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The transfer of 6-mercaptopurine in the dually perfused human placenta

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ABSTRACT

The immunosuppressant azathioprine is increasingly being used in pregnancy. The human placenta is considered a relative barrier to the major metabolite, 6-mercaptopurine (6-MP), and likely explains the lack of proven teratogenicity in humans. The aim of this study was to determine how the human placenta restricts 6-MP transfer using the human placental perfusion model. After addition of 50 ng/ml ($n = 4$) and 500 ng/ml ($n = 3$) 6-MP into the maternal circulation, there was a biphasic decline in its concentration and a delay in fetal circulation appearance. Under equilibrative conditions, the fetal-to-maternal concentration ratio was >1.0 as a result of ion trapping. Binding to placental tissue and maternal pharmacokinetic parameters are the main factors that restrict placental transfer of 6-MP. Active transport is unlikely to play a significant role and drug interactions involving, or polymorphisms in, placental drug efflux transporters are not likely to put the fetus at risk of higher 6-MP exposure.

1. Introduction

The immunosuppressant azathioprine (AZA) is increasingly being used in pregnancy for the treatment of autoimmune diseases or for organ transplant patients [1]. Peak incidence of autoimmune diseases, including inflammatory bowel disease (IBD) and systemic lupus erythematosus, occurs in women during the childbearing years [2] and successful pharmacologic therapy in these women has improved the feasibility of pregnancy [3]. Treatment with AZA is required into pregnancy to prevent relapse of the disease or organ rejection; thus, treatment can also minimize adverse fetal effects associated with the underlying maternal disease [3].

Several studies have investigated the safety of AZA in pregnancy. A review of 27 case series and three large retrospective and prospective studies did not find an increased risk for major malformations associated with the use of AZA in pregnancy [1,3–5]. However, one recent study reported a trend for increased malformations and an increased risk specifically for ventricular/atrial septal defects after prenatal AZA exposure [6]. An increased risk for prematurity and lower birth weight has also been reported, but this may be related to the underlying maternal disease [1,6]. Although evidence supports the relative safety of AZA in pregnancy compared to the untreated disease [5], safety concerns still arise from its mechanism of action and its toxicity during pregnancy in animal studies [3]. Over eighty percent of women with IBD have unwarranted concerns about medications, including AZA, in pregnancy and fears surrounding medications are greater than the effect of IBD exacerbation [7]. Moreover, some physicians still choose not to administer AZA during pregnancy or to discontinue treatment in the third trimester [8].

After oral administration, AZA is extensively (>80%) cleaved into 6-mercaptopurine (6-MP) in the blood [3]. 6-MP is a purine analogue, and after further conversion into active metabolites, it can inhibit *de novo* purine ribonucleotide synthesis leading to inhibition of cell proliferation. Furthermore, 6-MP can be cytotoxic after incorporation of its metabolites into cellular DNA. Since 6-MP targets rapidly dividing cells, the developing fetus would theoretically be expected to be sensitive to its cytotoxicity. However, even high dose 6-MP was not found to be useful as a single-agent medical abortifacient in early pregnancy, leading the authors to suggest that this drug does not alter cell division in trophoblastic tissues [9].

The observed safety of AZA in pregnancy likely results, in part, from the placenta limiting fetal exposure to the main metabolite, 6-MP. Indeed, only 1–2% of maternal 6-MP concentrations are found in cord blood and the human placenta is considered to be a relative barrier to 6-MP and its metabolites [10,11]. However, the mechanism of how the placenta restricts 6-MP transfer to the fetus is unknown. 6-MP is a substrate for several drug transport proteins, including breast cancer resistance protein (BCRP) [12]. BCRP is highly expressed in the placenta and is localized to the brush

Abbreviations: 6-MP, 6-mercaptopurine; AZA, azathioprine; BCRP, breast cancer resistance protein; CNT, concentrative nucleoside transporter; TPMT, thiopurine methyltransferase; MRP5, multidrug resistance protein 5; F:M, fetal to maternal; hCG, human chorionic gonadotropin; ENT, equilibrative nucleoside transporter; AUC, area under the curve.

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border (maternal-facing) membrane of the syncytiotrophoblast [13]. The importance of BCRP and other placental transporters in limiting fetal exposure to various drugs has been demonstrated in both human and animal models [14,15]. The objective of this study was to determine the mechanisms by which the placenta restricts 6-MP transfer. Understanding these mechanisms will allow for prediction of fetuses that may be at risk of higher exposure to 6-MP as a result of drug interactions with, or polymorphisms in, placental drug efflux proteins.

2. Methods

2.1. Materials

6-Methylmercaptopurine (6-MMP) and 6-thioxanthosine were obtained from MP Biomedicals (Solon, Ohio). 6-Thiouric acid was obtained from US Biological (Swampscott, MA) and toluene from EMD Chemicals (Gibbstown, NJ). HPLC-grade methanol was ordered from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Sigma (St. Louis, MO). The water used for all experimental procedures was obtained from a Milli-Q Advantage A10 Ultrapure Water Purification System (Millipore, Billerica, MA).

2.2. Placental perfusion

The dual perfusion of a placental lobule was previously described by Miller et al. [16] and adapted in our laboratory [15,17]. Term placentas were obtained immediately after elective caesarean sections from healthy mothers with uncomplicated pregnancies from Mount Sinai or St. Michael's Hospitals in Toronto, Ontario. Research ethics board approval at each hospital was obtained and maternal consent was obtained prior to the surgery. The placentas were transferred to the lab in heparinised ice-cold phosphate-buffered saline where a vein/artery pair supplying a clearly identifiable cotyledon was chosen for cannulation [17]. Maternal and fetal circulations were established within 30 min of delivery.

The perfusate consisted of M199 tissue culture medium (Sigma, St. Louis, MO) containing heparin (2000 U/l), glucose (1.0 g/l), kanamycin (100 mg/l), and 40,000 molecular-weight dextran (maternal 7.5 g/l; fetal 30 mg/l). Antipyrine (1 mM) was added to the maternal circulation as a marker of passive diffusion. Flow rates were maintained at 2–3 and 13–14 ml/min in the fetal and maternal circuits, respectively. The maternal perfusate was equilibrated with 95% O_2, 5% CO_2 and the fetal with 95% N_2, 5% CO_2. Maternal and fetal circuits were maintained at pH 7.4 and 7.35, respectively, by the addition of small volumes of sodium bicarbonate and hydrochloric acid. This pH mimics the slightly more acidic fetal circulation observed *in vivo* [18]. The temperature of the circuits and the perfusion chamber was kept at 37 °C.

Each perfusion consisted of a 1 h closed pre-experimental control period, followed by a 3 h closed experimental period, and a final 1 h closed post-control period. The post-control period was omitted from the perfusions where 6-MP was added to only the maternal circulation since this allowed measurement of drug binding to placental tissue. The perfusates in both circulations were replaced with fresh media prior to each of the 3 periods. During the pre- and post-control periods, samples were taken every 15 min to analyze glucose and oxygen consumption, and human chorionic gonadotropin (hCG) secretion as measures of tissue viability. These measures were taken every 30 min during the experimental period. Tissue viability measures were calculated as previously described [15]. Fetal reservoir volume and fetal perfusion pressure were monitored as an indicator of tissue integrity. pH, pO_2, and pCO_2 were monitored using a blood gas analyzer (Radiometer ABL 725, Copenhagen, Denmark). During the experimental period, 50 ng/ml or 500 ng/ml 6-MP was added to the maternal circulation only or simultaneously to both the fetal and maternal circulations. 50 ng/ml is the maximum concentration obtained after oral administration of AZA in adult renal transplant recipients [19,20]. Samples were taken for measurement of 6-MP every 10 min for the first half-hour and every half-hour following. The perfusion was terminated at any time if there was a >3 ml/h loss in fetal reservoir volume.

The expected fetal to maternal (F:M) ratio at steady state resulting from ion trapping – given the change in pH between the fetal and maternal circulations – was calculated using a modified form of the Henderson–Hasselbalch equation that incorporates the two pK_a of 6-MP:

$$\frac{F}{M} = \frac{1 + 10^{(pK_{a2} - pH_F)} + 10^{(pK_{a1} + pK_{a2} - 2pH_F)}}{1 + 10^{(pK_{a2} - pH_M)} + 10^{(pK_{a1} + pK_{a2} - 2pH_M)}}$$

This equation was derived by assuming conservation of mass and rapid equilibration between the neutral and protonated forms of 6-MP (6-MP, 6-MP^{+}, and 6-MP^{++}). Only the un-ionized form is assumed to cross the membrane. Data are expressed as mean ± SEM unless otherwise indicated. F:M ratios were compared using a two-tailed Student's *t*-test.

2.3. Sample analysis

Perfusate samples were stored at −20 °C until analysis. 6-MP and its metabolites (6-MMP, 6-thioxanthosine, 6-thiouric acid, 6-thioguanine) were extracted using a method adapted from Lennard and Singleton [21]. 500 μl of perfusate was added to 500 μl of 6 mM DL-dithiothreitol and 500 μl of 1.5 M H_2SO_4 in duplicate. Since 6-TG was not detected in any of the perfusions, it was further used as an internal standard (100 ng/ml). One of each sample was heated at 100 °C for 1 h for acid hydrolysis of the thionucleotide metabolites back into the parent thiopurine and for extraction of 6-methylmercaptopurine [21]. Samples not being heated were kept on ice. After cooling on ice, 500 μl of 3.8 M NaOH was added followed by 6 ml of isoamyl alcohol (170 mM) and phenylmercury acetate (1.3 mM) in toluene and placed in an automatic rotator for 15 min. The mixture was centrifuged at 2500 × *g* for 15 min at room temperature and the toluene layer was added to a new tube. 200 μl of 0.1 M HCl was added and vortexed for 2 × 1 min. 70 μl of the aqueous phase was injected onto a Atlantis T3 Column (3 μm, 4.6 mm × 150 mm) protected with a guard cartidge (3 μm, 4.6 mm × 20 mm)(Waters, Milford, MA). The mobile phase consisted of acetonitrile, methanol, and KH_2PO_4 (0.02 M; pH 2.25) (3:1:96, v/v/v) [22] and was filtered through a 0.45 μm filter. The flow rate was 1.0 ml/min.

The HPLC system (Shimadzu, Columbia, MD) consisted of a solvent delivery pump (LC-10AT), auto injector (SIL-10A), degasser (DGU-14A), controller (CBM-20), and dual wavelength UV detector (SPD-20A). 6-MP was monitored at 313 nm; 6-MMP at 303 nm; and 6-thioguanine, 6-thioxanthosine, and 6-thiouric acid at 342 nm. The chromatograms were acquired and analyzed using Shimadzu Class-VP Software, Version 7.4 SP2. The limit of quantification was 2 ng/ml for 6-MP and 6-thioguanine and 5 ng/ml for 6-MMP, 6-thiouric acid, and 6-thioxanthosine.

3. Results

A total of 16 cotyledons from different placentae were perfused with 6-MP and the physical parameters for the perfusions are given in Table 1. The mass of the perfused cotyledons ranged from 10.22 to 32.0 g. Throughout the experiments, measures of placental viability, integrity, and function remained within normal ranges and were not significantly different between the control and experimental phases (Table 1). There was no significant difference between experimental and control periods in the fetal arterial pressures. The rate of antipyrine appearance in the fetal circulation during the experimental period was equal to the rate of disappearance from the maternal circulation with values of 0.030 ± 0.002 and 0.032 ± 0.003 μmol/g/min, respectively.

After addition of 50 ng/ml 6-MP into the maternal circulation only, there was a biphasic decline in its concentration from this compartment (Fig. 1A); an initial rapid decline during the first 30 min, followed by a slower decline in the final 150 min. There was a delay in the transfer of 6-MP in the fetal circulation as measurable concentrations of 6-MP appeared only after 30 min. This delay suggests uptake and retention of 6-MP by the placental tissue. After 3 h, the mean fetal concentration of 6-MP was 8.8 ± 1.71 ng/ml and the mean fetal to maternal (F:M) concentration ratio was 0.44 ± 0.06. After addition of the higher 6-MP concentration (500 ng/ml), there was a similar biphasic decline from the maternal circulation (Fig. 1B). 6-MP was detected in all three perfusions at 20 min, which is more rapid compared to the perfusions at the lower concentration. After 3 h, the mean fetal concentration of 6-MP was 91.48 ± 7.50 ng/ml and the mean F:M ratio was 0.57 ± 0.04.

When equal concentrations (50 ng/ml or 500 ng/ml) were added to both fetal and maternal circulations at the start of the experimental period, the fetal to maternal (F:M) ratio increased over time (Fig. 2). The F:M ratios of 6-MP at the end of the 180 min experimental period were 1.21 ± 0.08 and 1.19 ± 0.04 for 50 ng/ml and 500 ng/ml, respectively. The F:M ratios increased significantly from time 0 to 180 min at both the 50 and 500 ng/ml concentrations ($p = 0.007$, $p = 0.02$, respectively). 6-MP is a basic drug with two pK_a's ($pK_{a1} = 7.77$, $pK_{a2} = 11.17$) [23], and basic drugs are more ionized in the fetal circulation since it is slightly more acidic than the maternal (7.35 vs. 7.40, respectively)[18]. The expected F:M ratio at equilibrium based on ion trapping alone was calculated to be 1.22.

Table 1
Placental viability parameters and metabolic capacity throughout the perfusions (n = 16 cotyledons from independent placentae) (mean ± SEM).

Viability parameter	Pre-control	Experiment	Post-control
Fetal arterial inflow pressure (mmHg)	40.36 ± 1.11	40.71 ± 1.08	42.91 ± 1.81
hCG production (mIU/g/min)	57.52 ± 10.12	39.62 ± 6.83	40.86 ± 13.07
Oxygen (μmol O_2/g/min)			
Transfer	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Delivery	0.48 ± 0.05	0.48 ± 0.04	0.45 ± 0.07
Consumption	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.04
Glucose consumption (μmol/g/min)	0.42 ± 0.05	0.36 ± 0.04	0.38 ± 0.06

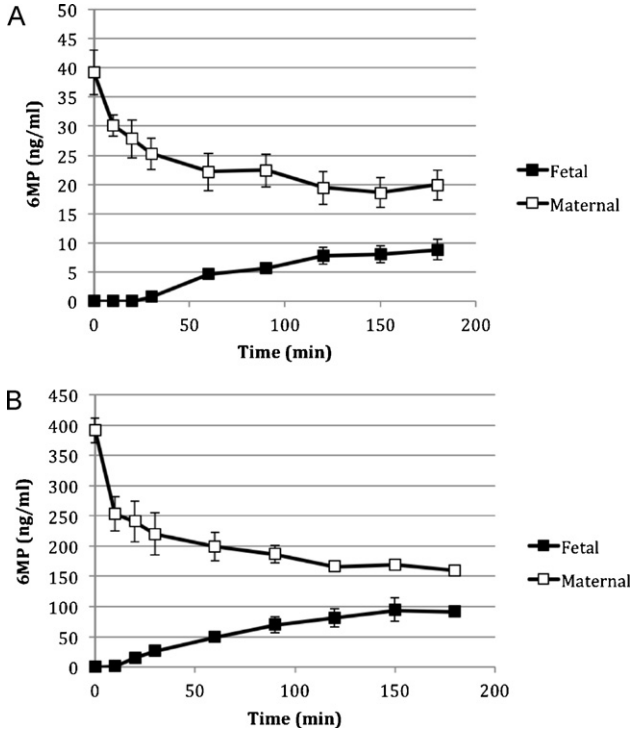

Fig. 1. Maternal-to-fetal transfer of 6-mercaptopurine after dual perfusion of a single placental lobule. 6-mercaptopurine was added to the maternal circulation at (A) 50 ng/ml (n = 4) or (B) 500 ng/ml (n = 3) and transfer was determined for a period of 180 min. Data are shown as mean values ± SEM at each time point.

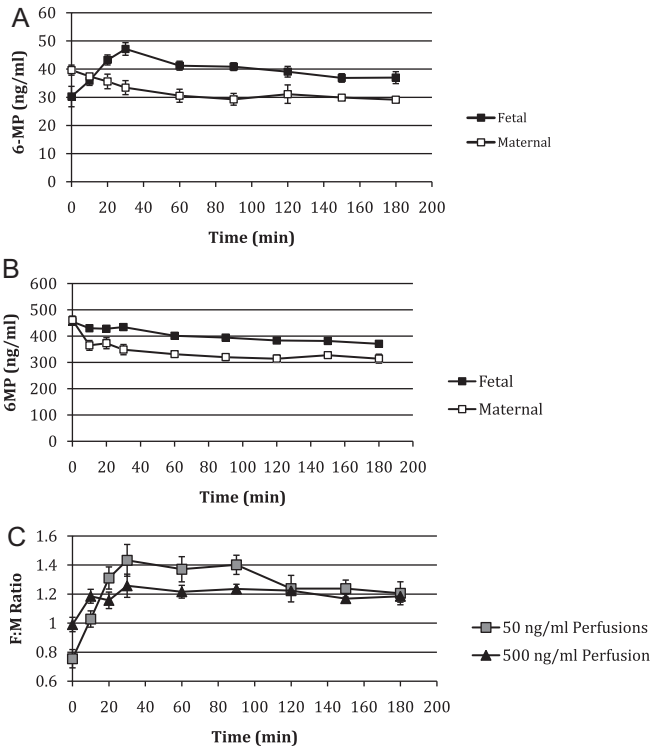

Fig. 2. Concentrations of 6-MP in the fetal and maternal circulations after perfusion of a single placental lobule under equilibrative concentrations of (A) 50 ng/ml (n = 5) and (B) 500 ng/ml (n = 4). (C) The fetal to maternal (F:M) concentration ratios for the perfusion under equilibrative conditions. Data are shown as mean values ± SEM at each time point.

After perfusion with 50 ng/ml 6-MP, the metabolites including 6-MMP, 6-thioguanine, 6-thioxanthosine, 6-thioinosine, and 6-thiouric acid (and the related thionucleotide metabolites) were not detected in the fetal nor the maternal circulations. At the ten-fold higher 6-MP concentration of 500 ng/ml, only 6-MMP was detected with a mean concentration of 11.75 ± 2.02 and 8.65 ± 5.97 ng/ml in the fetal and maternal circulations, respectively, at 180 min. Two of the seven perfusions at 500 ng/ml had 6-MMP below the limit of detection. The tissue-to-maternal perfusate ratio was 0.49 ± 0.08 at the end of 180 min of perfusion (n = 7). The tissue-to-maternal perfusate ratio was not significantly different between the 50 ng/ml and the 500 ng/ml perfusions.

4. Discussion

The human placenta is considered a relative barrier to 6-MP and its metabolites [10,11,24] and our study demonstrates that placental binding and not active transport limits transfer. Limited fetal exposure to 6-MP may in part explain why studies have not shown conclusive evidence that 6-MP is teratogenic [1,5,6]. Understanding the mechanism of this limited transfer will allow for prediction of fetuses that may be at risk for higher exposure or for prediction of potential drug interactions.

After introduction of a clinically relevant concentration into the maternal circulation, there was a rapid decline in maternal levels, however, there was a delay in appearance of 6-MP in the fetal circulation. This delay can be attributed to the uptake and binding by the placental tissue. After addition of a 10-fold higher concentration, there was also a delay in the appearance of 6-MP in the fetal circulation. The appearance of 6-MP in the fetal circulation was more rapid for 500 ng/ml, suggesting saturation of tissue binding sites. Although binding to the tissue delayed placental transfer, the tissue to maternal perfusate ratio at the end of the perfusion was low. This low ratio is similar to the placenta to maternal plasma ratio observed *in vivo* after therapeutic abortions in the ninth to fifteenth week of gestation [10]. The human placenta does have a capacity to sequester 6-MP and thus decrease transfer to the fetus [10].

To determine if there is active efflux of 6-MP into the maternal circulation, we introduced the drug into both fetal and maternal circulations as this methodology has been used previously to identify placental efflux [15,25]. After 180 min of perfusion, the F:M ratio was above 1.0. Under equilibrative conditions at 50 ng/ml and 500 ng/ml, the term placenta was not able to concentrate 6-MP into the maternal circulation. This suggests that efflux of 6-MP into the

maternal circulation is not responsible for or plays a very minor role in the limited placental transfer of 6-MP. The F:M ratio at the end of the 3 h experimental period at both 50 ng/ml and 500 ng/ml was not significantly different from the calculated F:M ratio based on ion trapping. This supports ion trapping as the mechanism for the observed F:M ratio at steady state.

During pregnancy, nucleoside transporters transfer nucleotides from the mother to the fetus. Equilibrative nucleoside transporters (ENTs) 1, 2 and concentrative nucleoside transporter 2 (CNT2) have been localized to the brush-border membrane of the syncytiotrophoblast [26]. 6-MP is a substrate for the ENTs [27], however, these are energy-independent transporters and are only capable of equilibrative (not concentrative) transport [28] and are not likely to play a role in the transfer under equilibrative conditions. Only mRNA expression (not protein) of CNT2 was demonstrated in the human placenta [26], thus it is unlikely that CNTs would play a major role in the F:M distribution of 6-MP. 6-MP is also a substrate for multidrug resistance protein 5 (MRP5) [29,30]. MRP5 is localized to the basolateral membrane of the syncytiotrophoblast and also on fetal endothelial cells [31] and could thus transport 6-MP to the fetal circulation. However, the expression of MRP5 decreases in term compared to pre-term placentas [31], thus it is unlikely that MRP5 plays an important role in our perfusion model.

Our results show that active efflux of 6-MP is not the mechanism responsible for limited placental transfer. After introducing 6-MP in the maternal circulation only, the fetal 6-MP concentration was approximately half of the maternal. This is higher than that observed *in vivo* where 6-MP transfer to the fetus is limited [10,11]. The placental perfusion model is a useful model for determining transport mechanisms, but does not always correlate well with *in vivo* data if non-placental pharmacokinetic variables are important factors in fetal exposure [32,33]. Two non-placental pharmacokinetic factors for 6-MP, namely distribution and clearance, are likely to be important in the limited transfer *in vivo*. First, distribution of 6-MP includes binding to placental tissue as demonstrated in our experimental model, but also 6-MP is rapidly converted into intracellular metabolites. Because of the intracellular location, the metabolites are not free to cross the placenta *in vivo* [24]. Second, maternal metabolism of 6-MP and its corresponding short $t_{1/2}$ of 1 h [34,35] will limit fetal exposure by decreasing the maternal area under the curve (AUC). Saarikoski and Seppala [10] suggested that maternal metabolism of 6-MP was important in limiting exposure as they observed that 6-thiouric acid, an inactive metabolite, was the main metabolite transferred to the fetus. Metabolism by the placenta does not likely play an important role in limited fetal 6-MP exposure as in our perfusions 6-MMP was the only metabolite detected and only after perfusion with 500 ng/ml of 6-MP. *In vivo*, 6-MMP was not detected in RBC from cord blood in 3 infants; however, the limit of detection in this study was not provided [11]. Furthermore, 6-MMP is not stable in blood samples and may have degraded before the samples were analyzed [36].

A limitation of our placental perfusion model is that it uses term placentas. Generalizations to earlier gestational ages are difficult, including extrapolations to the embryonic period where the placental structure varies. Our results suggest that the distribution of 6-MP and its short maternal $t_{1/2}$ are the key factors limiting transfer. These factors are unlikely to change in the different stages of pregnancy and monitoring maternal plasma for 6-MP and its metabolites would prevent elevated drug concentrations during pregnancy [11]. Another limitation to our study is that there is no information available regarding plasma concentrations of 6-MP during pregnancy. The clinically relevant concentration used in our study was determined in adults taking AZA at similar doses to those administered during pregnancy. Although the K_m for BCRP and 6-MP has not been published, a linear rate of transfer was observed at higher concentrations than those utilized in our study [12].

5. Conclusions

Pharmacological therapy with AZA during pregnancy is needed to prevent relapse of autoimmune diseases or rejection of renal transplants. Studies looking at the safety of AZA and its major metabolite, 6-MP, have demonstrated that the benefit of treating the disease outweighs the risk of maintaining treatment during the pregnancy [37]. Additional large epidemiological studies and meta-analyses are needed to provide conclusive evidence regarding the teratogenicity of 6-MP [5], especially to account for the pharmacogenetic variability in thiopurine S-methyltransferase (TPMT) [38]. The placenta acts as a relative barrier to 6-MP and its metabolites [10,11,24], and our results suggest that maternal pharmacokinetic factors and placental binding limit transfer. These findings support monitoring maternal plasma for elevated levels of 6-MP and metabolites are useful in preventing potential fetal toxicity [11]. Our results also suggest that active transport of 6-MP into the maternal circulation is not an important mechanism. Therefore, polymorphisms or drug interactions involving active drug transport proteins in the placenta are unlikely to leave a fetus more vulnerable to 6-MP exposure.

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