GENX INHIBITS P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN TRANSPORT ACTIVITY AND EXPRESSION AT THE BLOOD-BRAIN BARRIER IN SPRAGUE-DAWLEY RATS

Alicia Carolyn Richards

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Approved by:

Linda S. Birnbaum

Louise Ball

Ronald Cannon

ABSTRACT

Alicia Carolyn Richards: GenX Inhibits P-glycoprotein and Breast Cancer Resistance Protein Transport Activity and Expression at the Blood-Brain Barrier in Sprague-Dawley Rats
(Under the direction of Linda S. Birnbaum)

GenX (CAS No. 62037-80-3) is a replacement for perfluorooctanoic acid (PFOA) as a chemical precursor in fluoropolymer manufacturing. The compound is a persistent contaminant in drinking water and air. To address the potential for CNS toxicity, we investigated the effects of low concentrations (0.01 – 1000 nM) of GenX on ATP-Binding Cassette transporter activity and expression at the blood-brain barrier. Using an *ex vivo* steady-state luminal fluorescence-based assay, we found that 1 - 1000 nM GenX rapidly inhibited P-glycoprotein (P-gp, ABCB1) and Breast Cancer Resistance Protein (BCRP, ABCG2) activity in SD rats and in human ovarian cancer cells. Inhibition of efflux transport reduces the neuroprotective function of the BBB, potentially exposing the CNS to toxic substrates. If GenX has a similar effect in other P-gp- and BCRP-expressing tissues, then changes in pharmacokinetics of xenobiotics could occur.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ACUC	Animal Care and Use Committee
BBB	Blood-brain Barrier
BCRP	Breast Cancer Resistance Protein
DEQ	Department of Environmental Quality
DMSO	Dimethyl Sulfoxide
EU	European Union
HPLC	High Performance Liquid Chromatography
IV	Intravenous
KO	Knock-out
MRP2	Multidrug Resistance Protein 2
NBD-CSA	[N-ε(4-nitrobenzofurazan-7-yl)-d-Lys8]-cyclosporin A
NC	North Carolina
NIEHS	National Institute of Environmental Health Sciences
PBS	Phosphate-buffered Saline
PFAS	Poly and per fluoroalkyl substances
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane sulfonic acid
PFTE, Teflon	Polytetrafluoroethylene
P-gp	P-glycoprotein
PPAR	Peroxisome proliferator
ppm	Parts per million

ppt	Parts per trillion
REACH	Regulation for the Registration, Evaluation, Authorization and Restriction of Chemicals Act
SD	Sprague-Dawley
TDAR	T cell-dependent antibody responses

CHAPTER 1. INTRODUCTION

Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy) propanoate (CAS number: 62037-80-3, GenX) is the ammonium salt of the hexafluoropropylene dimer acid. It is a member of a class of compounds called per- and polyfluorinated alkyl substances (PFAS) (PubChem). Because of its surfactant properties, GenX and related substances are useful as a polymerization processing aids in the production of fluoropolymers. Under the Regulation for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) Act, the European Union (EU) regulated and began the phase-out of perfluorooctanoic acid (PFOA) due to its human and environmental exposure in addition to increasing reports of toxicity in laboratory animals. Fluoropolymer manufacturers introduced GenX as a PFOA replacement in the production of polytetrafluoroethylene (PTFE, Teflon), which creates nonstick coatings on cookware. GenX is also a constituent in food packaging and firefighting foams. GenX and other short-chain or ether linked PFAS were chosen to minimize concerns about persistence and toxicity.

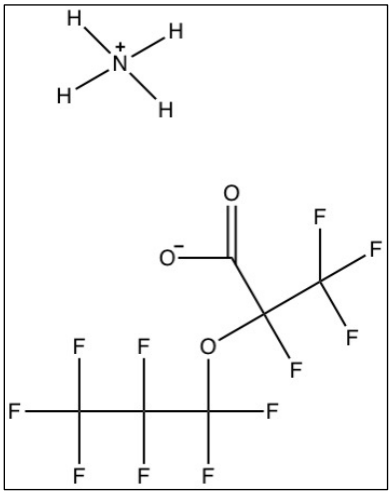

Figure 1*. GenX Structure*

GenX is structurally related to PFOA, but it is distinguished by an ether linkage and a carboxylic acid group at the second carbon (**Figure 1**). GenX is amphipathic, due to its lipophilic hydrocarbon chain tail and hydrophilic head. Because of its strong carbon-fluorine bonds and steric hindrance, this structure is both stable and unlikely to be metabolized. GenX is a crystalline solid when associated with an ammonium ion. It has a molecular weight of 347.084

g/mol, is highly soluble in water, and is a strong acid with a pKa of 3.82 ("<Chemours Dordrecht Evaluation of substances used in genx tech.pdf>,").

Table 1. *GenX chemical characteristics*

Characteristic	
Chemical Formula	$C_6H_4F_{11}NO_3 \cdot NH_4^+$
Molecular Weight	347.084 g/mol
Melting Point	208 °C
Freezing Point	-21 °C
Vapor Pressure	0.012 Pa
Solubility in Water	Aqueous 1000 g/L<
Density	1118 g/L
Dissociation Constant	pKa: 3.82

GenX has a production volume of 10-100 tons per year in the EU, but its worldwide production volume is unknown ("<Chemours Dordrecht Evaluation of substances used in genx tech.pdf>,"). It enters the environment through the manufacturing process in liquid and aerosolized effluent. GenX contaminates rivers in the Netherlands, Germany, and China at concentrations of 0.210 nM, 0.248 nM, and 8.9 nM, respectively (Heydebreck, Tang, Xie, & Ebinghaus, 2015). In a study on acute and chronic aquatic toxicity of GenX, researchers determined the 96-hour LC50 for rainbow trout was >96.9 mg/mL. At 120 mg/L, GenX did not produce immobility or sublethal effects in *Daphnia magna*, growth inhibition in algae, or changes in *Daphnia* reproduction or rainbow trout hatching (Hoke, Ferrell, Sloman, Buck, & Buxton, 2016). In addition, GenX did not bioaccumulate in any of the test species (*Daphnia*, trout, carp, and algae). These results suggest that GenX is unlikely to pose significant aquatic hazard, but it will be persistent in environmental media.

In June 2017, the EPA detected GenX at multiple sites in Cape Fear River Watershed in North Carolina (NC) downstream of a fluoropolymer manufacturer (Chemours Dupont) (Sun et al., 2016). According to the NC Department of Environmental Quality (DEQ), GenX was present

in finished drinking water at concentrations from 340-1100 ppt (approximately 1-3 nM) (NCDEQ, 2018). NC DEQ reported that the Fayetteville, NC facility releases approximately 2,700 pounds of GenX into the air per year (NCDEQ, 2018). The chemical is also present in private well water and some local food products, such as oysters and honey. Following state direction, the manufacturer ceased further discharge of GenX, and levels of GenX in drinking water began to decline. There are no mandatory federal or state regulatory requirements concerning GenX at the time of this publication, but NC set a health guideline of 140 ppt (0.40 nM) GenX in drinking water. This guideline is intended to be protective for bottle-feeding infants, children, nursing and pregnant women, and adults (NCDEQ, 2018).

Human exposure to GenX most likely occurs through contaminated drinking water, air, and potentially in food. There are no blood or urine concentration data available in the U.S. population. In an occupational biomonitoring study in the Netherlands, serum concentrations of GenX ranged from below the limit of detection to 486 nM with a mean concentration in serum of 47 nM (Vandenberg, 2018). Estimations of this population's environmental exposure were not provided.

In the manufacturer's study of the toxicokinetics of GenX, Gannon et al. described oral and intravenous routes of exposure in rats, mice, and cynomolgus monkeys. In the oral kinetic studies, they administered a single dose of 10 or 30 mg/kg GenX (water vehicle, PO) to Sprague-Dawley (SD) rats (3/sex/dose) and to Crl:CD1 mice (3/sex/dose) (Gannon et al., 2016). GenX was rapidly absorbed (absorption half-life of 0.21 h in males and 0.46 h in females), and the C_{max} of 50,000 ng/mL in rats occurred less than 24 hours following oral administration of 10 mg/kg of the compound. The test compound was eliminated rapidly in urine following biphasic kinetics without undergoing metabolism. The alpha phase half-life following oral dosing was 2.8 and 0.2

hours for male and female rats, respectively, indicating a moderate sex difference in the rate of elimination. In the *intravenous* (IV) studies, they dosed Crl:CD SD rats (6/sex/dose) with 10 or 50 mg/kg GenX (PBS, single tail vein injection), as well as cynomolgus monkeys (3/sex, 10 mg/kg, PBS, peripheral vein injection). All test species showed similar blood concentration curves and elimination of the parent compound, suggesting that humans will behave similarly.

In a 2-year oral dosing study of GenX in SD rats, Caverly Rae et al. investigated the carcinogenicity and toxicity potential of GenX, using doses of 0, 0.1, 1, or 50 mg/kg in male rats and 0, 1, 50, or 500 mg/kg in female rats (Caverly Rae et al., 2015). They observed rapid absorption and elimination of the unmetabolized compound in urine. In the 50 mg/kg males and the 500 mg/kg females, GenX decreased red cell mass and increased some liver enzymes indicative of liver injury. The high dose (500 mg/kg) females displayed kidney, tongue, and stomach changes, in addition to hepatocellular adenoma and carcinoma. At the high doses (50 and 500 mg/kg) GenX exposure resulted in neoplastic lesions of the liver, pancreas and testes in the male SD rats and in livers of female SD rats. Liver damage was attributed to peroxisome proliferator alpha (PPARα) agonism (Caverly Rae et al., 2015).

Rushing et al. investigated markers of liver peroxisome proliferation and T cell-dependent antibody responses (TDAR) in C57Bl/6 mice in response to 28-day GenX exposure (Rushing et al., 2017). Females exposed to 100 mg/kg and males exposed to 10 mg/kg of the test compound exhibited increased hepatic acyl-CoA oxidase activity, indicative of peroxisome proliferation (Rushing et al., 2017). Liver weights were increased in both sexes at 10 and 100 mg/kg, and TDAR was significantly decreased in females at 100 mg/kg. PFOA had more potent effects on PPARα and TDAR than GenX. It is unlikely that the PFOA-mediated TDAR suppression was entirely due to peroxisome proliferation, as previous studies have shown that

PFOA decreases TDAR in PPARα knockout (KO) animals (DeWitt, Williams, Creech, & Luebke, 2016). Additional studies are needed to investigate if GenX's immunotoxicity is also independent of PPARα.

Although the potential for GenX to cause central nervous system (CNS) toxicity is unknown, previous research showed that other PFAS (PFOS and PFNA) modulate ATP-binding cassette (ABC) transporter activity and expression at the Blood-Brain Barrier (BBB) (More et al., 2017). The BBB regulates the movement of endogenous and exogenous compounds into and out of the brain through a network of brain microvessels. Tight junction protein complexes connect endothelial cells and prevent the paracellular movement of compounds. Pericytes, astrocytes, neurons, and a basal membrane surround the abluminal side of the capillary, helping to form a physical barrier. Enzymes on the luminal-facing membrane of endothelial cells add an additional layer of chemical protection. Selective channels and carrier proteins located on both sides of the capillary membrane control transcellular transport (Fricker, 2016).

Finally, active transporter proteins, or ATP-binding cassette (ABC) proteins, located on the luminal side of the endothelial cells regulate the efflux of endogenous and exogenous substrates. These proteins protect the brain from potentially harmful chemicals by transporting them into the blood. Once in the blood, the chemicals flow to the kidney or liver where they are metabolized and eliminated in urine or bile. ABC transporters perform barrier functions in other tissues, such as kidney, gastrointestinal tract, liver, placenta, and testis. P-glycoprotein (P-gp, ABCB1, MDR1), Breast Cancer Resistance Protein (BCRP, ABCG2), and Multidrug Resistance Protein 2 (MRP2, ABCC2) are three examples of ABC transporters that transport a wide range of pharmaceuticals, chemotherapeutics, xenobiotics and endogenous molecules (**Table 2**). ABC

transporter-inhibiting drugs can increase the efficacy of chemotherapeutics and other drugs that normally cannot enter the target organ.

Table 2. *Examples of endogenous and exogenous substrates for ABC transporters*

Transporter	Endogenous Substrates	Exogenous Substrates
P-glycoprotein	Phospholipids	Cyclosporine A, digoxin, doxorubicin, tamoxifen
Breast Cancer Resistance Protein	Dietary flavonoids, porphyrins, estrones	Doxorubicin, irinotecan, methotrexate, prazosin, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), statins, topotecan
Multidrug Resistance Protein 2	Glucuronide and glutathione conjugates, bilirubin	Cisplatin, doxorubicin, irinotecan, methylmercury-N-acetyl-L-cysteine, methotrexate, ochratoxin A, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), statins

(Jonker et al., 2005), (Jemnitz et al., 2010), (Biotechnology)

In a previous study, 10 nM perfluorooctane sulfonic acid (PFOS) and 10 nM perfluorononanoic acid (PFNA), two longer chain perfluoroalkyl surfactants, induce the activity of P-gp, BCRP, and MRP2 (More et al., 2017). However, shorter chain PFAS (10 nM concentrations of perfluorobutyric acid and perfluorohexanesulfonic acid) had no effect on P-gp activity. Information on the toxicity of GenX is crucial in its risk assessment. Although the concentration of GenX in human biological matrices remains unknown, exposure through water, air, or food is likely. Perfluorinated chemicals are highly persistent in the environment, so understanding the risk of these chemicals prior to their release is important. Due to the high likelihood for human exposure, persistence, and potential for toxicity, we designed this study to investigate the effects of GenX on transport activity and expression of P-gp, BCRP, and MRP2 in SD rats.

CHAPTER 2. METHODS

Chemicals

The fluorescent P-glycoprotein substrate N-" (4-nitrobenzofurazan-7-yl)-D-Lys cyclosporine A (NBD-CSA) was custom synthesized. We purchased the fluorescent Breast Cancer Resistance Protein substrate BODIPY-Prazosin from Life Technologies, and the fluorescent MRP2 substrate sulforhodamine 101 free acid (Texas Red) from Sigma-Aldrich. The specific P-glycoprotein inhibitor, PSC-833 (3S,6S,9S,12R,15S,18S,21S,24S,30S,33S)-1,4,7,10,12,15,19,25,28-nonamethyl-33-[(E,2R)-2-methylhex-4-enoyl]-6,9,18,24-tetrakis (2-methylpropyl) 3,21,30-tri(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31 undecazacyclotritriacontane-2,5,8,11,14,17,20,23,26,29,32-undecone) was kindly provided by Novartis. Specific BCRP inhibitor KS-176 (N-[4-(2-Hydroxyethyl)phenyl]-2-[(4-nitrobenzoyl)amino]-benzamide) and specific MRP2 inhibitor MK-571, 5-(3-(2-(7-Chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid sodium salt were purchased from Sigma-Aldrich . Solutions of specific fluorescent substrates and inhibitors were prepared at the concentrations 1000 times stronger DMSO (**Table 3**), then diluted in isolation buffer.

Table 3. *Transporter-specific fluorescent substrates and inhibitors*

Transporter	Fluorescent Substrate	Concentration	Specific Inhibitor	Concentration
P-glycoprotein	NBD-CSA	2 µM	PSC-833	10 µM
BCRP	BODIPY-Prazosin	2 µM	KS-176	10 µM
MRP2	Texas Red	2 µM	MK-571	20 µM

Isolation buffers were prepared 4-48 hours prior to the experiment and are stored in a 4 ℃ refrigerator. Isolation buffer was prepared using the following recipe: 500 mL phosphate-buffered saline (PBS) containing calcium and magnesium, 0.055 g sodium pyruvate (Sigma-Aldrich, St. Louis, MO), and 0.45 g glucose (Sigma-Aldrich, St. Louis, MO). Isolation buffer containing 1% BSA solution was prepared using the following recipe: 500 mL PBS containing Calcium and Magnesium, 0.055 g sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 0.45 g glucose, (Sigma-Aldrich, St. Louis, MO), and 5.0 g of Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO). The two bottles of isolation buffer were placed on a shaking platform to mix them. A 30% Ficoll solution was prepared by combining 40 mL of Isolation Buffer (not containing BSA) and 15 g of Ficoll (Sigma-Aldrich, St. Louis, MO) and mixed using a magnetic stir plate for 30 minutes. During the experiment, isolation buffers and the 30% Ficoll solution are stored on ice.

Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate (CAS No. 62037-80-3, GenX) was purchased from Synquest (Alachua, FL) in powder form. The concentrations of GenX tested include 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, and 1 μM. 1000X stock solutions were prepared in DMSO in 4 mL glass vials for immediate use. We vortexed for 10 seconds immediately after mixing and prior to formation of the next solution. A 1:1000 dilution was then used in the treatment of GenX dissolved in PBS. After each addition of GenX, the vial was vortexed for 5 seconds.

Animals

The Animal Care and Use Committee at National Institute of Environmental Health Sciences (ACUC-NIEHS) approved all the animal protocols. We conducted all experiments as per ACUC-NIEHS guidelines. Retired breeder male Sprague-Dawley rats (age 9 months; Taconic) and female Sprague-Dawley rats (age 13 weeks; Taconic) were housed separately

under a 12-h light/dark cycle in a temperature controlled facility and allowed access to food and water *ad libitum*. Animals were euthanized using carbon dioxide inhalation and decapitation with guillotine. We isolated brain capillaries using the protocol described below for immediate use in transport assays, or they were flash frozen in liquid nitrogen and stored in a -80 ℃ freezer for use in Western Blotting.

Capillary Isolation in Adult Rats

Procedures for capillary isolation for use in *ex vivo* or *in vivo* transport assays have been outlined previously (Chan & Cannon, 2017). Some modifications have been made to the method to optimize the number of capillaries, minimize contamination, and improve repeatability. Four rats per treatment group were used in *in vivo* assays; 2-6 rats were used in *ex vivo* assays; and 5 rats were used for Western Blotting. After euthanizing the rats via CO_2 inhalation, we severed the head at the base of the skull with a guillotine. Using a scalpel, we scored the skull from between the eyes to the base of the neck. We spit the skull with a Littauer bone cutter (Fine Science Tools, cat. no. 16152-15) along the score mark, and removed the brain with a spatula. We stored the brains in 50-ml Falcon polypropylene conical tubes (Becton, Dickinson and Company) containing 20 mL cold isolation buffer.

In a large (100×15 mm) Polystyrene petri dish (Becton, Dickinson and Company) containing ice cold isolation buffer and halved the brain using the edge of a spatula. Using forceps (Dumont no. 7: fine and Iris, curved; Fine Science Tools), we isolated cortical gray by removing the meninges, white matter, choroid plexus, olfactory lobes and midbrain. We stored the remaining cortical gray matter in a clean 50mL Falcon tube containing 15mL of isolation buffer stored in ice. We then transferred all of the collected gray matter and approximately 5 mL of the isolation buffer from the tube into a small petri dish (30×15 mm) and minced using a razor blade until in less than 1 mm^3 pieces. The minced brain tissue was rinsed into size C 55-ml tissue

grinding vessel (prechilled on ice with the pestle) using a 7-ml bulb polyethylene transfer pipette (USA Scientific, cat. no. 1020-2500) and ice-cold isolation buffer. We rinsed the small petri dish and sides of the grinding vessel with additional isolation buffer.

We placed the grinding vessel in a 250 mL beaker containing ice. Using a pestle (Size C serrated, Thomas Scientific, cat. no. 3431F25) (clearance: 150 to 230 μm) to a drill, we homogenized up and down slowly (approximately 5-10 seconds per up and down motion) for a total of 25 strokes at a speed of 20 RPM. We used 1 mL of isolation buffer to rinse remaining homogenized tissue on the pestle into the grinding vessel. We poured the homogenate into a pre-chilled 15 ml KONTES dounce tissue grinder (VWR, cat. no. 885300-0015), and we dounced 8-10 times using the pestle (size B; clearance: 165 to 889 μm). Then, we poured the mixture into a 50-ml polycarbonate centrifuge tube with polyethylene cap (Beckman Coulter, cat. no. 363664) and added 22mL of 30% Ficoll PM400 solution to the tube, inverting at least 6 times.

Then, we centrifuged at 7000 rpm, 4℃, for 24 minutes using a Sorvall RC-5B centrifuge using an SS-34 rotor. Removing the tubes carefully, we placed the tubes back on ice. We poured off the top layer of fat and supernatant, leaving behind a red pellet. Then, we wiped away any remaining fat on the sides of the tube with a Kimwipe, being careful to not disturb the pellet. Immediately we added 1-3 mL of ice cold isolation buffer containing 1% BSA and resuspended the pellet using a transfer pipette. Once it was fully dissolved into the BSA solution, we added another 15 mL of BSA solution and store on ice.

Then, we secured a 300-μm Spectra/Mesh woven nylon filter to a 50 mL Falcon tube with a cap which has been cut with a hole in it. The entire filtering process was performed on ice. For every two adult rat brains, we prepared one 50mL Falcon tube with a 30 μM pluriStrainer unit and store on ice. We washed the nylon filter with the 1% BSA solution to reduce surface

tension, then transferred the capillary-rich solution from the Beckman centrifuge tube through the mesh using a transfer pipette. We removed the mesh filter. Then, we washed the pluriStrainers with the 1% BSA solution before pouring the filtered capillary-rich solution through them.

Once all the capillary-rich solution was poured through the filter, we removed the filter and flipped it over on top of a clean, open 50-mL Falcon tube. We used a transfer pipette to rinse the back side of the filter with one pipette-full 1% BSA solution, lightly touching filter with the tip of the pipette. We repeated this action two more times. Then, we rinsed the top side of the filter into the tube, using another pipette-full of 1% BSA solution. We repeated this action two times. We then poured the remaining solution in the tubes with the pluriStrainers through an additional pluristrainer to catch any capillaries that may have passed through the other filters. We then rinsed the additional pluriStrainer into the “clean capillaries” tube using the same process described above. We then centrifugde the filtered capillary-rich solution for 7 minutes at 1300 RPM in a ThermoFisher Sorvall RC 6+ Centrifuge.

We poured off the supernatant, leaving behind a red pellet, and wiped the sides of the tube with a Kimwipe. We resuspended the pellet in ice cold isolation buffer (not containing 1% BSA) and transfer the pellet to a 15 mL Falcon tube. Once the pellet was fully mixed into the isolation buffer, we added additional buffer solution for a total volume of approximately 8 mL. We centrifuged a second time for 9 minutes at 1300 RPM. If there was not a distinguishable pellet after this spin, we vortexed the tube briefly and centrifuge it again for 7 minutes at 1300 RPM. We poured off the supernatant and resuspend the pellet in 500 µL of isolation buffer. We added a drop to a microscope slide and observed the capillaries. We added enough isolation buffer for 75-95 µL of capillary solution per chamber slide. We added the capillary solution to

the chamber slides and waited 30 minutes before adding 1 mL of room temperature isolation buffer.

Capillary Isolation in Pups

To isolate capillaries from rat pups (8h – 14 days old), the process is similar to the process in adult rats, but the differences are noted below. Four to five brains per treatment group were used in our *in vivo* assay. The skin and skull are much thinner, so only a scalpel or razor blade are necessary to open the skull and slide out the brain. We removed meninges if present. We skipped the step using the homogenizing drill, since the brain tissue is much more fragile. We reduced the number of douncing motions from 10 to 5. We then poured the dounced mixture into a Beckman centrifuge tube and added 1:2 volume of 30% Ficoll solution. After the 7000 RPM centrifugation, we removed the supernatant and add approximately 10 mL of 1% BSA solution. We omitted the nylon filter step and just filtered using one pluriStrainer per 5 brains. Only one 1300 RPM centrifugation for 7 minutes was necessary prior to applying the capillaries to the slides.

***Ex vivo* Transport Assay**

After adding 1 mL of room temperature isolation buffer to each slide, we began adding the treatments as soon as possible. Each experiment included a control slide, which was treated with 2 mL of isolation buffer with 0.1% DMSO, and an inhibited slide, which was pre-treated with a transporter-specific inhibitor for 30 minutes before adding the fluorescent substrate with the inhibitor. The treated slides received 2 mL of isolation buffer prepared with the desired concentration of GenX (solutions were prepared as described previously). Fluorescent substrates were added 45 minutes prior to imaging on a 710 Zeiss confocal microscope.

To investigate if the effects seen on transport activity were reversible or irreversible, a 100 nM solution of GenX was prepared in the same manner as for a time course. After 1-2 hours, the

solution was removed from the chamber slide. One mL of isolation buffer was added then removed, then this process was repeated to rinse any remaining GenX from the slide. 2 milliliters of GenX were then added until it was time to add the fluorescent substrate. Control and inhibited slides were also rinsed twice to simulate the rinse on the treated capillaries

***In vivo* Transport Assay**

Four age-matched male Sprague-Dawley rats (13 weeks, Taconic) were dosed with 0.03 µmol/kg of GenX dissolved in water by oral gavage, and four rats were treated with vehicle. Four age-matched female Sprague-Dawley rats (13 weeks, Taconic) were dosed with 0.3 µmol/kg of GenX dissolved in water by oral gavage, and four rats were treated with vehicle. After three hours, all rats were euthanized by CO_2 euthanasia, and blood was collected by cardiac puncture. They were then decapitated and the capillaries from each treatment group were isolated separately as described previously. All tools used in decapitation were cleaned in deionized water prior to being used on the next treatments group or were single-use. After capillaries were isolated, they were immediately added to the slides. For each treatment group, three slides received the fluorescent substrate for P-gp, BCRP, and MRP2, and three slides received a 30-minute pretreatment with a specific inhibitor before being treated with the fluorescent substrate.

Western Blotting

Capillaries were isolated as described previously and resuspended in 2 mL of isolation buffer. The 2 mL were split into two 50-mL Falcon tubes. We added 5 µL of 100 µM (GenX powder in DMSO) to a vial containing 4 mL of room temperature isolation buffer. This solution was added to the vial containing the capillaries suspended in 1 mL of isolation buffer. In another tube, we added a solution of 5 µL DMSO and 4 mL of room temperature isolation buffer. Every thirty minutes, we opened the caps of the tubes to allow for oxygenation of the capillaries. After

3 hours, we centrifuged both treatment tubes at 1300 RPM for 7 minutes (ThermoFisher, Sorvall RC 6+ Centrifuge), poured off the isolation buffer solution, and submerged them in liquid nitrogen. We stored the tubes in a -80 °C freezer until they were needed.

We prepared the lysis buffer by combining 6 ml of Cellytic MT Lysis buffer (Sigma-Aldrich, St. Louis, MO) and 1 ml of 7X protease inhibitor [one pellet (Roche Diagnostics, Mannheim, Germany)/1.5 ml of 1X PBS]. We then directly added 150 µL of lysis buffer to resuspend the brain capillary pellet. We transferred this volume to a microcentrifuge tube (Beckman) and repeated this process for a total volume of 300 µL of lysis buffer and capillaries in the microcentrifuge tube. We vortexed the tubes for 10 seconds every five minutes and stored them on ice. After one hour, we centrifuged the tubes at 10,000 g for 30 minutes (Eppendorf centrifuge, 5430 R). Then, we pipetted off the denucleated supernatant into a new microcentrifuge tube, and we centrifuged that supernatant at 100,000 g for 90 mins (Beckman Coulter, Optima MAX-XP Ultracentrifuge). Then, we pipetted off the cytoplasmic supernatant into a new microcentrifuge tube. The pellet left behind is the isolated membrane protein fraction. We resuspended the nuclear pellet and the membrane pellet in 30 µL of lysis buffer, and protein concentrations were determined by Bradford Assay.

To perform the Bradford assay, we pipetted 5 µL of each standard or protein sample of unknown concentration into the appropriate microplate wells. Then, we added 250 µL of Coomassie Reagent to each well and mix with plate shaker for 30 seconds, and incubated at room temperature for 10 minutes. We measured the absorbance at or near 595 nm with a FLUOstart Omega Microplate Reader (BMG Labtech, Cary, NC). We subtracted the average 595 nm measurement for the blank replicates from the measurements for all other samples and replicates. We prepared a standard curve by plotting the average blank-corrected 595 nm

measurement for each BSA standard versus its concentration in ug/mL. We used the standard curve to determine the protein concentration of each unknown sample.

After the protein concentrations have been determined by Bradford assay, we determined the volumes necessary to generate 25 µL total volume of sample, NuPAGE LDS Sample Buffer at 4X concentration, NuPAGE Reducing Agent at 10X concentration, and the remaining of deionized water. We denatured the samples at 80 ℃ in a GeneMate Digital Dry Bath for 10 minutes. We prepared 1000 mL of Running Buffer by adding 50 mL of 20X NuPAGE SDS Running Buffer and 950 mL of deionized water in a graduated cylinder and mixing on a stir plate. We removed 200 mL of running buffer and added 500 µL of NuPAGE Antioxidant. We prepared 1000 mL of transfer buffer by adding 50 mL of NuPAGE Transfer Buffer (20X), 1 mL of NuPAGE Antioxidant, 100 mL of methanol, and 849 mL of Deionized water to a graduated cylinder and mixing on a stir plate.

We removed the NuPAGE Gel from the pouch and rinsed the gel cassette with deionized water, before peeling off the tape from the bottom of the cassette. Gently we pulled the comb out of the cassette in one smooth motion. Then, we rinsed the sample wells with 1X NuPAGE SDS Running buffer. We inverted and shook the gel to remove the buffer. We repeated this action two more times. We oriented the two gels in the Mini-Cell such that the notched “well” side of the cassette faced inwards toward the Buffer Core. We seated the gels on the bottom of the Mini-Cell and locked into place with the Gel Tension Wedge. The, we filled the Upper Buffer Chamber with a small amount of the running Buffer to check for tightness of seal. Once we ensured the seal was tight, we filled the upper buffer chamber with enough 1X running buffer to fill the chamber without overflowing.

We loaded the samples and two molecular weight markers into each well using gel-loading pipette tips. Then, we filled the lower buffer chamber with 600 mL of the appropriate 1X running buffer. We ran the electrophoresis under constant 200 V for 35 minutes. After complete, we shut off power, disconnected the electrodes and removed gel from the XCell SureLock Mini-Cell. Then, we separated each of the three bonded sides of the cassette by inserting the gel knife into the gap between the two plastic plates that make up the cassette. We cut off the foot of the gel. The notched "well" side of the cassette should face up. We pushed down gently on the knife handle to separate the plates and repeated on each side.

We soaked the PVDF membrane for 30 seconds in methanol, and briefly rinsed in deionized water, then placed it in a shallow dish with 50 mL of 1X NuPAGE Transfer Buffer for several minutes. We placed a piece of pre-soaked filter paper on top of the gel, with the edge above the slot in the bottom of the cassette. We wet the surface of the gel with transfer buffer and placed the pre-soaked transfer membrane on the protein side of the gel. Then, we placed another pre-soaked filter paper on top of the membrane and rolled out any bubbles with a roller. We placed three soaked blotting pads into the cathode core of the blot module and oriented the sandwich such that the gel is closest to the cathode core. Then, we placed three additional soaked blotting pads on the other side of the sandwich. Finally, we held the blot module together firmly and slid it into the guide rails on the lower buffer chamber. We inserted the gel tension wedge so that its vertical face is against the blot module and locked it into place by pulling the lever forward.

Then, we filled the blot module with 1X NuPAGE Transfer Buffer until the gel/membrane assemble was totally covered. We attached the lid to the cell and then attached the cathode and anode cords to the power source. We ran the transfer under the following conditions:

25 V constant (began at 330 mA, ended at 130 mA) for 90 minutes. After the transfer was complete, we removed the membrane from the box and rinsed with 1X PBS three times. We then applied a 50/50 Licor blocking buffer and 1X PBS (25 mL) for 30 minutes on a plate shaker.

We removed the blocking buffer and rinsed the membrane with 1X PBS. We then applied an 4 mL solution of primary antibodies. In the 4 mL solution was 20 μL (1:200) rabbit P-gp primary (Ab170904), 20 μL (1:200) rat BCRP primary (ab24115), 0.8 μL (1:5000) mouse Actin (Sigma) and 4 μL Tween. The membrane was vacuum sealed with this solution in a plastic envelope, and it was placed on a plate shaker in a 4 °C refrigerator overnight.

The next day, we removed the membrane from the envelope and poured off the primary solution. We rinsed the filter with 1X PBS then placed it in a container with 1X PBS and rocked on a plate shaker for 30 minutes. This was repeated three times. Then we poured off the PBS and added 20 mL secondary treatment, which included 2 μL AlexaFluor 647 goat anti-mouse Actin secondary antibody, 2 μL AlexaFluor 647 goat anti-rabbit P-gp secondary antibody, and 2 μL Licor 800 goat anti-rat BCRP secondary antibody. After 90 minutes, we removed the secondary treatment and imaged the membrane in a digital imager.

Statistical Analysis

Adult *in vivo* experiments used 4 age-matched control and 4 treated animals, pooled. The pup *in vivo* experiment containted 4-5 pup brains pooled per treatment group. *Ex vivo* experiments required 2-6 rat brains, depending on the number of treatment groups needed. All brains for *ex vivo* treatment were pooled, then divided into slides for treatment. We repeated male *ex vivo* experiments at least twice. All *in vivo* data (in adult and young rats) have been performed once. Western blotting experiments were performed once. The human cell line experiments were repeated three times and reported as an average of the experiments.

Average luminal fluorescence intensity of each in focus and intact capillary was quantified using ImageJ software. Approximately 10-15 images were taken for each treatment group and controls, and 15 ≤ capillaries per group were analyzed. We adjusted the means of the treated and control groups for the average fluorescence intensity of the group treated with a specific inhibitor. In all cases, the capillaries treated with MK-571 were not significantly different from controls, so these results were reported unadjusted. The adjusted and unadjusted means of fluorescence intensity were analyzed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). The data were subjected to one-way ANOVA and multiple comparisons. Statistical difference compared to control are reported as follows: ****, significantly different than control, $p \leq 0.0001$; ***, significantly different than control, $p \leq 0.001$; **, significantly different than control, $p \leq 0.01$; *, significantly different than control, $p \leq 0.05$; ns, not significantly different than control $p > 0.05$. The y axis is plotted as specific or non-specific average fluorescence intensity. Error bars represent statistical error of the mean (SEM).

CHAPTER 3. RESULTS

P-glycoprotein Transport Activity and Expression

We first examined the effects of the concentration of GenX on P-glycoprotein using an *ex vivo* steady-state luminal fluorescence assay in male and female Sprague-Dawley rats. In males, all concentrations of GenX tested significant decreased P-gp transport activity, from 1 nM to 100 nM, following a 3-hour *ex vivo* exposure (**Figure 2A**). In females, a 3-hour *ex vivo* exposure to GenX decreased P-gp transport activity from 10 nM to 1000 nM (**Figure 2B**).

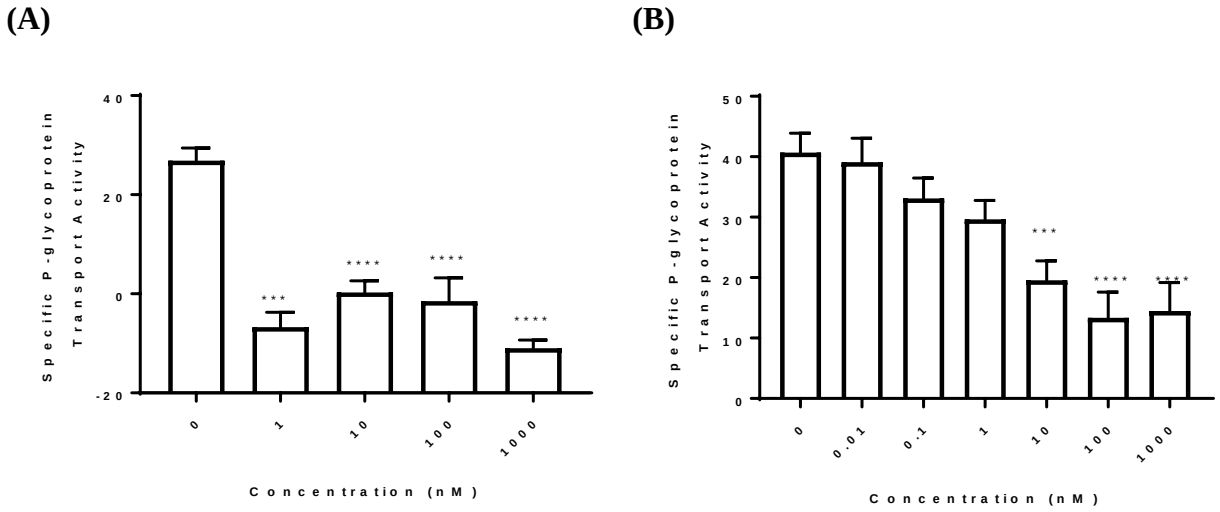

Figure 2. *Specific P-gp transport activity versus concentration of GenX treatment (3h, ex vivo). GenX-mediated decreases in P-gp activity were concentration-dependent in both (A) males and (B) females.*

In order to understand how GenX affects P-gp transport activity over time, we applied 100 nM GenX to capillaries *ex vivo* for 15 minutes up to 4 hours. In males, GenX inhibited P-gp transport activity after 15 minutes of treatment, and fully inhibited after 1 hour (**Figure 3A**). Preliminary data show that GenX inhibited female P-gp transport activity by 30 minutes, with

full inhibition occurring after 2 hours. GenX elicits a nonlinear decrease in P-gp transport activity in both males and females.

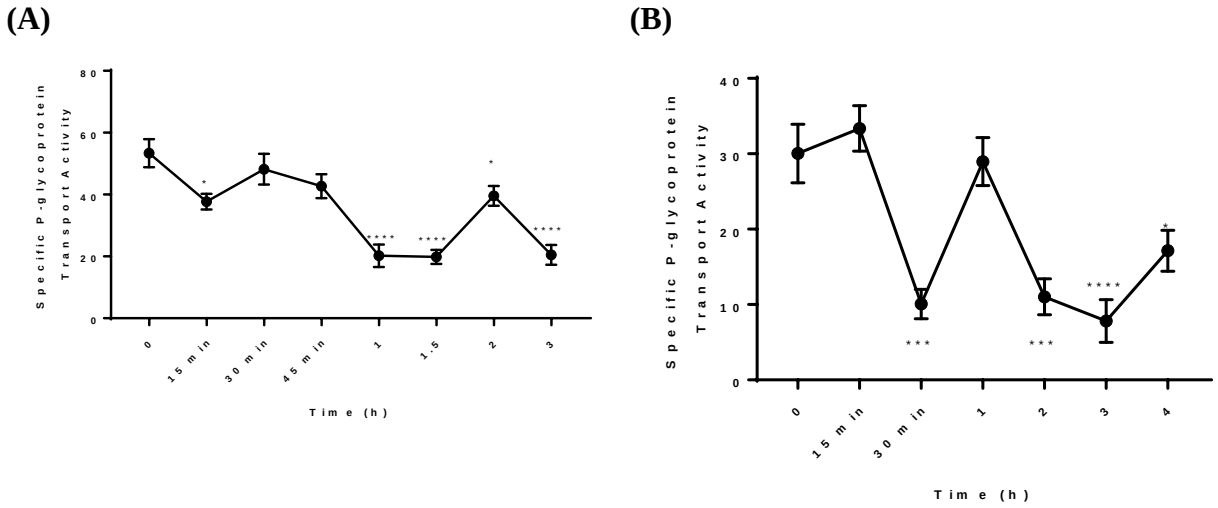

Figure 3. *Specific P-gp transport activity versus duration GenX treatment (100 nM,* ex vivo*). GenX-mediated decreases in P-gp transport activity were time-dependent in both (A) males and (B) females.*

To understand whether the effects of GenX on P-gp transport activity are reversible, we applied a 1-hour treatment of 100 nM GenX (*ex vivo*) then removed it and rinsed the capillaries. Then, one, two, and three hours following removal of the treatment, we measured the level of specific P-gp transport activity. One hour after removing the treatment, P-gp transport activity returned to baseline level in males (**Figure 4**).

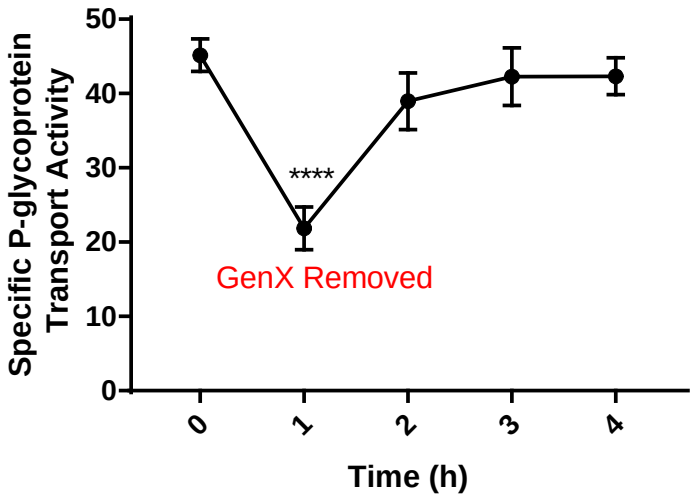

Figure 4. *Specific P-gp transport activity versus time. GenX-mediated decreases in P-gp transport activity are reversible in males (1h* ex vivo *100 nM treatment).*

Changes in transport activity are not necessarily indicative of changes in amount of protein present in the membrane. To quantify changes in protein expression, we treated male and female SD rat brain capillaries *ex vivo* with 100 nM GenX for 3 hours prior to performing Western Blotting. When normalized for GLUT1 or Actin, P-gp expression in males and in females was significantly decreased in the GenX-treated groups (**Figure 5**).

(A) **(B)**

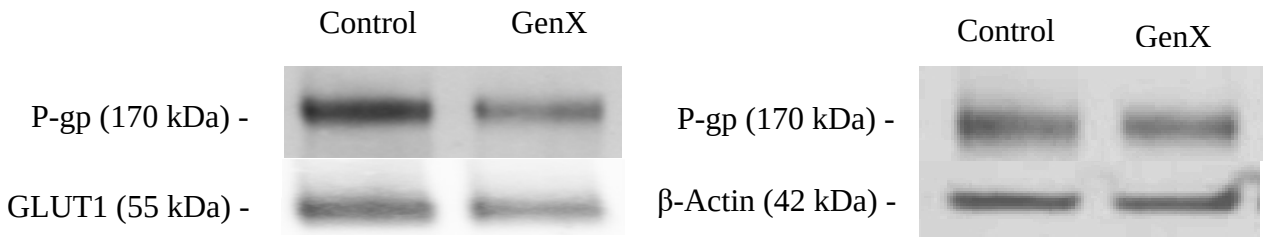

Figure 5. *P-gp expression in male and female at brain capillary membranes, following ex vivo 3-hour exposure to 100 nM GenX. GenX decreased P-gp expression in (A) male and (B female capillaries.*

We then wanted to know whether the decreased expression and activity of P-gp in rat cells treated *ex vivo* would also occur in human ovarian cancer cells. This cell line expresses only P-gp, which protects the cell under normal conditions from Adriamycin, a toxic chemotherapeutic P-gp substrate. When we exposed the cells to 100 nM GenX and increasing concentrations of Adriamycin, the percent cell survival significantly declined compared to Adriamycin-only treatment (**Figure 6**). GenX-only treatment had little to no effect on cell survival.

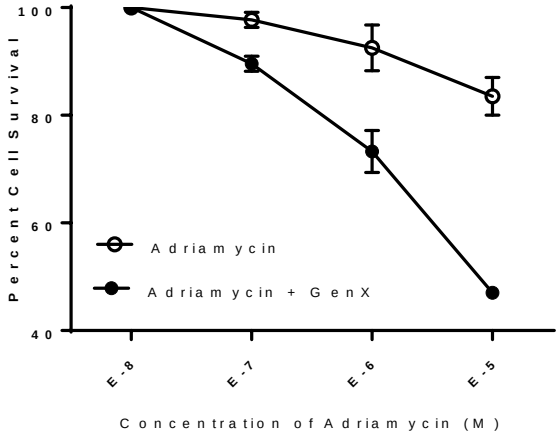

Figure 6. *Percent P-gp expressing human ovarian cancer cell survival versus concentration of Adriamycin. Co-treatment of GenX and Adriamycin increases cytotoxicity compared to Adriamycin alone.*

Next, we investigated the effects of an *in vivo* exposure to GenX in adult rats. We treated males with 0.03 nmol/kg GenX or water vehicle and females with 0.3 nmol/kg GenX or water vehicle for 3 hours, then removed the brains and performed the transport assay. In males, we observed no significant change in P-gp transport activity. In contrast, we observed a significant increase in P-gp transport activity in females.

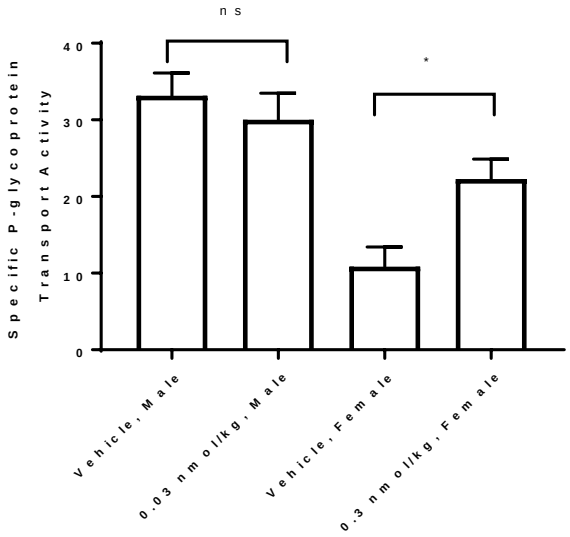

Figure 7. *Specific P-gp transport activity versus concentration of GenX (3h* in vivo *treatment with 0.03 nmol/kg male or 0.3 nmol/kg female). (A) P-gp transport activity does not change in the presence of GenX in males, but (B) increases in females.*

We then decided to investigate the effects of gestational and lactational repeated exposures to GenX in female pups. The dams were exposed from gestational day 8 (GD8) to postnatal day 4 (PND4) with 0.028 mmol/kg or 0.086 mmol/kg (10 mg/kg or 30 mg/kg) GenX in water. We saw a significant decrease in P-gp activity in PND4 female pup P-gp transport activity at the highest dose we tested (0.086 mmol/kg) (**Figure 8**).

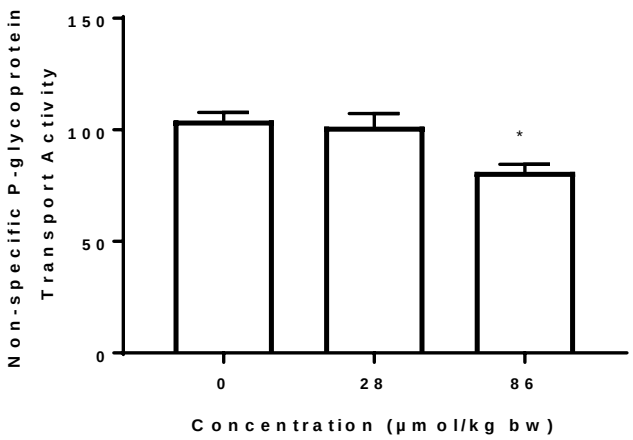

Figure 8. *Specific P-gp transport activity versus concentration of GenX treatment (GD8-PND4,* in vivo*, 28 µmol/kg or 86 µmol/kg). P-gp activity decreases in female PND4 pups in the presence of the highest dose of GenX.*

Breast Cancer Resistance Protein Transport Activity

We were also interested in the effects of GenX on Breast Cancer Resistance Protein (BCRP). Using a steady-state luminal fluorescence assay, we found that a 3-hour *ex vivo* exposure to increasing concentrations of GenX inhibits BCRP transport activity. In males, we observed inhibition from 1 nM – 1000 nM, and in females inhibition occurred from 0.1 nM – 1000 nM (**Figure 9**).

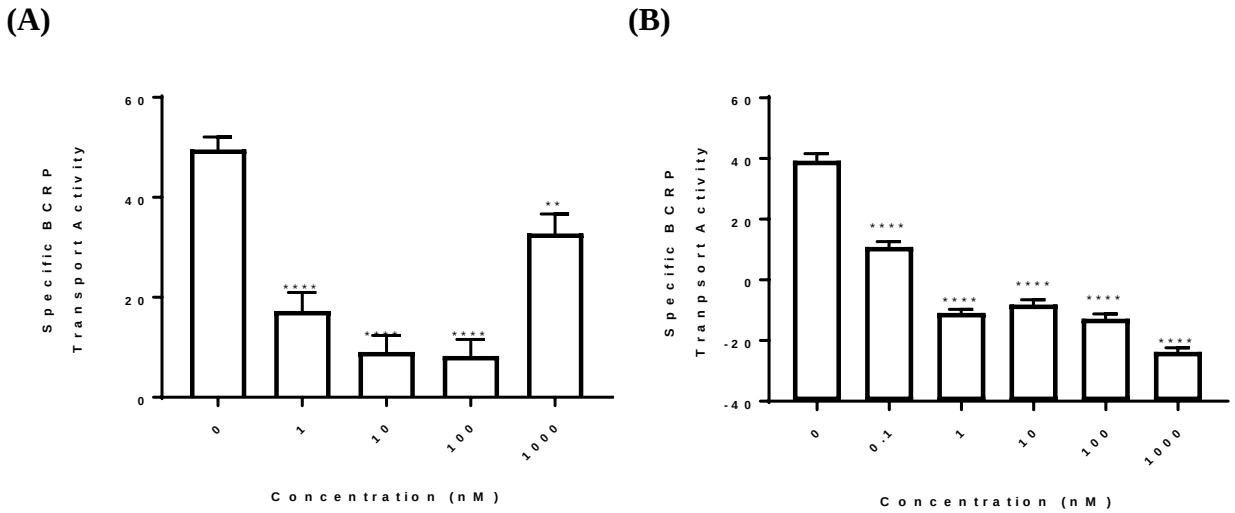

Figure 9. *Specific BCRP transport activity versus concentration of GenX treatment. GenX-mediated decreases in BCRP transport activity are concentration-dependent in (A) males and (B) females.*

We then looked at the effects of 100 nM GenX *ex vivo* exposure over time. In the presence of 100 nM GenX, *ex vivo* BCRP transport activity decreases beginning at one hour in both sexes (**Figure 10**). BCRP activity remains significantly inhibited until the latest time points we investigated in males (6h) and females (4h).

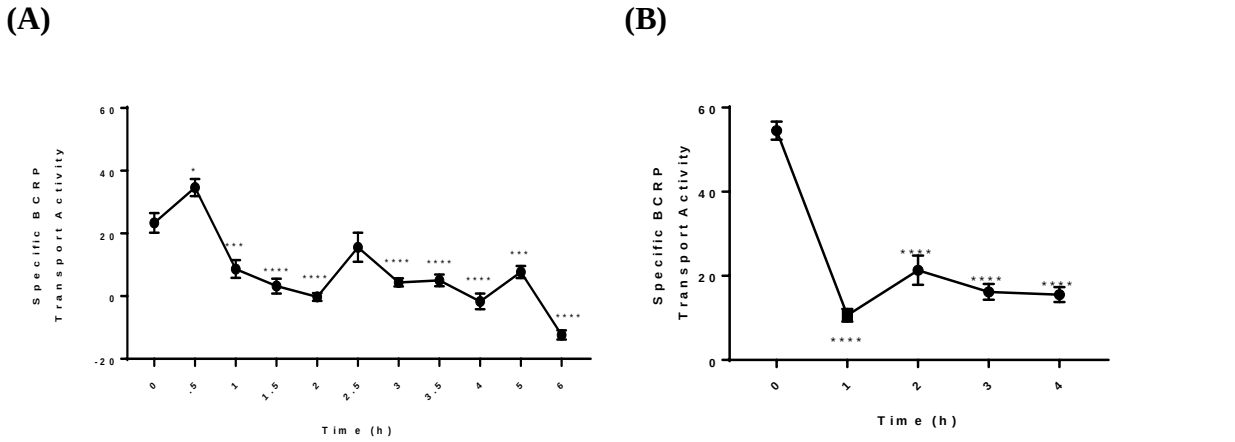

Figure 10. *Specific BCRP transport activity versus duration of treatment GenX (100 nM,* ex vivo). *GenX-mediated decreases in BCRP transport activity are time-dependent in (A) males and (B) females.*

To test the reversibility of GenX-mediated inhibition of BCRP, we exposed the capillaries to a 2-hour *ex vivo* treatment with 100 nM GenX. A treatment of 100 nM of GenX significantly inhibits BCRP transport activity after two hours. Up to 4 hours following removal, BCRP transport activity remains inhibited (**Figure 11**).

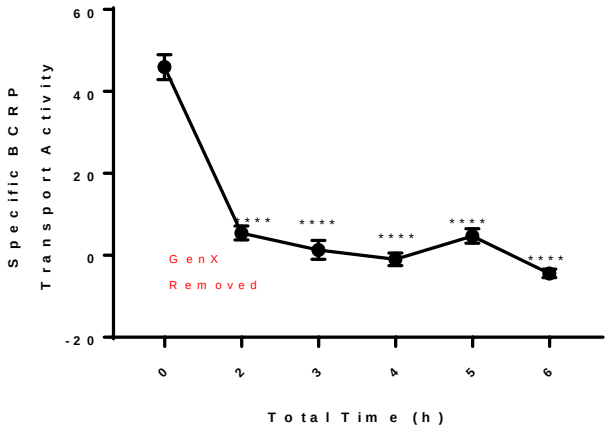

Figure 11. *Specific P-gp transport activity versus time. GenX-mediated decreases in P-gp transport activity are irreversible in males (2h* ex vivo *100 nM treatment).*

We then looked at the effects of an *in vivo* exposure to 0.03 nmol/kg GenX or water vehicle (4 rats/treatment group) in males and 0.3 nmol/kg GenX or water vehicle (4 rats/treatment group) in females. After 3 hours, we removed the brains and performed the transport assay. In males, we observed a significant decrease in P-gp transport activity (**Figure 12**). In females, we saw no change in P-gp transport activity (**Figure 12**).

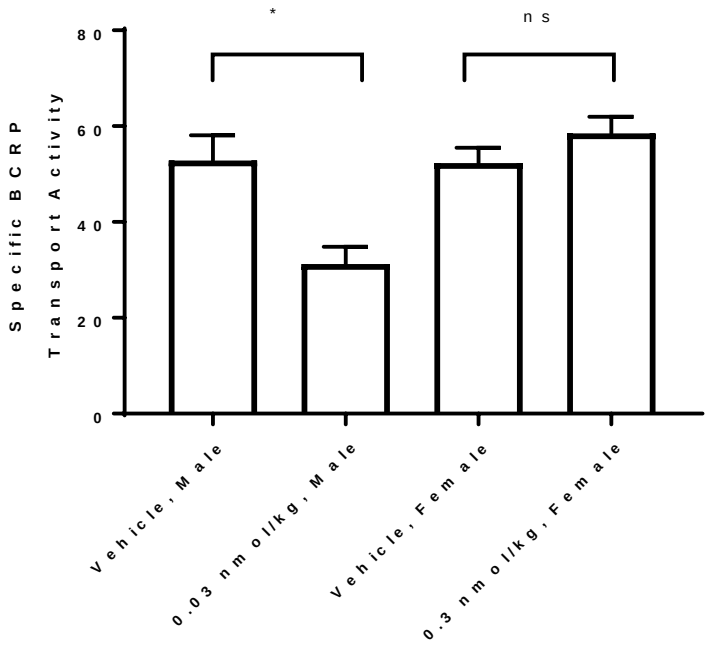

Figure 12. *Specific BCRP transport activity versus concentration of GenX (3h* in vivo *treatment with 0.03 nmol/kg male or 0.3 nmol/kg female). (A) BCRP transport activity decreases in the presence of GenX in males, but (B) does not change in females.*

Then, we decided to investigate the effects of *in vivo* developmental exposure to GenX on pup BCRP transport activity. We observed a significant increase in BCRP transport activity at the high dose female PND4 pups exposed gestationally/lactationally from GD8 to PND4 (**Figure 13**).

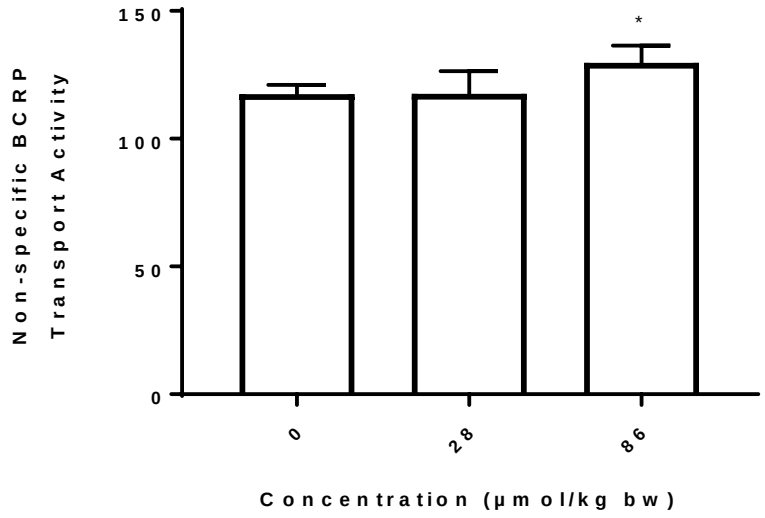

Figure 13. *Specific BCRP transport activity versus concentration of GenX treatment (GD8-PND4,* in vivo*, 28 µmol/kg or 86 µmol/kg). BCRP activity increases in female PND4 pups in the presence of the highest dose of GenX.*

Multidrug Resistance Protein 2 Transport Activity

Next, we investigated the effects of *ex vivo* GenX exposure on MRP2 transport activity. A 3-hour exposure to GenX did not change MRP2 transport activity significantly at concentrations of 1-100 nM in males (**Figure 14A**). However, at the highest concentration of GenX (1000 nM) in males, we observed a significant decrease in MRP2 activity. In females, preliminary data show that a 3-hour exposure to 1 nM decreased MRP2 significantly, but no other concentration caused a significant change (**Figure 14B**).

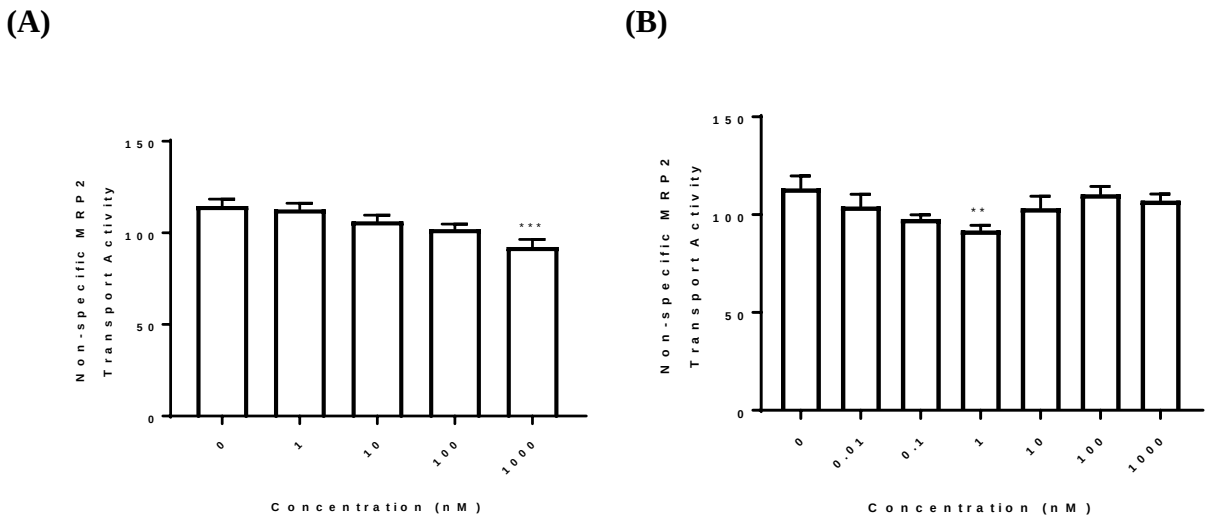

Figure 14. *MRP2 transport activity versus concentration of GenX treatment (3h* ex vivo*). MRP2 transport activity decrease significantly at (A) 1000 nM in males and (B) 1 nM in females.*

Then, we investigated the effects of a single concentration of GenX over a period of 4 hours on MRP2 transport activity. GenX significantly increases MRP2 transport activity following a 4-hour *ex vivo* exposure to 100 nM of GenX in males and females (**Figure 15**).

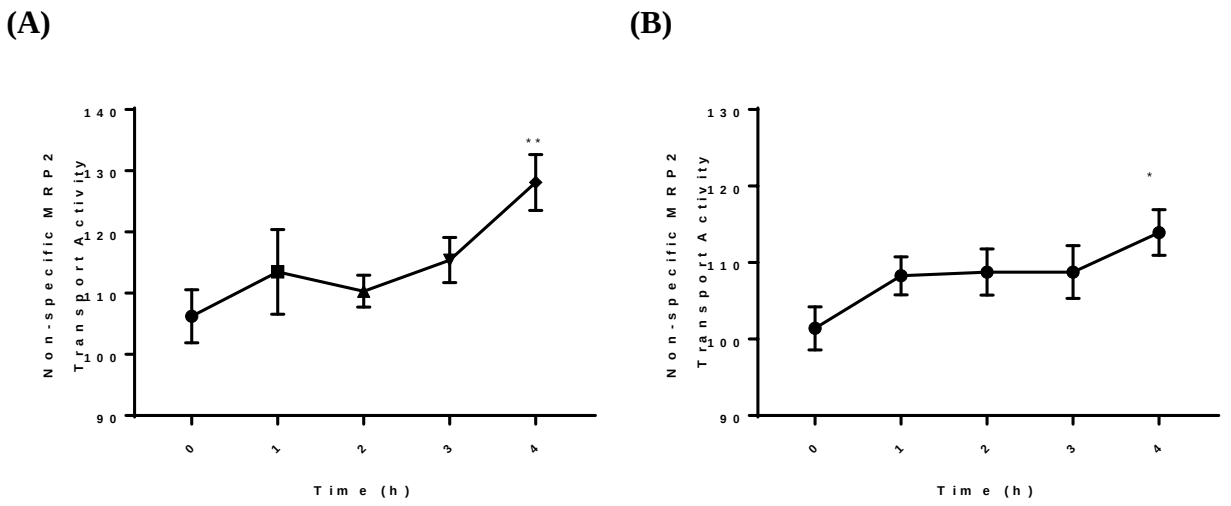

Figure 15. *MRP2 transport activity versus duration of GenX treatment (100 nM,* ex vivo*). MRP2 transport activity increases in (A) males and (B) females after 4 hours.*

Then, we looked at the effects of a 3-hour *in vivo* exposure to GenX in adult males and females. In both sexes, we observed no significant change in MRP2 transport activity (**Figure 16**).

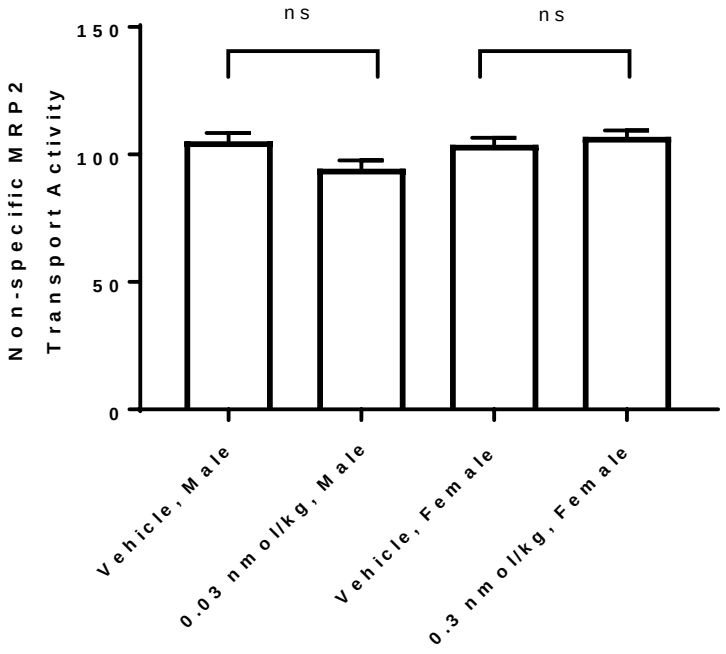

Figure 16. *MRP2 transport activity versus concentration of GenX (3h* in vivo *treatment with 0.03 nmol/kg male or 0.3 nmol/kg female). MRP2 transport activity does not change in the presence of GenX in (A) males or (B) females.*

Finally, we investigated the effects of GenX *in vivo* on MRP2 transport activity in female PND4 pups exposed developmentally. We observed no significant changes at either concentration (0.028 mmol/kg or 0.086 mmol/kg) compared to control (**Figure 17**).

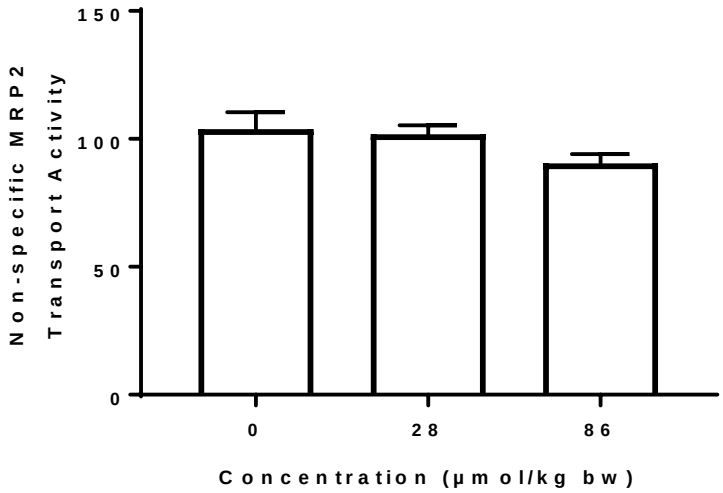

Figure 17. *MRP2 transport activity versus concentration of GenX treatment (GD8-PND4,* in vivo*, 28 µmol/kg or 86 µmol/kg). MRP2 transport activity does not change in female PND4 pups at any dose.*

CHAPTER 4. DISCUSSION

The aim of this study was to investigate the potential effects of GenX on the function and expression of ABC transporters at the blood-brain barrier in Sprague-Dawley rats. GenX is a known global contaminant in drinking water and air (Heydebreck et al., 2015; Sun et al., 2016). It shares structural similarities to PFOA and PFNA, which cause toxicity and carcinogenicity. These two PFAS induce P-gp and BCRP activity and expression at the BBB, likely through PPARα agonism (More et al., 2017). GenX is also a PPARα agonist (Caverly Rae et al., 2015), but is has a shorter carbon chain compared to PFOA and PFNA. No previous study has investigated the toxicity of GenX at low concentrations or its potential for CNS toxicity. We hypothesized that GenX would modulate P-gp and BCRP activity and expression at the blood-brain barrier. Changes in P-gp and BCRP activity and expression have implications for the pharmacokinetics of the substrates of these efflux transporters.

P-glycoprotein is the most well-characterized ABC efflux transporter we studied. It has broad substrate specificity. P-gp is expressed in many tissues, such as brain, liver, and gastrointestinal tract, and it serves a protective function by discharging potentially hazardous chemicals into extracellular space. In our study, we found that environmentally and biologically relevant concentrations of GenX inhibit P-gp transport activity and expression *ex vivo* in rat brain capillaries in both males and females. In male rats, the lowest concentration we tested (1 nM) inhibited P-gp transport activity. In female rats, 10 nM was the lowest concentration we tested that significantly inhibited transport activity, suggesting that female rats are potentially less sensitive to GenX when compared to males.

GenX (100 nM) rapidly inhibits P-gp in male and female rat capillaries *ex vivo* by one hour. For other molecules we studied, rapid inhibition occurred via direct signaling in less than 30 minutes (Cannon, Peart, Hawkins, Campos, & Miller, 2012), but inhibition occurring after approximately one hour coincided with a complex indirect signaling event (Mesev, Miller, & Cannon, 2017). Due to the nonlinear and rapid effects we observed, it is possible that GenX has a dynamic impact on indirect cellular signaling pathways. To understand if the GenX-mediated inhibition is reversible, we administered a 1-hour exposure to 100 nM GenX, then removed the treatment and quantified P-gp transport activity 1, 2, 3, and 4 hours following removal. In males, P-gp transport activity returns to baseline levels when GenX is removed. These data also indicate that a 1-hour exposure to 100 nM GenX does not damage or affect the integrity of the capillary membrane, since activity is able to return to baseline when GenX is removed. These experiments have not been completed in females.

We observed no change in P-gp transport activity following *in vivo* exposure to 0.03 nmol/kg in male rats. We extrapolated this dose based on the maximum concentration in blood reported in Gannon et al. (50,000 ng/mL, 10 mg/kg rat, oral), in order to create a maximum concentration of 100 nM in blood. The process of isolating capillaries following the *in vivo* exposure requires approximately 3 hours. Since our *ex vivo* studies demonstrated that P-gp transport activity is rapidly reversible, and since the half-life of GenX is approximately 3 hours, it is possible that the animals fully eliminated the test compound before euthanasia or that P-gp activity returned to baseline during the capillary isolation process. The kinetics of GenX at lower doses, such as the ones used in our experiments, are unknown. In female rats, we increased the dose tenfold to approximate 1000 nM C_{max} in blood, due to lower sensitivity to GenX *ex vivo* and the less potent *in vivo* response in males. Following a 3-hour exposure to 0.3 nmol/kg GenX,

female rat P-gp transport activity increased significantly. This response is different from what we observed *ex vivo*. We do not know if GenX has a reversible effect in females or how the estrous cycle affects transport activity.

We performed Western Blotting using capillaries treated *ex vivo* to assess the effects of 100 nM GenX on the expression of P-gp. The capillary protein purification methods we used were effective, and we were unable to use β-actin as a loading control since it was not expressed in males. For the males, we used GLUT1, a glucose transporter associated with endothelial cells in the BBB, as a loading control. β-actin was present in females, so we used it as a loading control in females. In both sexes, a 3-hour *ex vivo* treatment of 100 nM GenX significantly decreased P-gp expression. This finding suggests that the decreased P-gp activity could be due to the decreased expression of P-gp.

The second efflux transporter we investigated was Breast Cancer Resistance Protein. This ABC protein is "half-transporter," so it requires two units to function. More than half of pharmaceutical drugs and chemotherapeutics are substrates for BCRP, as well as a variety of xenobiotics and endogenous molecules. In our study, GenX was a potent inhibitor of BCRP. The lowest concentrations tested (1 nM in males, and 0.1 nM in females) decreased BCRP transport activity in rat brain capillaries. The response we observed was rapid (30 minutes) and non-linear in both males and females. The female data have not been repeated, but they show a similar response speed. GenX (100 nM, *ex vivo,* 2h) irreversibly inhibited BCRP transport activity. Since BCRP is dimeric, it is possible that the individual functional units rapidly degrade and require a longer period of time to recover. These experiments have not been performed in females.

In our *in vivo* experiments, we observed that a 3-hour exposure to 0.03 nmol/kg GenX in male rats caused significant inhibition of BCRP transport activity. These effects could be due to the irreversibility or the potency of the effects of GenX on this transporter. In females, a 3-hour exposure to 0.3 nmol/kg GenX caused no change in BCRP transport activity. We do not know whether GenX has an irreversible effect on BCRP activity in females, nor do we know the potential for the estrous cycle to impact BCRP expression or activity.

At 100 nM, preliminary data suggest that a 3-hour exposure to GenX decreases the expression of BCRP in isolated male brain capillaries treated *ex vivo*. These data support our finding that the same concentration of GenX lowers BCRP transport activity. We did not detect BCRP in our female capillaries. The literature indicates that BCRP expression is up to eight times lower than P-gp expression. In addition, 17β-Estradiol, which is present at significantly higher levels in females, inhibits BCRP (Mahringer & Fricker, 2010). So, a higher concentration of protein is likely necessary to detect BCRP in capillaries isolated from female rats in a Western Blotting.

The final ABC transporter we investigated was Multidrug Resistance Protein 2. MRP2 has the smallest active site of the three transporters we studied. In males, 1000 nM GenX (3h, *ex vivo*) and 100 nM GenX (4h, *ex vivo*) significantly decreased MRP2 transport activity. In females, 1 nM GenX (3h, *ex vivo*) slightly decreased MRP2 transport activity, however these experiments have not been repeated and no other concentration showed any change. Since effects were either not repeated, at high concentrations, or after a long incubation with the test compound, it is likely that the effects on MRP2 are due to an abnormal toxic response. Additionally, we saw no change in *ex vivo* MRP2 expression or *in vivo* MRP2 transport activity in males or females. These data suggest that GenX does not degrade the capillary membrane and

that the inhibition of P-gp and BCRP are specific to those transporters rather than a total inhibition of efflux transport.

We then used Adriamycin, a P-gp substrate chemotherapeutic, and a human ovarian cancer cell line containing P-gp (no BCRP or MRP2) to investigate the effects of GenX in human cells. In the absence of GenX, the efflux activity of P-gp protects the cells from Adriamycin toxicity. However when we exposed the cells to 100 nM GenX and increasing concentrations of Adriamycin, we observed increased cytotoxicity. These findings indicate that GenX inhibits human P-gp transport activity *in vitro*, allowing for a higher concentration of Adriamycin within the cell leading to cell death.

Our study was not without limitations. Firstly, this study did not investigate the mechanism underlying the GenX-induced P-gp and BCRP inhibition. Understanding GenX's mechanism of action could allow us to predict which PFAS will cause similar effects on transporter activity and expression at the BBB. We were also limited by the lack of knowledge surrounding the sex differences in transporter expression and the effects of estrous cycle on both activity and expression of efflux transporters. Since the estrous cycle includes known fluctuation of hormones that affect at least BCRP expression, future studies in female animals should track the animals stage in estrous for at least a week prior to the experiment and group animals by their stage. Preliminary data show that GenX significantly increases BCRP activity and decreases P-gp activity in female PND4 pups, whose mothers were dosed with 30 mg/kg GenX from GD8 to PND4. Method development is in progress to create a viable assay to study young animal transporter activity, but these data could indicate that maternal exposure to GenX can affect the BBB of lactating and gestating animals.

The studies presented here are the first to demonstrate the effects of GenX on efflux transport at the Blood-Brain Barrier. At biologically and environmentally relevant doses, GenX inhibits P-gp and BCRP transport activity and expression. P-gp and BCRP are common transporters for many chemicals, and alteration in their activity has the potential to change the pharmacokinetics of these drugs and endogenous molecules. Although GenX itself is unlikely to cause toxicity, the impaired ability of ABC transporters to maintain a protective barrier in the presence of GenX could lead to neurotoxicity. If the effects seen in brain are applicable to other organs where P-gp and BCRP are expressed, then These findings have implications for not only the general population, but also the clinical population. Patients being treated for cancer who are also ingesting water contaminated with GenX could experience increased drug delivery of their chemotherapeutics. Because PFAS are present in mixtures and contaminate the drinking water of people worldwide, more research into the individual substances and potential synergism will be crucial to estimating the risks posed to the public.

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