT, but not to its congener 3TC, upregulates BCRP in placenta and fetal liver limiting the disposition of the drug in the tissue. This could explain the lower fetal toxicity of AZT compared to 3TC even when both drugs were equally genotoxic for the mother.

The lower body weight of pups on GD 21 after oral chronic treatment

with AZT and 3TC found in the present study (Fig. 1B) agrees with the fetal toxicity observed in preclinical studies in rats (Figueiró Filho et al., 2002; Greene et al., 1996). On the other hand, unlike the present results, increased resorptions and intrauterine deaths were reported after *in utero* exposure to 30 mg/kg AZT (Christmas et al., 1995). Regarding 3TC, the significantly lower weight of the placenta (Fig. 1C) as well as the higher number of fetal resorptions (Fig. 1D), suggest that chronic *in utero* exposure to 3TC (30 mg/Kg) exerts marked developmental damage than AZT (60 mg/Kg), in Sprague-Dawley rats. This agrees with the higher DNA damage found in the fetal liver after *in utero* exposure to 3TC compared to AZT (Fig. 3). However, present results showed similar levels of DNA damage in maternal blood cells from AZT- and 3TC-treated animals (Fig. 2) suggesting restricted disposition of AZT compared to 3TC in the fetal liver.

In the present model, the drugs were administered in the time window in which P-gp, BCRP and MRP2 are expressed in placenta and fetal liver (Han et al., 2018; St-Pierre et al., 2004). Since AZT is a substrate of BCRP (Filia et al., 2017; Li et al., 2018; Neumanova et al., 2016), induction of this MDR transporter could restrict placental passage as well as facilitate fetal elimination of AZT. In this sense, induction of BCRP has been attained by sustained exposure to substrate drugs such as efavirenz in small intestine (Peroni et al., 2011) and blood-brain barrier (Roma et al., 2015) of rats. Upregulation of BCRP was also observed in human colonic cells after continuous exposure to imatinib (Burger et al., 2005). In contrast, other BCRP substrates such as abacavir (Pan et al., 2007) do not modulate BCRP expression (Gilmore et al., 2021). Present results showed that BCRP in also upregulated by AZT in placenta and fetal liver since a significant increase in protein abundance was found after chronic *in utero* exposure (Fig. 4F and G).

The lack of P-gp modulation in the placenta (Fig. 4D) and fetal liver (Fig. 4E) after chronic AZT exposure was not consistent with the previously reported induction of P-gp at the intestinal level caused by AZT (Quevedo et al., 2011). Tissue-specific modulation of P-gp has also been reported in diabetic rats (Zhang et al., 2011). In this sense, MDR transporters expression is controlled by ligand-activated transcription factors and nuclear receptors (Pavek and Smutny, 2014; Tocchetti et al., 2016). It is possible that AZT activates different regulatory pathways leading to different transcriptional control of P-gp depending on the context. Such analysis is beyond the scope of this work.

Moreover, no NRTIs have been described to modulate MRP2 protein or mRNA expression (Jemnitz et al., 2010; Neumanova et al., 2016). The previous evidence agrees with the lack of MRP2 protein abundance modulation in placenta and fetal liver after chronic *in utero* exposure to either AZT or 3TC found in the present study. In turn, AZT seems to increase the expression of mrp4 and mrp5 mRNA in human macrophages and lymphocytes reducing its own pharmacological activity (Jorajuria et al., 2004). Furthermore, polymorphic variants of MRP4 could alter the pharmacokinetics of AZT in humans (Anderson et al., 2006). Like MRP2, MRP4 localizes in the apical membrane of the syncytiotrophoblast (Joshi et al., 2016), but does not modulate AZT transplacental transport in the rat placenta (Neumanova et al., 2016). This is consistent with the low levels of MRP4 reported in the placenta and undetectable in the fetal liver even after chronic exposure to substrates (Huang et al., 2021; Sharma et al., 2013).

Since AZT is commonly administered in combination with other antiretroviral drugs, it is important to consider possible drug-drug interactions on membrane transporters that may modulate the placental transfer of drugs and increase fetal exposure to xenobiotics. The absence of modifications in the abundance of BCRP after chronic administration of 3TC (Fig. 4F and G) agrees with previous evidence from mice (Gilmore et al., 2021) and it supports that 3TC is not able to modulate BCRP expression. Moreover, we did not find modifications in either P-gp or MRP2 protein in the placenta or fetal liver after 3TC chronic *in utero* treatment (Fig. 4 B–E). Altogether, present results suggest that treatment with 3TC in pregnant women would not generate drug-drug interactions at the level of BCRP, P-gp or MRP2 substrates that could modify its accumulation in fetal tissues.

Distribution of AZT and 3TC to the fetal compartment has been shown to be fast with T_{max} occurring within the first hour post-administration (Alnouti et al., 2004; Huang et al., 1996). Present results showed that repeated *in utero* preexposure to AZT reduced its own accumulation in fetal liver just after 15 min post-administration accounting for a mechanism that could involve already induced transporters.

AZT disposition between mother and fetus has shown to be poorly modulated by BCRP when studied in term placenta of untreated rats (Neumanova et al., 2016) in which the transporter protein is almost undetectable (Filia et al., 2017). Congruently, we observed no change in AZT levels in the fetal liver of VEH-treated mothers when BCRP was inhibited by gefitinib (Fig. 5G–H). On the other hand, the induction of BCRP caused by repeated *in utero* exposure to AZT was associated with markedly decreased levels of the drug in the fetal tissue (almost 70% less than in VEH) while maternal blood concentrations remained unchanged, as depicted in Fig. 5E–F). Furthermore, levels of AZT were restored to values achieved in VEH by BCRP inhibition (Fig. 5G–H). These results demonstrated that BCRP induction in the placenta restricted AZT passage to the fetus. Moreover, the induction of BCRP in the fetal liver would facilitate the elimination of AZT, contributing to the low concentration of the drug in the tissue. As demonstrated previously for fetal brain (Filia et al., 2017), the results indicate a crucial detoxifying mechanism mediated by BCRP over AZT (Fig. 5). This is consistent with the loss of transplacental AZT transport asymmetry that has been reported in the rat due to elacridar-dependent BCRP inhibition (Neumanova et al., 2016). However, as reported by the authors, an interaction between AZT and P-gp could also be possible since elacridar is a non-selective inhibitor of BCRP and P-gp. Regardless of whether P-gp transports AZT, the results presented here show that repeated exposure to AZT did not modulate P-gp protein levels in the placenta or fetal liver. In addition, two results observed here reinforce the concept of selectivity on the modulatory effect on MDR transporters. The first is the absence of alterations in the accumulation of 3TC in the fetal liver under any of the treatments (Fig. 5C). The second is the lack of changes in AZT levels in the fetal liver after chronic exposure to 3TC (Fig. 5F).

5. Conclusions

We have found a selective and substrate-related induction of BCRP protein in the placenta and fetal liver that limits the distribution of xenobiotics, exerting a potent fetoprotective action. Founded on the pharmacokinetic and toxicity analysis of 3TC and AZT in maternal blood and fetal liver as well as on the results of ABC transporters expression in term placenta and fetal liver, we hypothesize that the decreased accumulation of AZT in the fetal liver compared to maternal blood is due to the induced efflux mechanism of BCRP and that it accounts, at least in part, for the lower incidence of DNA damage in the fetal liver compared to 3TC. It is important to note that extrapolation of placental toxicity from rats to humans should be done cautiously in the evaluation of risk factors since there are differences between rats and humans in terms of the placental histological structure, the fetal-maternal interface, and the function of the yolk sac (Furukawa et al., 2019). Although the doses used in this work led to 10-fold higher concentrations than those reported in maternal and umbilical cord blood in humans, this work shows that repeated *in utero* exposure to a substrate can significantly alter the profile of MDR transporters both in placenta and fetal tissue.

CRediT authorship contribution statement

Juan Mauricio Minoia: Investigation, Methodology, Formal analysis. **María Fernanda Filia:** Investigation. **Martín Ignacio Roma:** Investigation. **Fernanda Teresa De Fino:** Methodology. **Guillermo Javier Copello:** Methodology, Resources, Supervision. **Roxana Noemí Peroni:** Conceptualization, Methodology, Investigation, Formal

analysis, Validation, Project administration, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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