Title Page

Title:

Identification and Quantification of Paclitaxel and its Metabolites in Human Meconium from Newborns with Gestational Chemotherapeutic Exposure

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Conflict of Interest Statement
The authors have no conflicts of interest to declare relevant to this work.

Running Title: Quantification of Paclitaxel in Meconium

Abstract

Objective.

Cancer diagnosis during pregnancy occurs in 1 out of 1000 pregnancies with common malignancies including breast and hematological cancers. The pharmacology of chemotherapeutics, especially the transplacental disposition of currently utilized agents, is poorly described. This impedes the selection of agents for optimal therapy. We directly assessed fetal exposure by screening meconium from 23 newborns whose mothers had undergone treatment for cancer during pregnancy.

Study Design.

Meconium was collected from newborns whose mothers were diagnosed with cancer during pregnancy and underwent chemotherapy in the second or third trimester as part of the Cancer and Pregnancy Registry. Screening of 23 meconium samples for chemotherapeutics and known metabolites of chemotherapeutics was conducted by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Putative identification of paclitaxel and/or its metabolites was made in 8 screened samples. Identification was confirmed by quantification of paclitaxel, 3'-p-hydroxypaclitaxel, and 6α-hydroxypaclitaxel by stable isotope dilution-LC-HRMS.

Results.

Paclitaxel was detected at 399.9 pg/mg in meconium samples from newborn born to mothers that underwent chemotherapy during pregnancy. 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel were detected at 105.2 and 113.4 pg/mg meconium, respectively.

Conclusion.

Intact paclitaxel, and at least two of its major metabolites were detected in meconium, providing unambiguous confirmation of human fetal exposure. Variability in meconium levels between individuals may indicate a potential for reducing fetal exposure based on timing, dosing, and individual characteristics. This preliminary study may provide an efficient approach for examining the effects of cancer diagnosis during pregnancy on other outcomes by providing a measure of direct fetal exposure.

Introduction

Cancer diagnosis occurs in 1 in 1000 pregnancies. The incidence of cancer during pregnancy is expected to increase with the increasing age of childbearing and a higher incidence of several age-dependent malignancies. The most common malignancies complicating pregnancy are breast cancer, Hodgkin's and Non-Hodgkin's lymphoma, and melanoma. Termination of pregnancy has not been shown to improve patients' survival and consideration to treatment during pregnancy can be given. Patients along with their oncologists and obstetricians must weigh the maternal benefits of cancer therapy against the fetal/neonatal risks of treatment during pregnancy. The majority of fetal organogenesis occurs 3-10 weeks post conception. Studies of effects of chemotherapy exposure during the second and third trimester have been reassuring, where the majority detail the physical appearance at birth and general health during the first year of life [1-5]. Aviles, *et al.,* followed 84 children exposed to chemotherapy for maternal hematologic cancers. At a median age of 18.7 years old (range: 6–29 years) children underwent neurological and physical analysis [6]. There were no abnormalities among the chemotherapy exposed children compared to their unexposed siblings and unrelated controls. Interviews with teachers and developmental testing indicated that there were no learning disorders among the children. More recently, developmental testing has been performed on children exposed to chemotherapy *in utero* compared to the general population [7], to children from uncomplicated pregnancies matched for maternal and gestational age at birth and age at developmental testing, and to children born to mothers diagnosed with cancer during pregnancy but not treated with either chemotherapy or radiotherapy [8]. In all existing studies inclusion has been by maternal chemotherapy, but a more direct measure of fetal exposure and internalized dose to the fetus is lacking to date.

Pharmacokinetics of drugs in an obstetric population is complicated by changes in the mothers' pharmacokinetic parameters and in the unique placental/fetal compartments. Although maternal levels can be quantified by urine and blood analysis, direct fetal exposure is more difficult to ascertain. Lipophilicity, affinity

for transporters, molecular size of the agent, and variations in biotransformation lead to differential disposition across the maternal/fetal compartments and thus differential exposure for mother versus child, with the placenta strongly influencing disposition and metabolism of chemical exposures. Differential toxicokinetics and timing of exposure impacts the potential effects from maternal exposure, placental exposure, and direct fetal exposure [9-12]. Studies in non-human primate models have examined the pharmacokinetics of taxols (paclitaxel and docetaxel) in a limited number of pregnant baboons[13]. In the baboon model, transplacental disposition of chemotherapeutics was possible and fetal exposure to taxols occurred hours after infusion. Longer timepoints with docetaxel indicated a fetal reservoir, with higher fetal than maternal liver levels 72 hours after infusion. However, it is unknown whether fetal exposure occurs in human pregnancy and for which chemotherapeutics.

Meconium is the first stool of a newborn that passes in the first few days after birth but begins accumulation around the 13th week of gestation. Thereby, it provides a unique window into gestational metabolism and exposures, but is relatively understudied compared to blood and urine. Meconium also has the advantage of non-invasive collection within routine clinical practice, making it a potential useful biosample in epidemiologic studies of humans[14]. Human metabolism of paclitaxel occurs via cytochrome P450s to hydroxylated and epoxide metabolites, with high levels of excretion in the bile as well as urinary excretion[15]. Major metabolites include 3'-p-hydroxypaclitaxel, and 6α-hydroxypaclitaxel produced by CYP3A4 and CYP2C8, respectively[16]. To fill in the gap of the knowledge of human exposure *in utero* to chemotherapeutic agents, we utilized meconium sampled from newborns whose mothers underwent chemotherapy during pregnancy and detection by liquid chromatography-high resolution mass spectrometry (LC-HRMS). In a two-step approach we screened for the presence of major chemotherapeutics in meconium from newborns born to mothers who underwent chemotherapy during pregnancy. We then more rigorously quantified paclitaxel and its major metabolites in the positive meconium samples.

Materials and Methods

Study population

Since the Cancer and Pregnancy Regsitry was first created, 382 women have voluntarily enrolled and provided diagnostic and treatment information regarding their cancer during and after pregnancy. In addition, all participants have consented to having medical information requested at birth and then on a yearly basis for their children. Of these women, 268 have received chemotherapy during pregnancy. Since starting the current project, 30 women have enrolled and received chemotherapy, and 23 have agreed to collect meconium at the time of delivering their child. The majority of women in the study (20) were treated for breast cancer, 1 for colon cancer and 2 for Hodgkin's Lymphoma. As the study is ongoing, full un-blinding of treatment regimens is not possible. Mean gestational age at the first chemotherapy treatment was 20 +/- 4.8weeks, and mean number of days between last treatment during pregnancy and day of birth was 36 +/- 20.6 days.

Biosample Collection

Meconium was collected from newborns born to mothers who underwent chemotherapy during pregnancy. Patients treated with chemotherapy during pregnancy who consented to participate in this study were instructed to collect a single sample from the first 3 stools on day one of life. Meconium was collected on day 1 of life in all cases. Collection was performed by the mother herself, sent frozen in pre-labeled and provided packaging, then when received, were stored at -80°C until batched and shipped to laboratory for analysis.

Meconium was also collected from healthy newborns from the Hospital of the University of Pennsylvania in collaboration with the March of Dimes prematurity research center. Samples were collected by nurses in the hospital, stored at 4°C for under 24 hours, and then at -80°C until use. These samples were used for analytical method development, as negative control meconium, and as a matrix for spiking in analytes to generate positive control samples.

Chemicals

Water, methanol, ethyl acetate, methyl tert-butyl ether (MTBE), acetonitrile, and acetic acid were Optima LC-MS grade solvents from Fisher Scientific (Pittsburg, PA). Sodium chloride and paclitaxel was from Sigma-Aldrich (St. Louis, MO) and $^{13}C_6$-paclitaxel, 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel were from Toronto Research Chemicals (Toronto, Canada).

Meconium Analysis

Analysts were blinded to all sample identities during processing, including untargeted and targeted analysis. 3 aliquots of meconium were analyzed per newborn. Meconium analysis was conducted by liquid-liquid extraction with either ethyl acetate or MTBE. Briefly, 50 mg (average 50.6 mg) of meconium was weighed into 10 mL screw cap glass tubes. Wet weight was recorded to 0.1 mg on a balance with tolerance to +/- 0.01 mg. Samples were spiked with an internal standard to adjust for variation in extraction and analysis. For screening, 500 pg diclofenac (50 µL of 10 pg/ µL stock in methanol) was used as an internal standard to adjust for consistency of extraction and as an internal sample quality control. In quantitative analysis, 500 pg of $^{13}C_6$-paclitaxel (50 µL of 10 pg/ µL stock in methanol) was used as the internal standard. During screening experiments, ethylacetate and MTBE extraction were tested since a variety of extractions have been reported for the chemotherapeutics studied here. Briefly, ethylacetate extraction was conducted by adding 100 µL of saturated NaCl solution in water, 900 µL of water, sonicating in a bath sonicator for 10 minutes then adding 8 mL of ethyl acetate and vortexting for 30 minutes. MTBE extraction was similar except that 8 mL of MTBE was used. The organic layer was then removed and evaporated to dryness under nitrogen gas. Dried residue was re-suspended in 100 µL of 80:20 water: methanol

LC-HRMS analysis was conducted on an Ultimate 3000 quaternary UPLC equipped with a refrigerated autosampler (6° C) and a column heater (60° C) coupled to a Thermo QExactive Plus. LC separations were

conducted on a Waters XBridge C18 (3.5 µm, 100 Å, 2.1 x 100 mm). A multi-step gradient at 0.3 mL/min flow with solvent A (water 1% acetic acid) and solvent B (acetonitrile 1% acetic acid) was as follows: 10% B from 0 to 1 minute, increasing to 100% B from 1 to 5 minutes, holding 100% B until 12 minutes, then the column was returned to starting conditions and re-equilibrated at 20% B from 12 to 15 minutes. This gradient was sufficient to resolve an interfering peak (retention time of 5.5 min) in meconium samples that interfered with the detection of paclitaxel by HRMS. Column effluent was diverted to waste from 0 to 0.5 minutes and from 12.5 to 15 minutes. The mass spectrometer was operated in positive ion mode with a second generation heated electrospray ionization source (HESI-II) alternating between full scan (200-900 m/z) at a resolution of 70,000 and data independent analysis (MS/MS) at 17,500 resolution with a precursor isolation window of 0.7 m/z. In the targeted experiments MS/MS was conducted on the M+H of paclitaxel, $^{13}C_6$-paclitaxel, 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel. Source parameters were as follows: capillary temperature, 425 °C, probe temperature, 425 °C, sheath gas, 45 arb units, aux gas, 15 arbitrary units, sweep gas, 2 arbitrary units, spray voltage 4.0 kV, S-lens, 50. Targeted data analysis was performed in Xcalibur and TraceFinder software (Thermo Fisher, San Jose, CA).

Statistical analysis

Data was analyzed and summary statistics were calculated in Excel, using python, and/or with Graph Pad Prism (v7).

Results

Putative Detection of Paclitaxel and its metabolites in Human Meconium by LC-HRMS

Samples from 23 newborns born to mothers who underwent chemotherapy during pregnancy were screened by LC-HRMS for the presence of chemotherapeutics and their major metabolites. Meconium from 3 healthy newborns was used for method development, as a negative control, and as a matrix to spike known quantities of analyte to serve as positive controls. Surprisingly, a chromatographic peak putatively corresponding to intact paclitaxel was detected in meconium from 7/23 meconium samples, with putative detection of two paclitaxel metabolites but no parent drug in one other sample. Detection frequency was markedly lower with MTBE extraction (4/23) from meconium despite this being a common liquid-liquid extraction solvent for quantification of paclitaxel in other matricies[17].

Quantification of Paclitaxel and its Metabolites in Positively Screened Meconium Samples.

Quantitative analysis by mass spectrometry can be improved in sensitivity, specificity, accuracy and precision by using a stable isotope labeled analog of target analytes. $^{13}C_6$-paclitaxel is commercially available and was used instead of the surrogate internal standard diclofenac for quantitative isotope dilution-LC-HRMS. 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel were well resolved on our LC-HRMS method and were quantified using $^{13}C_6$-paclitaxel as an internal standard (**Fig. 1A**). Standard curves over the range of 4-4,000 pg/mg meconium were linear with R^2 values of 0.9949, 0.9964 and 0.9971 for paclitaxel, 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel, respectively. All positively detected samples were interpolated from these curves. LC-tandem MS was conducted to confirm the identity and specificity of all quantified analytes, with at least 5 unique fragments shared between standard and analytes in samples.

Quantification of paclitaxel across samples revealed a mean (standard deviation) of 399.9 (248.6) pg/mg in positively screened samples (**Fig. 1B**). 3'-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel were quantified at 105.2 (54.6) pg/mg and 113.5 (48.9) pg/mg, respectively. Spearman rank correlation was used to analyze the

correlations of the averaged values of paclitaxel and its metabolites in the meconium samples corresponding to the same individual. Correlation of paclitaxel with its metabolites was strong (Spearman r (p-value) for each pair paclitaxel/3'-p-OH-Pac 0.952 (p=0.00114) paclitaxel/6α-OH-Pac 0.857 (p= 0.001071)) and correlation between the two metabolites was strong at 0.952 (p= 0.00114).

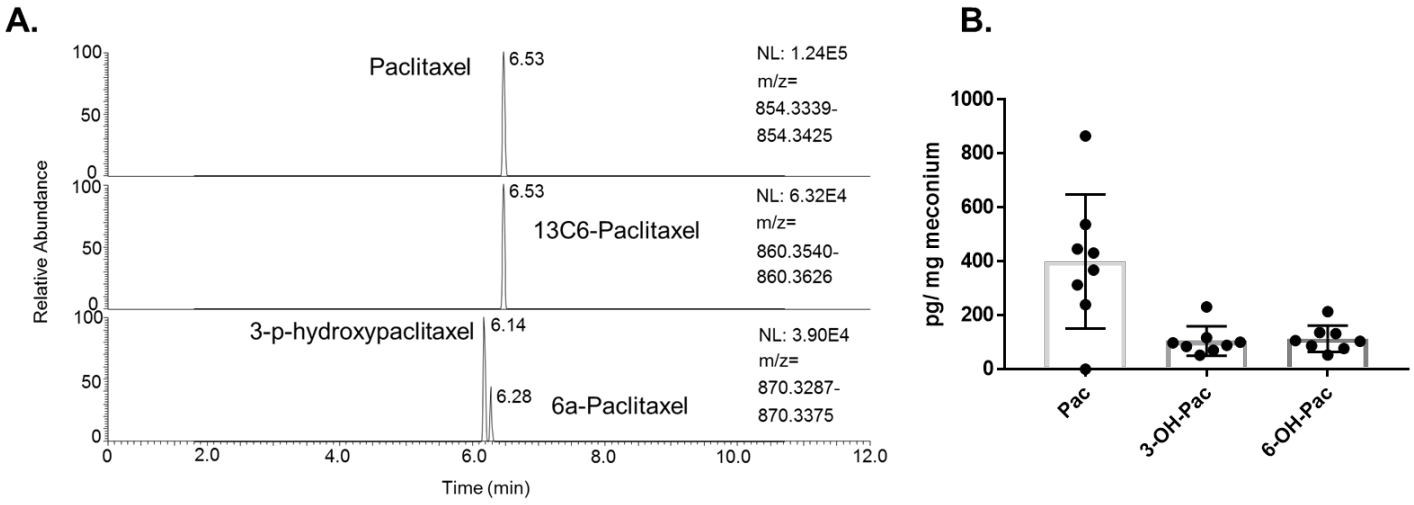

Figure 1. Quantification of paclitaxel in meconium. (A) Representative chromatogram of paclitaxel and its metabolites by isotope dilution-LC-HRMS in meconium. (B) Pg per mg wet weight of meconium for paclitaxel (Pac), 3'-p-hydroxypaclitaxel (3-OH-Pac), and 6α-hydroxypaclitaxel (6-OH-Pac) in 8 meconium samples. The mean of three averaged replicates per individual is shown with the standard deviation.

Discussion

Meconium provides a measure of fetal exposure from a biospecimen that can be collected non-invasively. This has been used to quantify maternal use of drugs of abuse and environmental exposures during pregnancy[14], anti-retroviral pharmacokinetics[18], metabolomics during gestational diabetes[19], and fetal sex steroid metabolism[20]. To our knowledge, this is the first meconium report of the transplacental pharmacokinetics of chemotherapeutics in humans. The finding of paclitaxel and its metabolites in meconium agrees with previous baboon studies [13]. Our findings also agree with the descriptions of fecal excretion of paclitaxel metabolites in

adults. However, the detection of intact paclitaxel in the meconium was surprising since we assumed that it would be predominantly bound to plasma proteins, metabolized during gestation, or actively exported by the placenta. This finding suggests that either meconium contents are protected from further metabolism and/or that the fetal/placental compartment lacks the requisite enzymes. We were also surprised to find paclitaxel and its metabolites in meconium since the literature suggests that paclitaxel is a substrate for P-glycoprotein, the major ATP-dependent efflux pump highly abundant in the placenta, and this would limit fetal exposure [9-11].

Of particular interest to interpretation in this study, Berveiller, *et al*., performed transplacental studies of paclitaxel and docetaxel in term placentas using a human perfused cotyledon placental model. Targeted maternal concentrations mimicked an infusion of 90 mg/m^2 of paclitaxel and 100 mg/m^2 of docetaxel used in clinical practice. Fetal transfer rate and placental uptake ratio were calculated as transport parameters. After perfusion, the cotyledons underwent extraction to quantify docetaxel and paclitaxel using reverse-phase high-performance liquid chromatography. Results showed that the transplacental transfer of paclitaxel and docetaxel were low and similar. Large heterogeneity was also noted across placentas, similar to the current study. An explanation offered by the authors was the differential expression of p-glycoprotein, a drug transporter which should decrease tissue accumulation in the fetus. Authors found both variability in expression according to both gestational age and between patients of similar gestational age [12]. Heterogeneity in the amount of paclitaxel and its metabolites in different individuals may indicate either differential maternal pharmacokinetics or different fetal pharmacokinetics. Variations in pharmacokinetic parameters of taxol have been reported from ABCB1 polymorphism and P-gp activity[21], and variations in toxicity have been reported by CYP3A4 genotype[22].

One limitation of our study is that we were unable to quantify maternal pharmacokinetic parameters during dosing in relation to meconium deposition. However, in all cases of maternal paclitaxel treatment, metabolites were detected in the meconium of their infants. Thus, meconium drug concentrations may be useful in designing more sensitive studies to detect potential effects of fetal exposure to chemotherapeutics during gestation, since the meconium levels indicate individual fetal level exposures.

Regardless, this study should be taken into context within the strong clinical evidence base of the effectiveness of taxol-based chemotherapy. The sequential use of Paclitaxel following completion of Doxorubicin/Cyclophosphamide has been life-saving in non-pregnant women. Pregnant women who complete Doxorubicin/Cyclophosphamide by the early third trimester could benefit from Taxol treatment rather than delaying this until after the delivery, or electively delivering a preterm infant in order to start taxanes postpartum. When looking at the developmental outcomes of infants exposed to chemotherapy, prematurity had a stronger negative influence on development than chemotherapy [7]. For this reason, using taxanes during pregnancy to expand the treatment period during pregnancy will decrease the incidence of preterm birth for pregnant women with cancer. The use of paclitaxel and docetaxel have been reported in human pregnancies with reassuring neonatal outcomes [23-30].

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Figure Legends.

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