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Understanding The Effects Of Endocrine Disruptors On The Glucocorticoid Signaling Pathway

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Abstract

Endocrine disruptors are exogenous chemicals that interfere with the functions of the endocrine system and can cause adverse developmental, reproductive, and neurological effects. Unlike toxic effects that may be evident immediately, disruption to the endocrine system may impact complex signaling pathways that take years or generations to discover. Current toxicology testing does not routinely incorporate endpoints that would identify potential endocrine disruptors. The objective of this study was to evaluate the endocrine disrupting potential of a broad range of industrial chemicals, specifically focusing on four chemicals with predicted activity on the glucocorticoid receptor (GR): 4-nonylphenol, bisphenol A (BPA), butylated hydroxytoluene (BHT), and phenolphthalein (PP). These chemicals are used in detergents, paints, pesticides, personal care products, and plastics. We have focused on glucocorticoids because these hormones play a critical role in reproduction, development, metabolism, and overall physiological homeostasis. Their actions are mediated by GR, a transcription factor that is necessary for life. Thus, endocrine disrupting chemicals that alter glucocorticoid signaling have the potential to alter the physiology of several organs and tissues. Human liver (HepG2) and Ishikawa (uterine) cell lines were used to study the effects of these chemicals on metabolism and reproduction, respectively. In Ishikawa and HepG2 cells, the industrial chemicals differentially regulated the transcript levels of both glucocorticoid responsive genes studied (*GILZ* and *PER1).* Interestingly, the effects of industrial chemical on gene expression varied by cell type. Additionally, the industrial chemicals altered the

phosphorylation status of two phosphorylated GR proteins (pGR-211 and pGR-226). A combination of chemicals causes unique effects compared to individual chemicals. Collectively, this suggests that the evaluated chemicals demonstrate gene- and cell-type specific effects. These findings demonstrate that the endocrine disrupting potential of commonly used industrial chemicals is an underappreciated potential source of toxicity.

Keywords: endocrine disruptors, glucocorticoids, industrial chemicals, toxicity, bisphenol A, butylated hydroxytoluene, phenolphthalein, 4-nonylphenol, chronic disease epidemiology

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1. Introduction

Endocrine disruptors are chemicals that disrupt the normal functioning of the endocrine system which can result in changes to hormone signaling and bodily functions [1]. While endocrine disruptors don't necessarily cause cell necrosis or immediate toxicity, they have been shown to have demonstrated effects on gene signaling and protein expression in low concentrations in animal models [2]. Endocrine disruptors can exert effects on hormone pathways in the body in various ways, including competing with endogenous hormones for substrate active sites, changing the rate of production and metabolism of endogenous hormones, altering the levels of co-activators, and changing the epigenetic makeup of cells [3]. Much of the research on endocrine disruptors has focused on industrial chemicals involved in plastic production as well as petroleum manufacturing [4, 5]. The annual global production of plastics and petroleum has increased from 50 million to 300 million since the 1970s, and the chemicals involved in these processes have been found to be endocrine-disrupting chemicals [6].

The potential effects of endocrine disruptors on the reproductive system are particularly important as endocrine disruptors can interrupt the delicate hormone signaling that is crucial to a variety of reproductive processes, including successfully reaching puberty in both males and females, creating a uterine environment receptable to embryo implantation, and carrying a pregnancy to term [7]. Reproductive homeostasis depends on tightly regulated interactions between organs, timing, stage of development, and hormone concentrations, and aberrant behavior due to exogenous chemicals can cause adverse downstream signaling and a variety of homeostatic and reproductive consequences [8].

Studies have documented that BPA and PBDEs, which are commonly used to increase the flexibility of plastic and vinyl, exert adverse changes on the neuroendocrine pathways fundamental to reproductive health [9, 10]. There is also evidence that endocrine disruptors induce aberrant changes to germ cells [11]. A significant body of research has been devoted to exploring the effects of BPA, a chemical found in plastics polycarbonate plastics and epoxy resins and often used in containers that store food and beverages. BPA exposure during a woman's reproductive years has been shown to compromise embryo implantation [12]. In Denmark, women under 40 working in the plastics industry were more likely to have sought fertility assistance than unexposed women of the same age [13].

The role of industrial chemicals, which are found ubiquitously and are a source of both environmental and occupational exposure, has been vastly understudied. Recent studies have shown the effects of industrial chemicals on the estrogen receptor, showing that industrial chemicals can act as endocrine disruptors and have the potential to alter reproductive signaling in uterine tissue [14]. Glucocorticoids, which are steroid hormones present in nearly every cell type and involved in many inflammatory processes, including several reproductive pathways, play a critical role in reproduction, development, metabolism, and overall physiological homeostasis [15]. Therefore, it is important to understand the potential effects that endocrine disruptors can have on glucocorticoid signaling at both the gene and protein levels of expression. This research will allow individuals to infer (from their level of exposure) the potential effects of these industrial chemicals on their metabolism and may provide women of reproductive age information related to the effects of exposure on their fertility. The specific effects of industrial chemicals on reproductive health and metabolism is a topic that has been

understudied in endocrine research. Thus, this project would lend invaluable information to the field of endocrinology and to the healthcare of those exposed to these environmental agents.

This project studied chemicals with reported glucocorticoid receptor activity determined by a variety of reporter assays (receptor binding, agonist/antagonist activity, response element binding) as documented in the ToxCast dashboard. Studies were performed with the following chemicals: 4-nonylphenol (4-NP), bisphenol A (BPA), butylated hydroxytoluene (BHT), and phenolphthalein (PP). Ishikawa (uterine) cells were utilized to examine the effects of endocrine disruptors on glucocorticoid signaling and reproductive. HepG2 (liver) cells were utilized to examine the effects of endocrine disruptors on glucocorticoid and metabolism. Both Ishikawa and HepG2 cells have demonstrated levels of glucocorticoid signaling that mediate inflammatory processes including reproductive function and metabolism.

2. Materials and Methods

2.1 Literature Search

A list of 139 chemicals with reproductive health hazard properties used in the industrial chemical industry was developed based on several regulatory and advisory lists, including the ToxCast database, Malaysia's Department of Occupational Safety and Health's Industry Code of Practice (ICOP), Germany's Substance Database (GESTIS), Japan's National Institute of Technology and Evaluation (NITE), and the European Chemicals Agency (ECHA). The reproductive classification was based on the Globally Harmonized System (GHS) for classification and labeling. We conducted a literature review in PubMed using the web browser Google Chrome and following query: “[chemical name] and ‘endocrine disruptor’” or “[CAS #] and ‘endocrine disruptor’”, notating the total number of published studies. A relevant hit was

defined as a published study that directly investigated the endocrine disrupting effects of the given chemical rather than include the chemical as part of the experimental design. The ToxCast dashboard, which covers 1000 high-throughput endpoints for over 9000 chemicals, was searched for active nuclear receptor endpoints, focusing on nuclear receptors with known functions in the reproductive system through the glucocorticoid receptor (GR). Chemicals with reported glucocorticoid receptor activity determined by a variety of reporter assays (receptor binding, agonist/antagonist activity, response element binding) as documented in the ToxCast database and demonstrating limited data related to their EDC effects were prioritized for *in vitro* experiments. Studies were performed with the following chemicals: 4-nonylphenol (4-NP), bisphenol A (BPA), butylated hydroxytoluene (BHT), and phenolphthalein (PP).

2.2 Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 Medium was purchased from Life Technologies Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO). Charcoal dextran-treated (stripped) FBS was purchased from Gemini Bio-Products (Sacramento, CA). TaqMan qRT-PCR primer-probes were purchased from Applied Biosystems (ThermoFisher Scientific, Waltham, MA). Dexamethasone (Dex; 1, 4-pregnadien-9α-fluoro-16α-methyl-11β, 17, 21-triol-3, 20-dione; ≥98% TLC), cortisol (Cort; 4-pregnen-11β, 17, 21-triol-3, 20-dione), and mifepristone (RU-486; 11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-1; 97% by TLC) were purchased from Steraloids (Newport, RI). Betamethasone (Beta; 9α-Fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione; 98.6% by HPLC) was purchased from MP Biomedical (Solon, OH). Butylated hydroxytoluence (2,6-di-tert-butyl-4-methylphenol) and

bisphenol A was purchased from Sigma Aldrich (St Louis, MO). Phenolphthalein was purchased from Fisher Scientific (Hampton, NH). 4-n-nonylphenol was purchased from Alfa Aesar (Haverhill, MA).

2.3 Cell culture

HepG2 cells were obtained from the Yale School of Medicine Department of Obstetrics, Gynecology, and Reproductive Sciences. Short tandem repeat analysis was conducted on HepG2 cells at the DNA Analysis Facility at Yale University to authenticate the cell line. Immortalized human uterine endometrial adenocarcinoma (Ishikawa) cells were obtained from ATCC (Manassas, VA). Both Ishikawa and HepG2 cell lines were grown in standard conditions with 5% carbon dioxide. Immortalized human uterine endometrial adenocarcinoma (Ishikawa) cells were maintained in RPMI 1640 supplemented with 5% FBS. Twenty-four hours prior to treatment, cell medium was changed to phenol red-free RPMI 1640 containing 5% stripped FBS. HepG2 cells were grown in DMEM supplemented with 10% FBS, 1.0 mM sodium pyruvate, 1 mM glutamine, and 100 U/mL penicillin/streptomycin. Twenty-four hours prior to experiments, cell medium was changed to phenol red-free DMEM containing 10% FBS, 1.0 mM sodium pyruvate, 1mM glutamine, 100 U/mL penicillin/streptomycin. Cells were treated with chemicals over a range of doses below the reported limit of toxicity.

2.4 RNA extraction

Total RNA was harvested from the Ishikawa and HepG2 cells using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA quantity and purity was assessed using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific) based on the absorbance ratios at 260 and 280 nmol/L and at 260 and 230 nmol/L.

2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA abundance was determined using a TaqMan One-Step procedure on the CFX Connect Real-Time System (Bio-Rad Laboratories, Herculues, CA) with predesigned TaqMan assays (Thermo Fisher Scientific). Quantitative RT-PCR was performed in a 10 µL reaction volume with the following thermocycling parameters: 48°C for 30 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. A standard curve was used to calculate expression values for each gene. The signal from each probe was normalized to the reference gene peptidylprolyl-isomerase B (*PPIB*). A total of 100 ng of total RNA was used as input. There were at least four biological replicates per treatment group and each gene primer probe was evaluated with a technical duplicate for each sample.

2.6 Protein Isolation and Western blotting

Tris-glycine SDS sample buffer supplemented with 2-mercaptoethanol was used to lyse cells. Total protein was quantified using the Pierce 660 nm protein assay kit (Thermo Fisher Scientific). 40 ng of protein from each sample was run on a stain-free, precast gel, separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 7.5% skim milk in Tris-buffered saline and probed overnight with primary antibodies against phosphorylated GR-211, phosphorylated GR-226, GR, and β-actin. The next day, membranes were washed with 0.1% Tween-20 in TBS and incubated with secondary antibody for 1 hour at room temperature. Immunoreactivity was visualized using the Odyssey LI-COR imaging system (LI-COR Biosciences, Lincoln, NE). Protein levels were normalized to β-actin expressed relative to control samples.

2.7 LDH Assay

Cells were treated with 1 µM chemical for 6 hours. Then, cell media was collected, plated on a 96-well plate, and assayed using an absorbance of 490 nm. Lactate acid dehydrogenase (LDH) release was measured using the Roche Cytotoxicity Detection Kit (Quebec, Canada) according to the manufacturer's instructions.

2.8 Bioinformatic Analysis

QIAGEN Ingenuity Pathway Analysis (Build 463,341 M, Version 42,012,434, Qiagen) was used to analyze datasets from NCBI GEO in order to extract overlapping genes and map them to canonical functional pathways. The p-value of the overlap was calculated by the right-tail Fisher's Exact Test. The relationship between chemical-associated genes and enriched diseases and functions was visualized using Cytoscape, an open-source software platform for integration, analysis, and visualization of networked data.

2.9 Statistical analysis

Data represent the average of at least three biological replicates and are presented as means ± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post-hoc analysis using Graph Pad Prism software version 7.0. Significance was determined as $*p < 0.05$ or $**p < 0.01$.

3. Results

3.1 Chemical Classification and Literature Review

To evaluate the potential impact to human reproduction and development, 139 chemicals used in petroleum manufacturing were examined via the ToxCast database according to their reproductive toxicity. The ToxCast database revealed that 40 of the 139 chemicals altered the activity of the nuclear receptors in reporter assays. 9 of the 40 chemicals altered the

activity of GR. We performed an additional literature search in PubMed for the 9 chemicals identified to alter GR activity and the term “glucocorticoid receptor.” We identified 4 chemicals that had limited published data related to their endocrine disrupting potential and glucocorticoid receptor activity and selected these chemicals for *in vitro* experimentation. The four chemicals chosen for experimentation were 4-nonylphenol (4-NP), bisphenol A (BPA), butylated hydroxytoluene (BHT), and phenolphthalein (PP).

In addition to their use in petroleum manufacturing, these four chemicals are found in a variety of products used across industries, including food additives, personal care products, oil and gas production, plastic manufacturing, dry cleaning reagents, and other manufacturing processes [14]. The chemical and CAS number, exposure source, exposure route, and exposure limit, molecular weight, and molecular structure were determined for each chemical (Fig. 1). The routes of exposure include inhalation, ingestion, and skin or eye contact. The 8-hour permissible exposure limit with time-weighted average as determined by the National Institute for Occupational Safety and Health (NIOSH) and the State of California's Occupational Safety and Health Administration are listed. NIOSH exposure limits were converted to molarity and are listed in Figure 1.

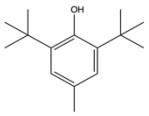
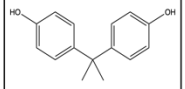
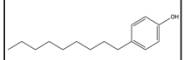
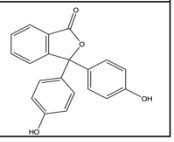
Chemical and CAS #	Exposure Source	Exposure Route	Exposure Limit	Molecular Weight (g/mol)	Molecular Structure
2,6-di-tert -butyl-p-cresol (Butylated hydroxytoluene) CAS #128-37-0	Used in food, cosmetics, pharmaceuticals, industrials, jet fuels, rubber, petroleum products, electrical transformer oil, embalming fluid, found in soft-necked garlics	Absorbed into the body by inhalation of its aerosol and by ingestion, ingestion, skin and/or eye contact	10 mg/c m	220.356	
4,4'-isopropylidenediphenol (Bisphenol A) CAS #80-05-7	Used as adhesives, sealants, flame retardants, paint additives, food packaging, ink, toner, toys, sporting equipment	Absorbed into the body by inhalation of its aerosol or ingestion	250 mg (skin contact)	228.291	
4-nonylphenol CAS #104-40-5	Pollutant from degradation of sewage surfactants, surfactant in cleaning and cosmetic products, spermicide in contraceptives	Inhalation, ingestion, skin/eye contact	0-0.5 mg/kg body weight	220.356	
Phenolphthalein CAS #77-09-8	Used medicinally as a cathartic, as a laboratory reagent and pH indicator, as a laxative,	Inhalation and dermal contact	N/A	318.328	

Figure 1. Description of the selected chemicals: 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene) (BHT), 4,4'-isopropylidenediphenol (bisphenol A) (BPA), 4-nonylphenol (4-NP), and phenolphthalein (PP), including the CAS number, description of the sources, routes, and limits of. Exposure limits are determined as the 8-hour permissible exposure limit with time-weighted average.

3.2 Baseline glucocorticoid activity in Ishikawa and HepG2 cells

Initial *in vitro* experimentation involved confirming glucocorticoid activity in both cell lines used in this study. *GILZ* and *PER1* are genes activated by the glucocorticoid receptor. Demonstrated *GILZ* and *PER1* activity confirms literature documentation of strong GR activity in these cell lines (Figure 2) [16, 17].

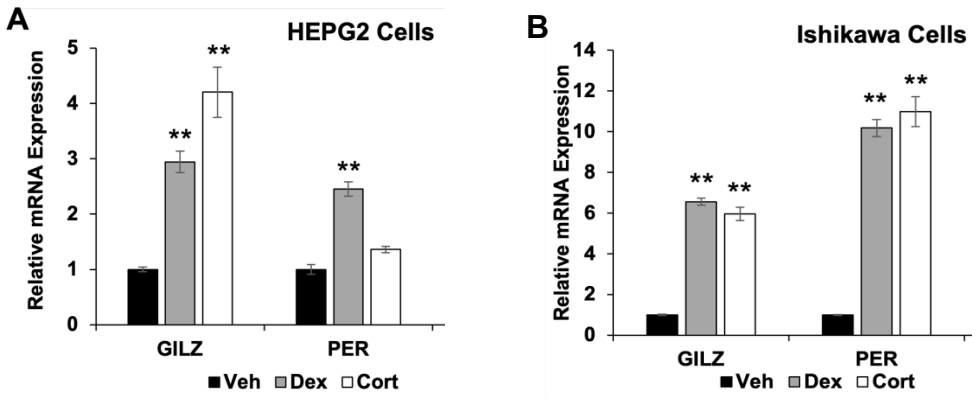


Figure 2. Demonstrated GR activity in HepG2 and Ishikawa cell lines treated with vehicle, dexamethasone, or cortisol and the expression of GR-responsive genes *GILZ* and *PER1*. A) HepG2 cell line GR activity. B) Ishikawa cell line GR activity.

3.3 LDH activity of selected chemicals

LDH assay was used to assess whether treatment of the chemicals induced cell death/cytotoxicity as a function of plasma membrane damage. Cells were treated with chemicals at 1 µM concentrations, and LDH activity after 6 hours was measured. None of the chemicals demonstrated cytotoxic effects in either HepG2 or Ishikawa cell lines.

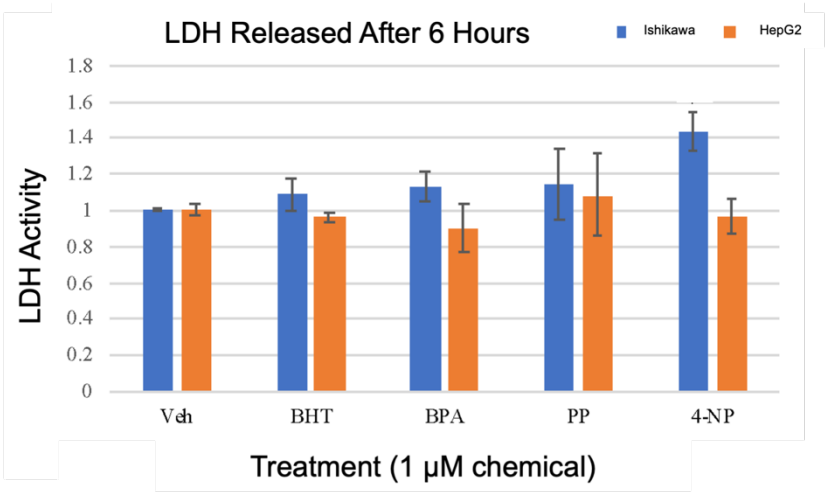


Figure 3. LDH activity from HepG2 and Ishikawa cell lines after being treated with 1 µM chemical for 6 hours.

3.4 *In vitro* exposure of selected chemicals in isolation and expression of endogenous

glucocorticoid-responsive genes

Each cell line was treated with 10, 100, or 1000 nM of each of the four selected chemicals for 6 hours (Figure 4). Gene expression of GR-responsive genes *GILZ* and *PER1* were measured. Treatments of 1000 nM BHT, 1000 nM 4-NP, 100 nM BPA, and 1000 nM BPA induced statistically significant upregulation of both *GILZ* and *PER1* in HepG2 cells. Treatment of 1000 nM PP induced statistically significant downregulation of both genes in HepG2 cells. The results differ by cell type. No significant change in gene expression was observed from BHT treatments in Ishikawa cells. Treatments of 1000 nM BPA, all concentrations of 4-NP, 100 nM PP, and 1000 nM PP were found to significantly upregulate *GILZ* and *PER1* expression in Ishikawa cells (Figure 4).

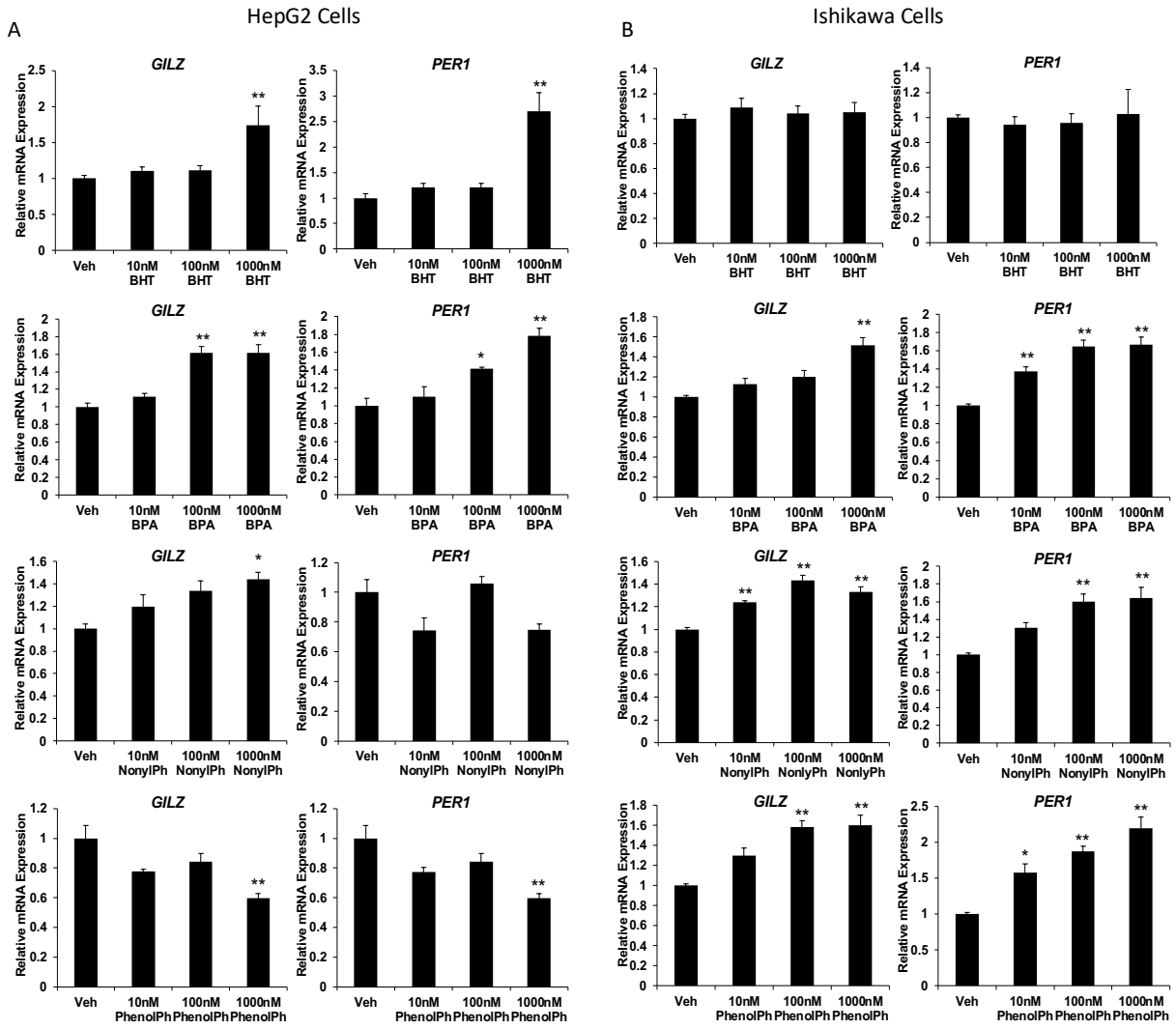


Figure 4. Gene expression data of *GILZ* and *PER1* genes from dose-response treatments of 10-1000 nM. A) HepG2 cells. B) Ishikawa cells.

3.5 *In vitro* exposure of select chemicals in combination and expression of endogenous glucocorticoid-responsive genes in HepG2 cells

Based on the results from the treatments with chemicals in isolation, the effects of chemicals in combination were examined through various dose response treatments. Chemical combinations were made based on results from Figure 4 to produce effects that would

theoretically supersede the effects of the chemical in isolation. In Figure 4A, we demonstrate that 10 nM of BPA or 4-NP alone doesn't significantly upregulate *GILZ* expression, but together they do. For the same treatment, there is no significant upregulation of *PER1*. Co-treatment of 4-NP and BHT also produces significant upregulation of *GILZ* expression that does not occur when the chemicals are treated in isolation. (Figure 5B). This combination does not produce significant upregulation of *PER1*. Treatment with 100 nM BPA alone induces significant upregulation of both *GILZ and PER1*, and co-treatment of BPA and BHT produces significant upregulation of both *GILZ and PER1* (Figure 5C). The results of the co-treatment of chemicals in HepG2 cells demonstrates that the effects of the chemicals differ by gene.

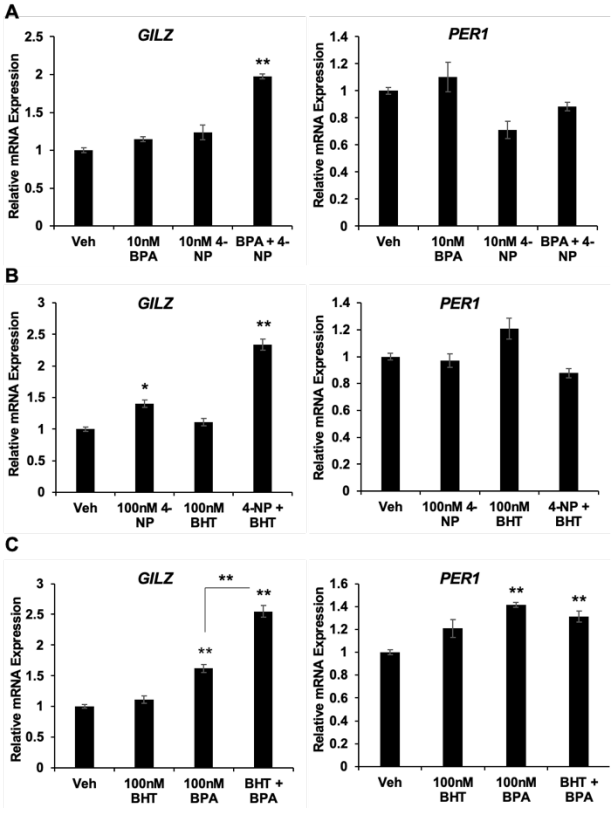


Figure 5. Gene expression data of *GILZ* and *PER1* genes from 10 nM chemical combination treatments in HepG2 cells. A) Gene expression from treatments of 10 nM BPA and 4-NP in isolation and combined for 6 hours. B) Gene expression from treatments of 100 nM 4-NP and BHT in isolation and combined for 6 hours. C) Gene expression from treatments of 100 nM BHT and BPA in isolation and combined for 6 hours.

3.6 *In vitro* exposure of select chemicals in combination and expression of endogenous glucocorticoid-responsive genes in Ishikawa cells

Based on the results from the treatments with chemicals in isolation (Figure 4), the effects of chemicals in combination were examined through various dose response treatments. Chemical combinations were made based on results from Figure 4 to produce effects that would supersede the effects of the chemical in isolation. In Figure 6A, co-treatment of 10 nM BPA and 10 nM 4-NP leads to downregulation of *GILZ* expression compared to the upregulation observed during treatment of 10 nM 4-NP alone. This co-treatment leads to significant upregulation of *PER1* expression. Co-treatment of 10 nM BPA and 10 nM PP leads to significant upregulation of both *GILZ* and *PER1* expression (Figure 6B). In Figure 6C, co-treatment of 10 nM BHT and 10 nM PP leads to downregulation of *GILZ* expression compared to the upregulation observed during treatment of 10 nM PP alone. This co-treatment leads to significant upregulation of *PER1* expression. Co-treatment of 10 nM 4-NP and 10 nM PP leads to significant upregulation of both *GILZ* and *PER1* expression (Figure 6D).

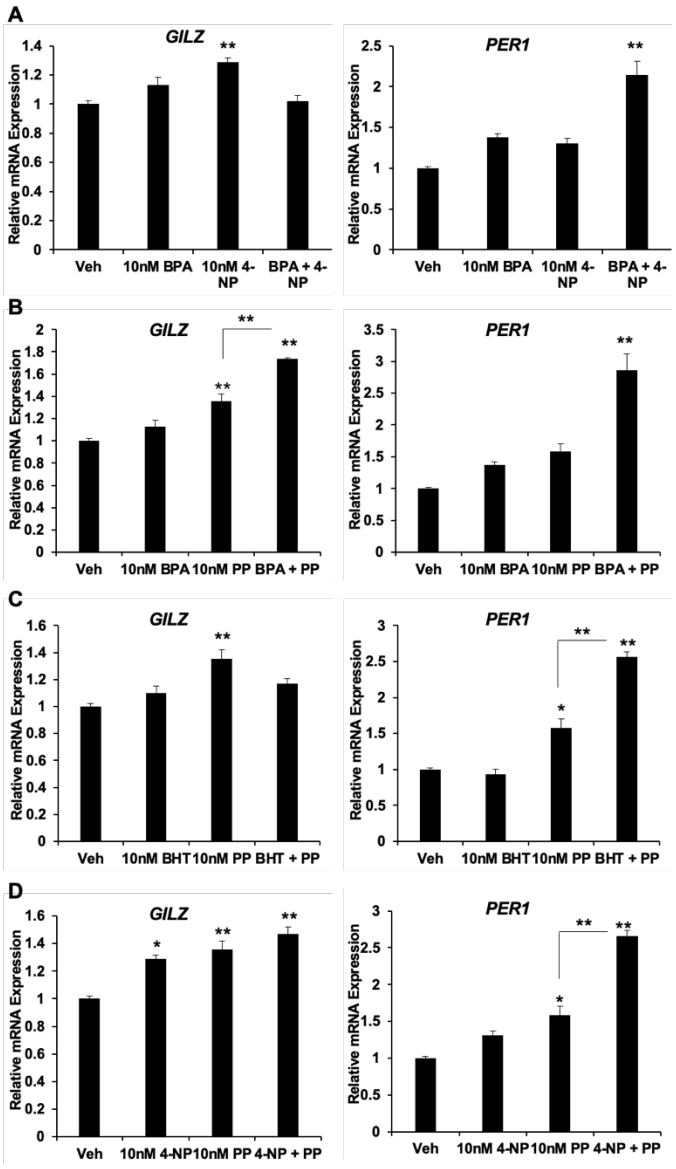


Figure 6. Gene expression data of *GILZ* and *PER1* genes from dose-response treatments of 10 nM chemical combinations in Ishikawa cells. A) Gene expression from treatments of 10 nM BPA and 4-NP in isolation and combined for 6 hours. B) Gene expression from treatments of 10 nM BPA and PP in isolation and combined for 6 hours. C) Gene expression from treatments of 10 nM BHT and PP in isolation and combined for 6 hours. D) Gene expression from treatments of 4-NP nM BHT and PP in isolation and combined for 6 hours.

3.7 *In vitro* exposure of artificial corticosteroid dexamethasone in combination with selected chemicals and expression of endogenous glucocorticoid-responsive genes in HepG2 and Ishikawa cells

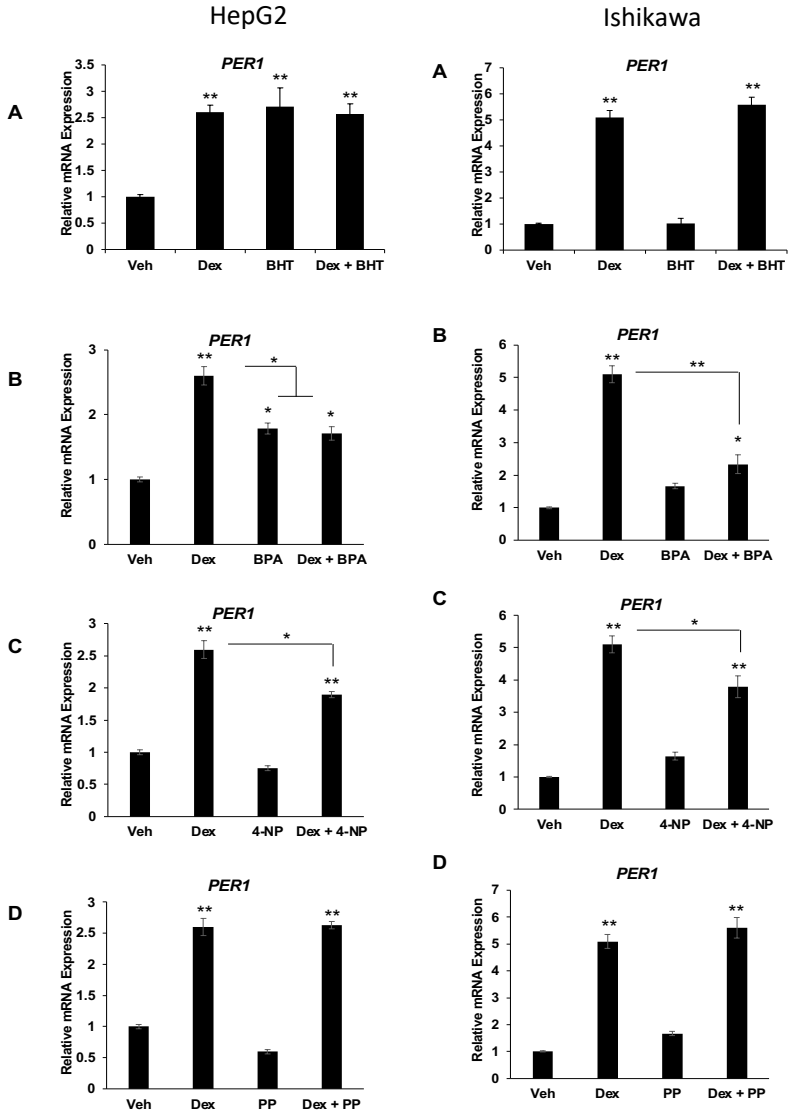


Figure 7. Gene expression data of *GILZ* and *PER1* genes from treatments of chemical and dexamethasone in isolation and then in combination (30-minute treatment of chemical and 6-hour treatment of dexamethasone) (HepG2 – left, Ishikawa – right). A) Gene expression from treatments of 10 nM BHT and dexamethasone in isolation and combined for 6 hours. B) Gene expression from treatments of 10 nM BPA and dexamethasone in isolation and combined for 6 hours. C) Gene expression from treatments of 10 nM 4-NP and dexamethasone in isolation and combined for 6 hours. D) Gene expression from treatments of 10 nM PP and dexamethasone in isolation and combined for 6 hours.

BHT did not attenuate the upregulatory effects of dexamethasone on *GILZ* and *PER1* expression in both HepG2 and Ishikawa cells (Figure 7A). BPA significantly downregulated the effects of dexamethasone on both *GILZ* and *PER1* expression in both HepG2 and Ishikawa cells (Figure 7B). 4-NP significantly downregulated the effects of dexamethasone on both *GILZ* and

PER1 expression in both HepG2 and Ishikawa cells (Figure 7C). PP did not attenuate the upregulatory effects of dexamethasone on *GILZ* and *PER1* expression in both HepG2 and Ishikawa cells (Figure 7D).

3.8 Protein expression from cells treated with selected chemicals and dexamethasone

Cells treated with 100 nM of chemical were probed for pGR-226 through Western blot. Dex, BHT, PP, and 4-NP significantly upregulated pGR-226 expression in both HepG2 and Ishikawa cells.

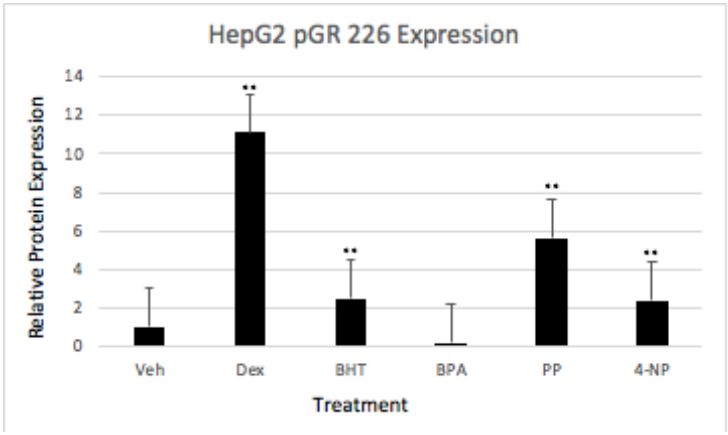


Figure 8. HepG2 cells treated with 100 nM of dexamethasone, BHT, BPA, PP, and 4-NP and probed for pGR-226 through Western blot.

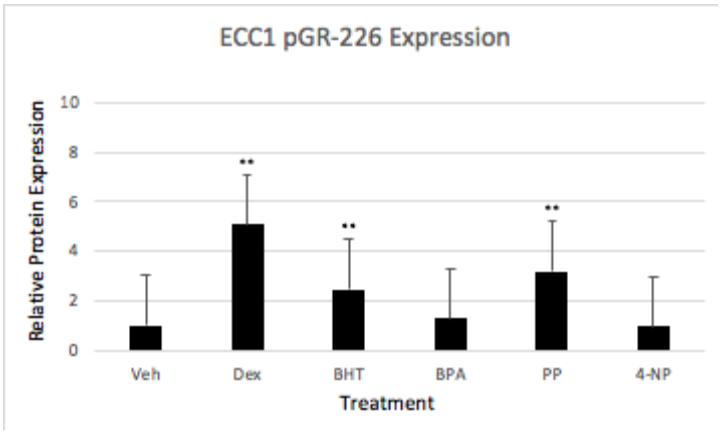


Figure 9. Ishikawa cells treated with 100 nM of dexamethasone, BHT, BPA, PP, and 4-NP and probed for pGR-226 through Western blot.

3.9 Bioinformatic gene networks regulated by selected chemicals in animal models

Ingenuity Pathway Analysis (IPA) was used to analyze data from rat liver cells treated with dexamethasone, BPA, 4-NP, and PP. IPA highlighted genes that were significantly

upregulated (green) or downregulated (red) according to treatments with the chemical (Figure 8).

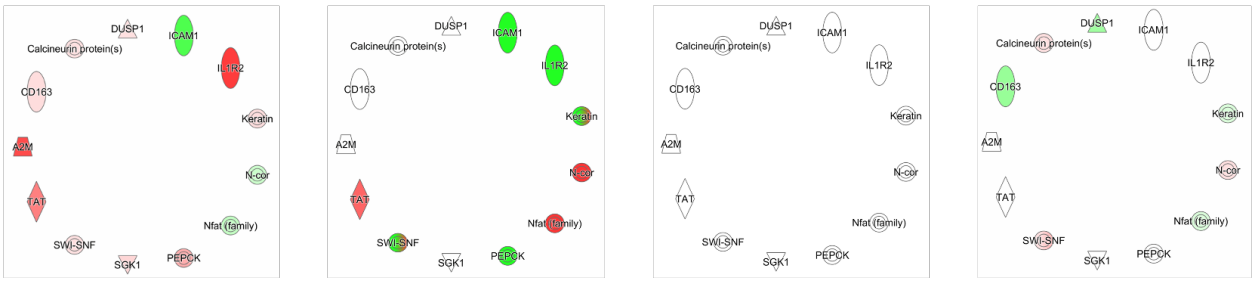


Figure 10. Genes regulated by chemical in rat liver cells. Genes regulated by dexamethasone, BPA, 4-NP, and PP were identified through the NCBI GEO database and analyzed through the Ingenuity Pathway Analysis (IPA) software. Genes upregulated are marked in green and genes downregulated are marked in red.

IPA was also used to generate heat maps linking the four chemical treatments to canonical pathways and molecular and cellular functions associated with the glucocorticoid receptor and related pathways (Figure 9). The chemicals have differential regulation on the pathways and molecular and cellular functions as seen by the range of activation z-scores. PP strongly downregulates calcium signaling while 4-NP has a higher activation score. Dexamethasone has a weak downregulatory activity effect while BPA has a weak upregulatory activity effect. Dexamethasone and BPA do not cause activity changes for Huntington's disease signaling while both dexamethasone and PP have high activity z-scores for that same pathway.

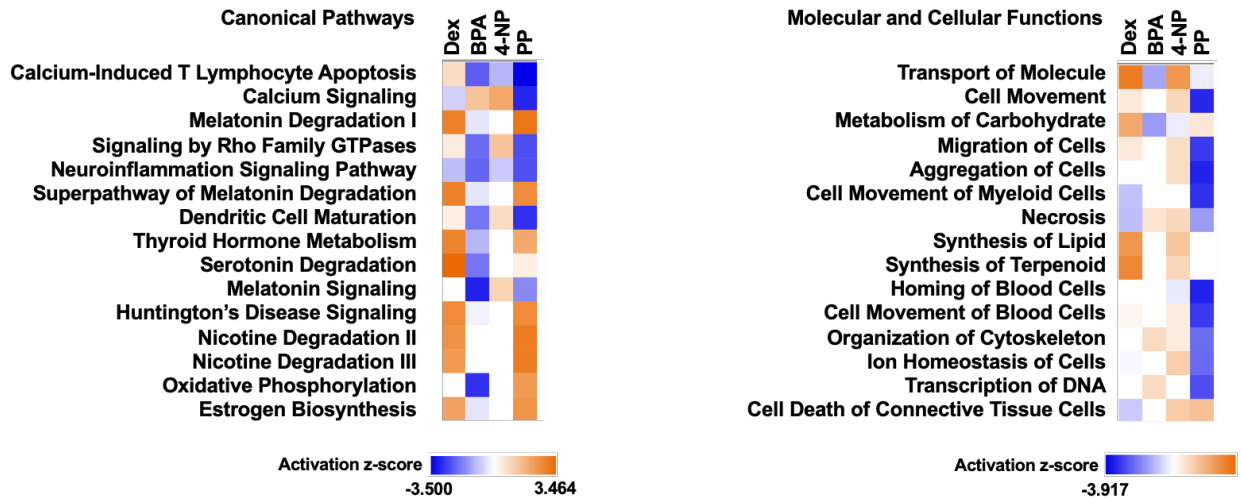


Figure 11. Rat liver gene expression data. Darker blue indicates downregulatory activity (inhibition) while darker orange indicates upregulatory activity (activation).

Discussion

Glucocorticoid activity was demonstrated in both Ishikawa and HepG2 cells in Figure 1, allowing our candidate genes of *GILZ* and *PER1* to be appropriate choices for measuring GR activity (Figure 2). LDH assays showed no cytotoxic effects of the selected chemicals on either HepG2 or Ishikawa cell lines, indicating that the endocrine disrupting effect is not immediately toxic (Figure 3).

While the selected chemicals don't display toxic effects, they do play a role in regulating GR-mediated gene expression levels in both cell lines (Figures 1-7). In HepG2 cells, BPA and BHT upregulate expression of both *GILZ* and *PER1* while PP downregulates expression of both genes (Figure 4). In HepG2 cells, 4-NP upregulates *GILZ* expression while not influencing *PER1* gene levels (Figure 4). Additionally, treatment of cells with multiple chemicals allows for upregulation of gene expression that cannot be achieved with treatment of one chemical alone. Co-treatment of 4-NP and BHT in HepG2 cells produces significant upregulation of *GILZ* expression

that does not occur when the chemicals are treated in isolation. (Figure 5B). Co-treatment of BPA and PP in Ishikawa cells produces significant upregulation of *GILZ* expression that only occurs when BPA is treated in isolation but not when PP is treated in isolation (Figure 6B). This demonstrates the ability for chemicals in combination to have greater effects than chemicals alone. In petroleum manufacturing and products that contain these endocrine disruptors, it is very common for multiple chemicals to be present.

Not only do the gene expression effects differ by chemical, but these results were also found to differ by cell type. BHT treatments in HepG2 cells upregulate *GILZ* and *PER1* expression while not having an effect in Ishikawa cells (Figure 4). We see similar patterns for other treatment types in which gene regulation in one cell type is either not present or attenuated in the other cell type (Figures 5, 6). This suggests differences in the GR binding complexes between liver and uterine cells which causes the chemicals to interact with GR differently.

It has been well documented that gene expression is fine-tuned to meet the needs of a cell [18]. The glucocorticoid receptor regulates target genes by associating with specific DNA binding sites, the sequences of which differ between genes [19]. These binding sites have been conventionally viewed only as docking sites, but recent work using structural, biochemical, and cell-based assays has shown that GR binding sequences, differing by as little as a single base pair, differentially affect GR conformation and regulatory activity [20].

Perhaps the treatment of the industrial chemicals alters the binding properties of GR which leads to functional differences in gene expression. GR utilizes hormones as allosteric effectors of their transcriptional regulatory activity, and additional inputs, such as phosphorylation, also affect GR function [20, 21]. Thus, the binding of selected chemicals from

competitive, uncompetitive, or noncompetitive binding to GR could alter the allosteric effects of GR and its ability to translocate to the nucleus [22]. Upon translocation, the chemically modified GR could differentially bind to DNA sequences in the nucleus which would cause differential activation of transcription factors which would ultimately lead to changes in gene expression between cell types.

Differences in the phosphorylation of the GR binding complexes between the Ishikawa (uterine) and HepG2 (liver) cells can also account for the difference in gene expression between the two cell lines. GR can be phosphorylated to different levels in each cell type. The pGR-226 protein levels were upregulated through treatment with 100 nM of dexamethasone, BHT, BPA, PP, and 4-NP in both HepG2 and Ishikawa cells (Figures 8, 9). [23]. The phosphorylation levels of pGR-211 have yet to be quantified and may be different from the levels of pGR-226 measured. Thus, GR may be more susceptible to conformational changes and phosphorylation from allosteric binding from chemicals in one cell type over another. Chemicals can thus act as partial agonists or antagonists and either interfere or upregulate naturally occurring GR signaling.

The chemicals could also be altering the epigenetic landscape of GR through conformational changes of the receptor. We have shown that treatment of cells with artificial corticosteroid dexamethasone as well as the selected chemicals can either interrupt, enhance, or antagonize the normal effects of dexamethasone (Figure 7). This suggests that not only can the chemicals bind to GR, but they can also compete with other hormones in various ways to cause downstream gene effects. This demonstrates the allosteric nature of GR in which multiple ligands can bind before the receptor translocates to the nucleus and binds to transcription factors [24].

Conclusion

Pending experiments in this project include synthesizing western blot data from to quantify fold changes in protein expression of phosphorylated GR-211 and evaluating the effects of co-treatment of the selected chemicals with RU486 (mifepristone). The antibodies targeted for the Western blot are two phosphorylated forms of GR, pGR-211 and pGR-226, which are two serine residues that are commonly phosphorylated before translocation of the receptor complex to the nucleus. Understanding changes in protein expression after treatment with the selected chemicals will allow us to see whether gene expression changes translate to changes at the protein level. The Western blots have been completed; quantification of pGR-211 remains.

Co-treatment of cells with the selected chemicals and RU486 is another pending experiment which will be critical to examining whether the treatments interfere or aid with the natural inhibition caused by mifepristone. RU486 is a known glucocorticoid receptor antagonist that interferes with steroid-mediated inflammatory activity [25]. The interaction of the selected chemicals with RU486 could either attenuate its inhibitory properties, increase the antagonist behavior, or have some other behavior. This experiment will further elucidate the interactions of the selected chemicals with other hormones in the body.

In summary, the four industrial chemicals selected altered the gene expression of GR-mediated elements in human uterine and liver cells. We have demonstrated that these chemicals exert gene-level changes that differ depending on cell type. Additionally, we have shown that chemicals in combination can exert greater gene effects than chemicals in isolation. Finally, our bioinformatics work has allowed for an expanded view of the various pathways and

molecular/cellular functions involved in these cell lines and the proteins involved in the shifted signaling because of the selected chemicals. As these chemicals are present in a variety of products used daily, this work has wide applications and can inform individuals on their level of endocrine disrupting chemical exposure and the risks associated with those exposures. This study focused on two GR-responsive genes, but we look forward to expanding to genome-wide studies so that a larger scope of canonical pathways connected to molecular and cellular functions can be derived. Our pending experiments on protein expression and treatment with a GR antagonist (RU486) will further elucidate the endocrine disrupting potential of industrial chemicals.

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