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Induction of ABCG2/BCRP restricts the distribution of zidovudine to the fetal brain in rats

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ABSTRACT

Safety concerns for fetus development of zidovudine (AZT) administration as prophylaxis of vertical transmission of HIV persist. We evaluated the participation of the ATP-binding cassette efflux transporter ABCG2 in the penetration of AZT into the fetal brain and the relevance for drug safety. Oral daily doses of AZT (60 mg/kg body weight) or its vehicle were administered between post gestational days 11 (E11) and 20 (E20) to Sprague-Dawley pregnant rats. At E21, animals received an intravenous bolus of 60 mg AZT/kg body weight in the presence or absence of the ABCG2 inhibitor gefitinib (20 mg/kg body weight, ip) and AZT in maternal plasma and fetal brain were measured by HPLC-UV. ABCG2 protein expression in placenta and fetal brain, as well as mitochondrial function and ultrastructure in fetal brain were also analyzed. *In utero* chronic exposure to AZT markedly induced ABCG2 expression in placenta and fetal brain whereas did not significantly alter mitochondrial functionality in the fetal brain. The area-under-the-concentration-time-curve of AZT significantly decreased in fetal brains isolated from AZT-exposed fetuses compared to control group, but this effect was abolished by ABCG2 inhibition. Our results suggest that the absence of mitochondrial toxicity in the fetal brain after chronic *in utero* administration of AZT could be attributed to its low accumulation in the tissue caused, at least in part, by ABCG2 overexpression. We propose that any interference with ABCG2 activity due to genetic, pathological or iatrogenic factors would increase the amount of AZT reaching the fetal brain, which could increase the risk of toxicity of this drug on the tissue.

1. Introduction

The nucleoside analog reverse transcriptase inhibitor (NRTI) zidovudine (AZT) continues to play a key role as a drug for the prevention of maternal-fetal transmission of HIV. Prophylactic administration of AZT in combination with a second NRTI and a viral protease inhibitor reduces the mother-to-child HIV transmission to 1–2% in developed countries (HIV/AIDS, 2013). Unfortunately, exposure to AZT *in utero* encompasses the risk of long term mitochondrial-related toxicity that might be linked to hematological and cardiac tissue alteration in children (Barret et al., 2003; European Collaborative Study, 2004; Pacheco et al., 2006; Sibiude et al., 2015).

Transplacental kinetics of drugs are frequently mediated by ATP-binding cassette (ABC) efflux transporters embedded in the maternal face of the placenta (Staud et al., 2012). P-glycoprotein (ABCB1), multidrug resistance-associated protein 2 and 5 (ABCC2, ABCC5), and breast cancer resistance protein (ABCG2) are well-described placental transporters affecting the passage of their substrates into the fetal circulation (Ceckova-Novotna et al., 2006; Hahnova-Cygalova et al., 2011; Meyer zu Schwabedissen et al., 2005a,b). In fact, a recent work reported in an *in situ* model of dually perfused rat placenta showed that ABCG2, and at lesser extent ABCB1, could be relevant for the passage of AZT from fetal to maternal side (Neumanova et al., 2016). Even though fetal-to-maternal plasma ratio of AZT is high (0.63–1.0) in Rhesus macaques and rats (Brown et al., 2003; Patterson et al., 1997), the transplacental passage of AZT markedly decreases whether AZT is co-administered with a BCRP substrate as acyclovir (Brown et al., 2003). Furthermore, it has been observed in Rhesus macaques that the accumulation of

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AZT is lower in fetal tissues expressing ABC transporters from early stages of morphogenesis, such as the brain (Saunders et al., 2013, 2012), compared to other fetal organs (Patterson et al., 1997). In fact, the central nervous system displays a relative resistance to nucleoside analogs such as AZT, in both treatment and toxicity when compared to other organ systems even considering that the developing brain is particularly sensitive to mitochondrial damage (McCann et al., 2012). In support of a possible participation of ABCG2 in this phenomenon, experimental evidence has pointed out that this transporter is expressed at higher levels and is functionally more active in the developing than in the adult brain (Ek et al., 2012). Additionally, ABCG2 expression has also been found in the fetal brain endothelium as early as post-gestational day 12 (E12) in rats (Kalabis et al., 2007).

Moreover, it was shown that the efflux of antiretroviral drugs by ABCG2 seems to become of relevance whether a high density of molecules are present in the cell membrane, as occurred either in transfected or transformed cell lines (Pan et al., 2007; Wang et al., 2004, 2003; Weiss et al., 2007) or under *in vivo* repeated exposure to a substrate as efavirenz (Peroni et al., 2011; Roma et al., 2015).

We propose that the low incidence of neurological damage associated with mitochondrial dysfunction in children exposed *in utero* to AZT (Ek et al., 2010) could be related, at least in part, to the restriction of AZT passage across both the placenta and the developing blood-brain barrier by induction of ABCG2. The aim of this study was to analyze whether ABCG2 restricts the accumulation of AZT in fetal brain after chronic *in utero* exposure in pregnant rats protecting against mitochondrial toxicity. We developed a model of chronic AZT administration leading to overexpression of ABCG2 in placenta and fetal brain that induced the efflux of AZT out of the fetal brain.

2. Materials and methods

2.1. Materials

AZT was provided by Laboratorios Richmond (Buenos Aires, Argentina). Gefitinib was obtained from Astra-Zeneca (Chesire, UK). Protease inhibitor cocktail (Complete mini, Roche Applied Science, Mannheim, Germany) and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO) and every other chemical not listed here were of the highest purity available and were used as received.

2.2. Methods

2.2.1. Animals and treatments. 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 NIH guidelines for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee Guidebook, 2nd ed., 2002). The 3Rs principle of Reduction, Refinement, and Replacement was considered in the design of the animal experiment and applied when possible. Animal treatment was carried out in accordance with the guidelines of the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT).

Pregnancy was confirmed by the presence of sperm in the vaginal smear the morning after matting and was considered post gestational day 0 (E0). Control pregnant rats at E11, E13, E15, E18 or E21 ($n = 3$ per group) were used in this study. The day of the assay, rats were anaesthetized with 1.2 g/kg urethane (i.p.) and subsequent doses of anesthesia were administered as needed. A laparotomy was performed and a small incision was made in the uterine wall to allow for sampling of the pups.

2.2.2. Treatments. Twenty-four pregnant rats were randomly divided into two groups, given either 60 mg/kg body weight zidovudine (AZT) or vehicle (0.05% ethanol in saline solution) from E11 to E20 orally by esophageal catheter. In E21, the animals from each group were again divided into two subgroups for the administration of the selective inhibitor of ABCG2 gefitinib (GFT, 20 mg/kg) or vehicle (dimethyl sulfoxide:propylene glycol:saline solution, in a 2:2:1 ratio), respectively, 30 min before the onset of pharmacokinetic assays. In this way, 4 groups were constituted: VEH/VEH, AZT/VEH, VEH/GFT, and AZT/GFT ($n = 6$ per group).

2.2.3. ABCG2 protein expression levels and localization. 2.2.3.1. Western Blot in placenta and fetal brain. Placenta (at E11, E15, E18, and E21) and fetal brain (E18 and E21) were collected from pregnant rats within 30 min of surgery and immediately stored at −70 °C. To prepare samples for western blotting analysis, total protein was obtained from tissues defrosted on ice and measured with bovine serum albumin (BSA, A7906 Sigma-Aldrich, USA) as standard (Lowry et al., 1951) and analyzed as previously described (Peroni et al., 2011). Briefly, placenta from E11 and E15 (pool of three, 30 μg of protein per line), E18 and E21 (30 μg of protein per line), and fetal brain from E18 and E21 (pool of three; 50 μg of protein per line) were loaded onto 8% SDS-polyacrylamide gels, subjected to electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were cut at molecular mass 52 kDa, based on the Kaleidoscope molecular weight standards (Bio-Rad, Hercules, CA, USA). The upper and lower portions were then incubated overnight at 4 °C with anti-rat ABCG2 antibody (M-70, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-ABCG2 antibody (BXP-21 clone 1/400, Santa Cruz Biotechnology, CA, USA, SC-58222) and anti-actin antibody (A2066, 1:1000; Sigma-Aldrich, USA), respectively. The immune complex was detected by incubation with the horseradish peroxidase-linked anti-rabbit antibody (sc-2004, 1/2000; from Santa Cruz Biotechnologies) or horseradish peroxidase-linked goat anti-mouse IgG (sc-2031, 1/2000; from Santa Cruz Biotechnologies) during 90 min. 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, USA).

2.2.3.2. Immunofluorescence in fetal brain tissue. Fetus obtained by cesarean surgery on E21 were decapitated and the brain were carefully removed from skull, washed in cold 0.1 M K_2HPO_4/KH_2PO4, pH 7.4 (PB), placed in a pre-chilled glass platen, and cleaned to remove meninges and choroid plexus. One hemisphere of each brain was postfixed in 4% paraformaldehyde/PB at 4 °C for 24 h, cryoprotected overnight in 30% sucrose/PB. Four serial hemi-coronal slices (20-μm thickness) at 3 mm (striatal level) from the frontal tip were performed with a rotary microtome cryostat (Thermo Scientific), mounted in gelatinized glass slides and stored at −20 °C until use. Two section of each series was stained with hematoxylin and eosin (H&E) according to standard protocols and observed at under low as well as 200 × magnification. For immunofluorescence, another two sections of each series were blocked with 5% fetal calf serum (FCS) in phosphate saline buffer (PBS) for 1 h prior incubation with mouse anti-ABCG2 antibody (BXP-21 clone 1/100, Santa Cruz Biotechnology, SC-58222) overnight at 4 °C. The BXP-21 antibody clone has previously been shown to specifically react with a 72 kDa protein in rat fetal brain (Ek et al., 2010). Slides were incubated with goat anti-mouse IgG (Fc specific)–biotin antibody (1/250 Sigma Aldrich, B7401) overnight at 4 °C, followed by 1 h incubation with DTAF-streptavidin (1/500 Jackson ImmunoResearch Laboratories Inc., TechFAQ #6) at room temperature. All antibodies were diluted in 3% FCS/0.3% Tween20 PBS and three washes with 0.3%-Tween20 PBS were performed between each step. Tissues were mounted with Mowiol and coverslipped. Control sections omitting primary antibody were prepared in each slide and were always blank. Photographs were taken with an optical microscope (Nikon Eclipse) equipped with fluorescent optics and a camera connected to a monitor and computer. Digital images were colour balanced with Adobe Photoshop 8.0 (Adobe