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# Evaluation Of The Endocrine Disrupting Potential Of Chemicals Used In Crumb Rubber: A Multi-Factorial Approach

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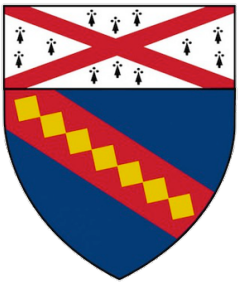

**ENVIRONMENTAL HEALTH SCIENCES**

# Evaluation of the Endocrine Disrupting Potential of Chemicals Used in Crumb Rubber:
## A Multi-Factorial Approach

**MASTER OF PUBLIC HEALTH**


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## Abstract

Crumb rubber, an infill substance made from repurposed tires, has been examined for its carcinogenic potential but no studies have looked at its endocrine disrupting potential to date. After prioritizing 306 chemical components of crumb rubber using computational models, four endocrine disrupting chemicals (EDCs) thought to possess estrogenic activity were chosen for further *in vitro* testing. In addition to these chemicals, a crumb rubber concentrated media (CRCM) was created for mixture analysis. Gene expression assays for estrogenic activity were conducted on Ishikawa, HepG2 and MCF-7 immortalized cell lines exposed to 4-tert-octylphenol, dibenz(a,h)anthracene, fluoranthene, chrysene and CRCM. Based on the findings of this study, crumb rubber is a potential mediating factor in harmful reproductive outcomes. Further discussion is needed to juxtapose these biological models with exposure assessment.

## Acknowledgements

I would like to express a special thanks to Dr. Shannon Whirledge for her lead and patience throughout the course of this research. Additionally, Andreanna Burman and Edwina Kisanga of the Whirledge lab for their assistance with experiments and lab companionship in the midst of failed experiments.

A token of gratitude to Ariana Yuen for supporting me in maintaining sanity, and to my lovely parents who innocently overestimate what I'm capable of.

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## Contents

## List of Tables

## List of Figures

**Evaluation of the Endocrine Disrupting Potential of Chemicals Used in Crumb Rubber: A Multi-Factorial Approach**

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## 1 | Introduction

According to the EPA, around 290 million tires are disposed of annually in the United States.[1] The disposal of tires poses unique issues to waste management due to their large size (75% void space), fire hazard potential, and natural breeding ground for mosquitoes.[2] For these reasons many states have regulated tire disposal; 40 states do not allow whole tires in landfills, and 12 states do not allow cut or shred tires.[3] With almost one scrap tire generated per person annually in the US,[4] the EPA and state governments alike have supported the repurposing of tires into consumer products. For example, the California Department of Resources Recycling and Recovery (CalRecycle) administers a tire grant program to divert waste products from landfills[5], and the state of Missouri issues waste tire playground grants with rewards up to $30,000 for installation of tire-derived materials.[6]

One of these repurposed products is known as “crumb rubber,” an infill substance utilized in synthetic athletic turf, as well as community parks and school playgrounds. With ease of upkeep and long-term savings compared to natural grass, this alternative has gained traction globally.[7] In the United States, there are over 12,000 synthetic turf fields and an additional 13,000 in Europe.[8] The infill material is composed of rubber polymer (40-60%), reinforcing agents (20-35%), aromatic extender oil (<28%), vulcanization additives, antioxidants, antiozonants, and

processing aids, such as plasticizers and softeners.[9,10] Consequently, the chemicals measurable in crumb rubber include polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), semi-volatile organic compounds (SVOCs), and metals, such as lead and zinc.[11]

One previous study identified the chemicals present in crumb rubber, characterized exposure mechanisms, and investigated the suspected carcinogenicity of the chemicals found in crumb rubber[12]. There have been no studies investigating the endocrine disrupting potential of these chemicals to date. An endocrine disrupting chemical (EDC) is an exogenous substance or mixture that alters the function(s) of the endocrine system by acting as a mimic or competitor to endogenous hormones in the human body. By disrupting overall hormonal equilibrium, EDCs can cause adverse health outcomes to an individual or its offspring. Topics of strongest evidence for endocrine disruption in the literature include obesity and diabetes, female reproduction, male reproduction, hormone-sensitive cancers in women females, prostate, thyroid and neurodevelopment and neuroendocrine systems.[13] EDCs are an emerging area of concern in the realm of environmental health risk assessment and toxicology and represent vast gaps in our understanding of exposures. Typically, chemicals are screened for their carcinogenic (genotoxic) potential but not specifically for hormonal disruption.

There are unexplained trends in reproductive dysfunction that coincide with the rise of industrial manufacturing and dissemination of widespread exposure to chemicals with endocrine disrupting potential. These trends include an average sperm count decrease of 59% from 1973 to 2011[14] and earlier start to puberty in women[15] shown to be correlated with increased levels of breast cancer[16] in epidemiologic findings. It is widely hypothesized that these trends are the result of exposure to endocrine disrupters, underscoring the need for increased understanding and identification of chemicals that could be acting via these mechanisms. While there are many potential effects of EDCs, estrogen receptor signaling is a well-characterized endpoint in wildlife and humans that is sensitive to the EDC exposure [reference]. Estrogen is a significant

factor in both male and female reproductive regulation; mainly, promoting the development and maintenance of female characteristics in the human body such as breasts and the regulation of both the reproductive and menstrual cycles. Moreover, EDCs often display a similar structure to that of endogenous estradiol ($E_2$), the form of estrogen most strongly related to development of cancer in females.

Being that there are over 140,000 chemicals in commerce with less than 10 percent screened for toxic endpoints, identifying and testing for endocrine disruption would add a further financial burden. However, emerging computational models allow for the strategic structural prioritization of chemicals to aid in weighting research targets.[18] This study provides a strategic two-pronged approach to conducting toxicological research on endocrine disruptors; first we utilized structural modeling and screening databases to prioritize the testing of potential EDCs among 300 crumb rubber chemicals, then we evaluated the endogenous estrogenic activity in a human uterine cell line to evaluate experimental endpoints in relation to computational findings. To account for the aggregated effect of crumb rubber as a mixture, a crumb rubber conditioned media (CRCM) was developed and evaluated in experimental endpoints.

## 2 | Materials and Methods

### 2.1 | Computational Prioritization.

Chemicals aggregated for carcinogenicity analysis by literature review in a previous study[12] were screened using the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP)[19] and the Collaborative Modeling Project for Androgen Receptor Activity (CoMPARA)[20] models built and validated by the EPA. These models are quantitative structure-activity relationship models that predict the effect on the estrogen and androgen receptors and their activity.

(i) CERAPP, the ER pathway model, demonstrates a balanced prediction accuracy of 95.2% against agonist reference chemicals and 97.5% when predicting the antagonist reference chemicals compared to the reference data sets.[19] After identifying 7,253 chemicals with quantitative endpoints by literature review (e.g. PC50, 50% of response induced by positive control or EC50, concentration of agonist that induces a response halfway between the baseline and maximum response), a subset of 36 active and inactive chemicals were chosen as the reference set. These 36 chemicals were chosen as a result of disagreement within the available literature pool being less than 20% between studies. The CERAPP model maps quantitative potency activity as categorical potent classes based on dose-response data: strong, moderate, weak, very weak, and inactive. Thresholds defining these five classes, for binding, agonist and antagonist activity, are defined as follows: (a) Strong: Activity concentration below 0.09 μM, (b) Moderate: Activity concentration between 0.09 and 0.18 μM, (c) Weak: Activity concentration between 0.18 and 20 μM. (d) Very Weak: Activity concentration between 20 and 800 μM, (e) Inactive: Activity concentration higher than 800 μM.

(ii) The AR pathway model (CoMPARA) was built by integrating 11 high-throughput screening ToxCast/Tox21 *in vitro* assays into a computational network. Multiple endpoints were utilized: receptor binding, coregulator recruitment, gene transcription and protein production on multiple cell types.[20] A chemical test set composed of 158 androgen active and inactive chemicals were sourced from a semi-automated systematic literature review and used as reference chemicals. CoMPARA has balanced accuracies of 95.2% for agonist and 97.5% for antagonist reference chemicals when compared to the reference set. Using the CoMPARA model, chemicals are given a consensus value which determines

whether or not there was a consensus of agonist or antagonist activity between reference data sets (0 for insignificant consensus, 1 for significant). Confidence scoring for this model was assigned by AR pathway model AUC scores, cytotoxicity information, and confirmation assay data. This model does not include threshold defining class categories.

Using the CERAPP and CoMPARA model, chemicals present in crumb rubber were prioritized based on a number of criteria. For ER relevant compounds, chemicals possessed an agonist consensus of 1, a concordance of >= 0.5, were within or above the “Very Weak potency category and lacked relevant EDC literature. AR relevant compounds possessed an antagonist consensus of 1, a concordance of >= 0.5, and a lack of relevant literature on EDC potential. Final chemicals selected were also chosen based on commercial availability.

### 2.2 | Literature Review and Active Site Identification

A literature search was conducted using the both the EPA’s Chemistry Dashboard interface (https://comptox.epa.gov/) and a targeted search via the “PubMed Abstract Sifter” using the following query: “ [CAS-RN] OR [chemical name] AND endocrine disruption OR female reproduction OR fertility OR estrogen”. Relevant papers were, allowing for the identification of crumb rubber chemicals with existing literature versus those that were understudied. Relevance was attributed to a conclusive understanding of the chemical’s endocrine via literature search when utilizing mammalian models or *in vitro* analysis, leaving 33 chemicals as seen in Figure 1. Active nuclear receptor assays associated with each compound were retrieved from ToxCast; compounds not registered in the database received a category of “N/A” under nuclear receptor bioactivity in Table 1.

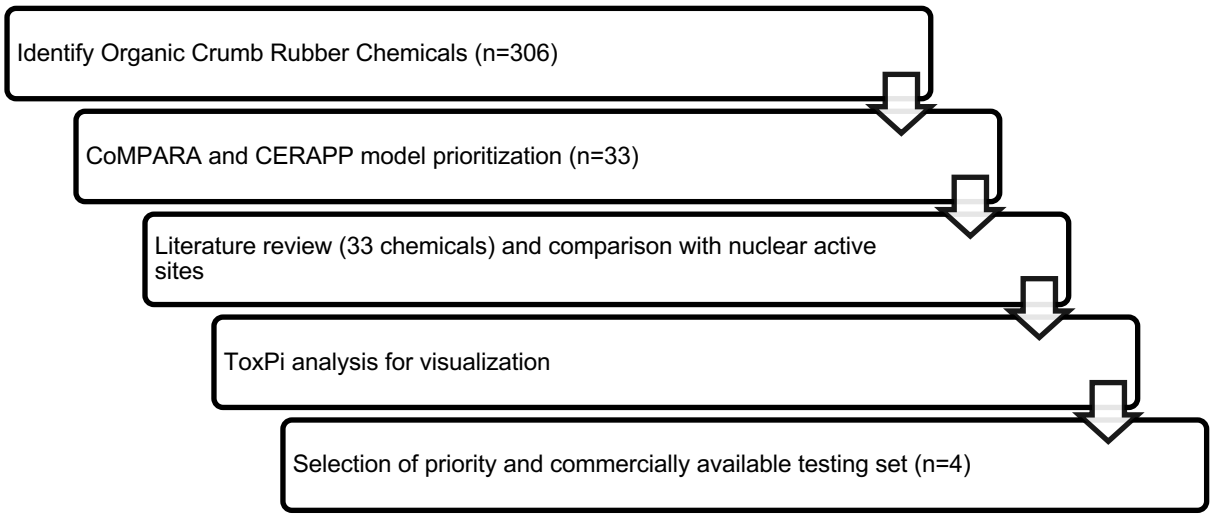



**Figure 1.** Overview of EDC chemical prioritization.

### 2.3 | ToxPi Analysis

The Toxilogical Prioritization Index (ToxPi) is a stand-alone, platform-independent Java application developed to enable integration of multiple sources of evidence.[21] ToxPi allows transparent visual rankings with weighted inputs of each data source to facilitate prioritization tasks Using ToxPi software. The culmination of CERAPP and CoMPARA model data along with active site detection were visualized for the 33 prioritized chemicals. In looking at the total estrogenic agonist effect of chemicals, the following data points were included in the ToxPi analysis: (1) presence of an active ER binding site, (2) presence of an active AR binding site, (3) estrogenic concordance binding quotient, (4) androgenic concordance binding quotient, (5) estrogenic agonist quotient and (6) androgen antagonist quotient.

### 2.4 | Reagents

RPMI Medium 1640 (11835-030; Gibco), phenol red-free RPMI (11873-030, Gibco) and DMEM were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS, F2442-500ML) was purchased from Sigma-Aldrich (St. Louis, MO). Charcoal dextran-treated (stripped) FBS was

purchased from Gemini Bio-Products (Sacramento, CA). Estradiol was purchased from Steraloids Inc. (Newport, RI). TaqMan qRT-PCR primer-probes were purchased from Applied Biosystems (ThermoFisher Scientific, Waltham, MA). Anti-Actin Antibody was purchased from Milipore (MAB1501; Temecula, MA) and Estrogen Receptor alpha (D8H8) Rabbit mAb from Cell Signaling Technologies **(**86445; Danvers, MA; Table S1). Goat Anti-Rabbit IgG, IRDye® 800CW Conjugated secondary antibody (926-32211) and Goat anti-mouse IRDye 680RD Goat anti-Mouse IgG secondary antibody (926-68070) were both obtained from LI-COR Biosciences (Lincoln, NE; Table S1). The four EDCs used in this study were purchased from Sigma Aldrich and are as follows: 4-tert-octylphenol (TCI, T0144; purity 95.0% by GC), fluoranthene (TCI, F0016; purity 98.0%), chrysene (Supelco, 40074; 1000ug/mL acetone, TraceCERT® Grade by HPLC) and dibenz(a,h)anthracene (TCI, D0145; purity 98.0% by GC).

**2.5 | Crumb Rubber Concentrated Media (CRCM)**

50 g of Al's Crumb Rubber (ALS-LC) purchased from Amazon.com was added to 500 mL of RPMI-1640 Phenol Red-free with 5% Stripped Heat inactivated FBS media or DMEM with 10% Stripped Heat Inactivated FBS media. Samples were kept at room temperature or in a water bath at 37°C for 24 hours. Media with crumb rubber was shaken periodically during incubation at room temperature or 37°C. Crumb rubber particles were removed from the media using vacuum filtration.

**2.6 | LDH cytotoxicity assay**

Cells seeded on 6-well plates were treated for 24 hours with Veh (1XPBS), 1000 nM of each selected EDC or 100% CRCM concentration. Following treatment, conditioned media was collected and centrifuged at 1000 g for 5 minutes. Supernatant was assessed for LDH activity using the Cytotoxicity Detection Kit (CYTODET-RO), purchased from Sigma Aldrich (Roche Diagnostics, Mannheim, Germany), according to protocol. Briefly, 100-µL conditioned media

was incubated with 100 µL reaction mixture for 15 minutes at room temperature, the reaction was stopped with 50 µL stop solution, and samples were read on a Vmax microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm wavelength.

### 2.7 | Ishikawa, Hep62 and MCF-7 cell culture

The immortalized Ishikawa uterine human endometrial adenocarcinoma cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). Ishikawa and MCF-7 cells were then grown at 37°C in a standard tissue culture incubator, with 95% humidity and 5% carbon dioxide. Cells were maintained in RPMI Medium 1640 supplemented with 5% FBS. 24 hours before treatment, media was changed to phenol red-free RPMI containing 5% charcoal-stripped heat-inactivated FBS.

Ishikawa controls were treated with chemical diluent, at no greater than 0.01% Et-OH or DMSO. Chemical-treated cells were treated with either 100 nM or 1000 nM of 4-OP dissolved in Et-OH, fluoranthene dissolved in dimethyl sulfide (DMSO), chrysene dissolved in DMSO or Dibenz(a,h)anthracene dissolved in DMSO for 6 hrs.

Immortalized HepG2 human liver cells were obtained from Dr. Vasilis Vasiliou at the Yale School of Public Health, Department of Environmental Health Sciences. Cells were grown at 37 C in a standard tissue culture incubator, as described above. Cells were maintained in DMEM medium supplemented with 10% FBS.

CRCM was applied in solution concentrations of 6.25%, 12.5%, 25%, 50% and 100% for 6 hrs to all three cell types – Ishikawa, HepG2 and MCF-7.

### 2.8 | Quantitative RT-PCR (QRT-PCR)

Total RNA was extracted from 6-well plates using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer protocol with the deoxyribonuclease (DNase) treatment performed on sample column. Isolated RNA was then assessed for purity and yield by

evaluating the A260/280 and A260/230 using the NanoDrop One Spectrophotometer (ThermoFisher Scientific, Waltham, MA). An absorbance ratio for 260/280 of 1.8 was utilized for the identification of adequate samples and the 260/230 absorbance cutoff is 1.6. cDNA was synthesized from 100 ng of total RNA using the One-Step RT-PCR Universal Master Mix Reagent (ThermoFisher Scientific). Using the CFX Connect (CFX384, Bio Rad, Hercules, CA) thermocycler, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using predesigned primer-probe sets (ThermoFisher Scientific) in a total 10-uL reaction volume. Phases for thermocycling of the samples were sequenced: 48 C for 30 min, 95 C for 10 min, 40 cycles of 95 C for 15 seconds and finally 60 C for 60 seconds.

For each of the gene primer-probe sets utilized, technical duplicates were compared to a standard curve and normalized against the chosen reference gene, peptidylprolyl isomerase B (*PPIB)*. *PPIB* was unaffected by EDC treatment. Analysis provided by this method is presented as a fold change, with ANOVA or Student's t-test to follow. Three to five biological replicates are analyzed per treatment group.

**2.9 | Western blotting**

Following treatment with Tris-glycine SDS sample buffer supplemented with 2-mercaptoethanol (BME), total protein was quantified using the Pierce 660 nm protein assay kit (Thermo Fisher Scientific). SDS-PAGE separation was used to separate equivalent amounts of protein from each sample prior to being transferred to a nitrocellulose membrane. Membranes were blocked with 7.5% skim milk in Tris-buffered saline (TBS) and proved overnight with primary antibodies against β-actin and ERα (Table S1). 24 hrs later, membranes were washed with 0.1% Tween-20 (TBS-T) in TBS and incubated with secondary antibody for 1 hour at room temperature (Table S1). The Odyssey LI-COR imaging system (LI-COR Biosciences, Lincoln, NE) was used to visualize immunoreactivity. Protein levels were normalized to β-actin and expressed to control samples.

### 2.10 | Statistical analysis

Data are presented as mean ± SE using a minimum of three biological replicates. Statistical significance was determined by ANOVA with Tukey's post hoc analysis. Statistical significance is reported as either $p < 0.05$ (*) or $p < 0.01$ (**).

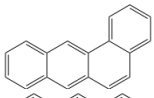
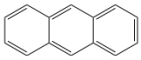
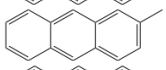
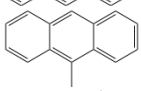
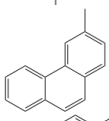
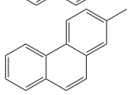
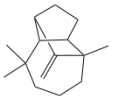
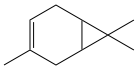
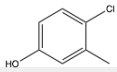
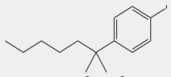
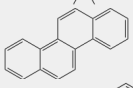
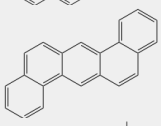
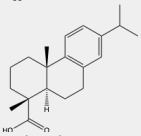
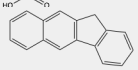
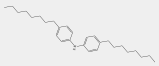
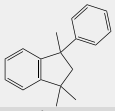
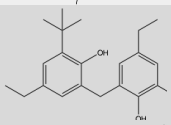
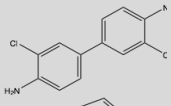
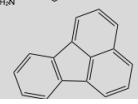
## 3 | Results

### 3.1 | Prioritization of chemicals in crumb rubber for further analysis

Based on the output from the CERAPP and CoMPARA models for the 304[12] chemicals found in crumb rubber, 33 chemicals were prioritized for their ER agonist or AR antagonist activity and binding coherence (Table 1). Compounds were prioritized if they had either a positive ("1") AR antagonist consensus and an AR concordance >= 0.5 or a positive ER agonist concordance ("1") and an ER agonist concordance >= 0.5.

The prioritized chemicals are indicated in Table 1 (*). As a comparison, the chemical structures of endogenous hormones, 17β-estradiol (E2) and total testosterone (TST), were included below in Figure 2. Additional visualization was done in ToxPi software, shown in Figure 3 below. Each value within the pi was scaled from 0 to 1; for agonist or antagonist activity this value was an output of the consensus and concordance values, presence of active nuclear receptors was classified as 1 and absence as 0. For the purposes of this study, we selected four chemicals for initial assays. Based on the ToxPi visualization, chrysene and fluoranthene demonstrate similar activities on AR antagonist and ER agonist models. However, previous fluoranthene research has identified active nuclear receptors while chrysene nuclear receptor data is not available. Dibenz(a,h)anthracene demonstrated activity in all six categories but lacked literature to support these findings. 4-tert-octylphenol (4-OP) was also a strong chemical in terms of categorical indicators and a literature review for 4-OP found 46 relevant papers on

estrogenic activity and EDCs. Both the contrasting data and similarities between chemicals made these four our candidates for the starting set, outlined in Table 2.

| CASRN | Chemical name | Structure | AR antagonist consensus | AR antagonist concordance | ER agonist consensus | ER agonist concordance | Nuclear receptor bioactivity (Yes, No, N/A) | Total relevant literature |
|---|---|---|---|---|---|---|---|---|
| 56-55-3 | Benz(a)anthracene |  | 0 | 0.5263 | 1 | 0.7 | Yes | 5 |
| 120-12-7 | Anthracene |  | 0 | 0.85 | 1 | 0.666666667 | Yes | 0 |
| 613-12-7 | Anthracene, 2-methyl- |  | 0 | 0.5789 | 1 | 0.6 | N/A | 0 |
| 779-02-02 | Anthracene, 9-methyl- |  | 0 | 0.6 | 1 | 0.6 | N/A | 0 |
| 832-71-3 | 3-Methylphenanthrene |  | 0 | 0.6316 | 1 | 0.6 | N/A | 0 |
| 2531-84-2 | 2-Methylphenanthrene |  | 0 | 0.5556 | 1 | 0.6 | Yes | 0 |
| 475-20-7 | junipene (longifolene) |  | 0 | 0.9048 | 1 | 0.555555556 | Yes | 0 |
| 13466-78-9 | 3-carene |  | 0 | 1.0 | 1 | 0.555555556 | Yes | 0 |
| 59-50-7 | 4-Chloro-3-methylphenol |  | 0 | 0.9048 | 1 | 0.5 | Yes | 4 |
| 140-66-9 | 4-tert-octylphenol* |  | 1 | 0.6842 | 1 | 1 | Yes | 46 |
| 218-01-9 | Chrysene* |  | 1 | 0.6 | 1 | 0.7 | N/A | 2 |
| 53-70-3 | Dibenz(a,h)anthracene* |  | 1 | 0.5789 | 1 | 0.666666667 | Yes | 1 |
| 1740-19-8 | Dehydroabietic acid |  | 1 | 0.5714 | 1 | 0.666666667 | N/A | 2 |
| 243-17-4 | Benzo(b)fluorene |  | 1 | 0.7895 | 1 | 0.6 | N/A | 1 |
| 101-67-7 | Benzenamine, 4-octyl-N-(4-octylphenyl)- |  | 1 | 0.6363636364 | 1 | 0.6 | Yes | 0 |
| 3910-35-8 | 2,3-dihydro-1,1,3-trimethyl-3-phenyl-1H-indene |  | 1 | 0.611111111 | 1 | 0.5556 | N/A | 0 |
| 88-24-4 | Phenol, 2,2'-methylenebis(6-(1,1-dimethylethyl)-4-ethyl- |  | 1 | 1.0 | 0 | 0.6364 | Yes | 0 |
| 91-94-1 | 3,3'-Dichlorobenzidine |  | 1 | 0.8 | 0 | 0.8 | Yes | 0 |
| 206-44-0 | Fluoranthene* |  | 1 | 0.7895 | 0 | 0.5556 | Yes | 0 |

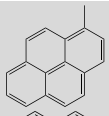
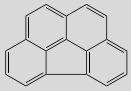
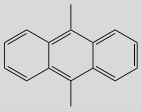
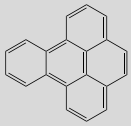
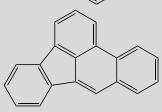
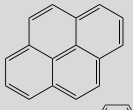
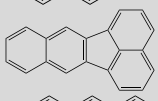
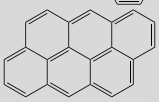
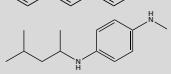
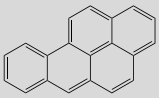
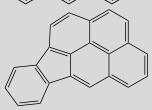
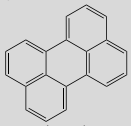
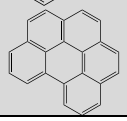
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2381-21-7 | Pyrene, 1-methyl- |  | 1 | 0.7222222222 | 0 | 0.5455 | Yes | 0 |
| 0203-12-3 | Benzo(ghi)fluoranthene |  | 1 | 0.705882353 | N/A | N/A | N/A | 0 |
| 781-43-1 | 9,10-Dimethylanthracene |  | 1 | 0.7 | 0 | 0.6667 | N/A | 0 |
| 192-97-2 | Benzo(e)pyrene |  | 1 | 0.6666666667 | 0 | 0.5 | Yes | 1 |
| 205-99-2 | Benzo(b)fluoranthene |  | 1 | 0.65 | 0 | 0.5556 | Yes | 1 |
| 129-00-0 | Pyrene |  | 1 | 0.631578947 | 0 | 0.5 | Yes | 5 |
| 0207-08-09 | Benzo(k)fluoranthene |  | 1 | 0.631578947 | 0 | 0.5556 | N/A | 6 |
| 191-26-4 | Anthanthrene |  | 1 | 0.625 | N/A | N/A | N/A | 0 |
| 793-24-8 | 1,4-Benzenediamine, N-(1,3-dimethylbutyl)-N'-phenyl- |  | 1 | 0.6111111111 | 0 | 0.9 | Yes | 0 |
| 50-32-8 | Benzo(a)pyrene |  | 1 | 0.611111111 | 0 | 0.5 | Yes | 19 |
| 193-39-5 | Indeno(1,2,3-cd)pyrene |  | 1 | 0.611111111 | 0 | 0.5 | Yes | 0 |
| 198-55-20 | Perylene |  | 1 | 0.611111111 | 0 | 0.5 | Yes | 0 |
| 191-24-2 | Benzo(g,h,i)perylene |  | 1 | 0.5625 | 0 | 0.5 | Yes | 0 |

**Table 1. CoMPARA and CERAPP results for 33 prioritized chemicals**. *Prioritized following targeted literature review. ER agonists are non-shaded rows, EDCs suspect of both estrogenic and AR antagonist activity are shaded light grey and progressively, those with solely AR antagonist properties are shaded in the darkest grey.

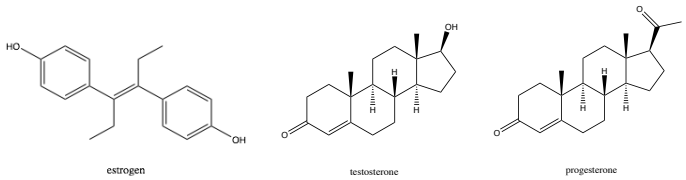



**Figure 2. Chemical structures of estrogen, testosterone and progesterone.** Serving as a visible reference to EDC structures.

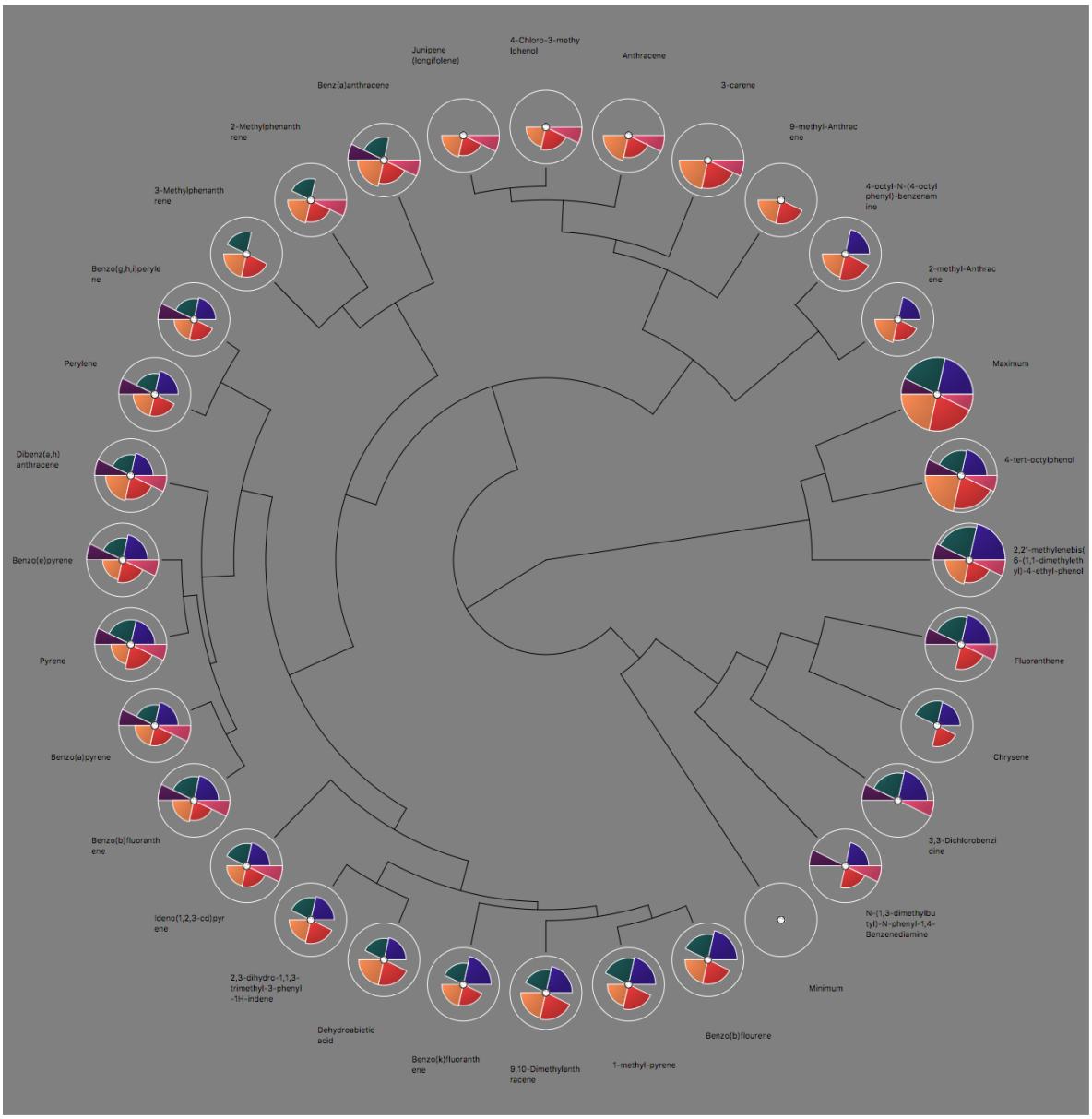



**Figure 3.** ToxPi prioritization of selected chemicals according to potential estrogenic activity modifying parameters. The following key indicates color variables; Red: ER agonist, orange: ER binding, pink: active ER receptor, blue: AR antagonist, green: AR binding, purple: active AR receptor.

| | ER agonist | ER binding | Active ER receptor | AR antagonist | AR binding | Active AR receptor | Proven literature |
|---|---|---|---|---|---|---|---|
| 4-OP | | | | | | | |
| Dibenz(a,h)anthracene | | | | | | | |
| Fluoranthene | | | | | | | |
| Chrysene | | | | | | | |

**Table 2.** Comparison of four selected EDCs for further study. Green indicates presence and red absence of.

### 3.2 | Cytotoxicity screening of four prioritized EDCs and CRCM.

To determine whether or not selected EDCs and CRCM induced cell toxicity, released lactate dehydrogenase (LDH) was measured in the media of Ishikawa cells treated with 100% CRCM, 1 uM of chrysene, 1 uM dibenz(a,h)anthracene, 1 uM 4-OP or 1 uM fluoranthene. Compared to the control, release of LDH was increased as a result of exposure to CRCM, suggesting that exposure to crumb rubber induces cell death in Ishikawa cells. Increases were seen as a result of exposure to 1 uM concentrations of dibenz(a,h)anthracene, 4-OP and fluoranthene (Figure 4) at a 6-hr treatment interval. No difference was seen upon treatment with 1 uM chrysene. CRCM treatment induced cell death and dibenz(a,h)anthracene, fluoranthene and 4-OP as CRCM constituents demonstrated cytotoxic behavior (Fig. 4), although not to the height of the conditioned medium.

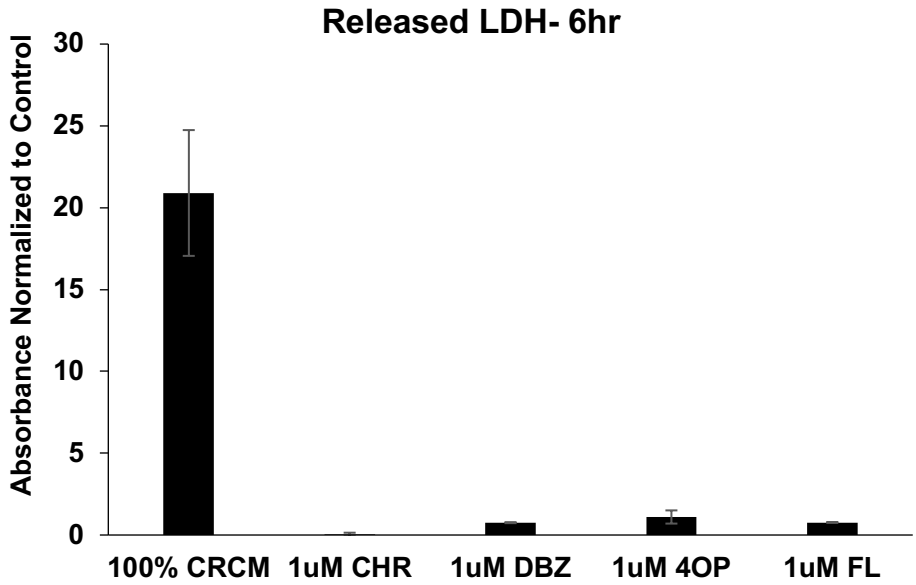



**Figure 4.** Response of cytotoxicity compared to control of 100% CRCM, 1 uM chrysene, 1 uM dibenz(a,h)anthracene, 1 uM 4-OP, or 1 uM fluoranthene as measured by lactate dehydrogenase (LDH) release in media. Assays are graphed as the ± SEM of 4 independent experiments. * $p < .05$ and ** $p < .01$ as determined by ANOVA.

### 3.3 | EDC activity as indicated by mRNa expression levels of *Pgr*, *Nppc*, and *Greb1* in the Ishikawa cells.

To evaluate estrogenic response, the expression of three known estrogen-responsive genes was evaluated in the immortalized Ishikawa cell line following treatment with the four prioritized EDCs: 4-OP, fluoranthene, chrysene and dibenz(a,h)anthracene for 6 hrs with 10 nM, 100 nM or 1000 nM concentrations with *Ppib* as the control. Response to treatment was compared to the response of Ishikawa cells to 10 nM $E_2$. Expression of well-known ER target genes, progesterone receptor (*Pgr*), growth regulating estrogen receptor binding 1 (*Greb1*), and natriuretic peptide C (*Nppc*)[22,23] was quantified by qRT-PCR. $E_2$ treatment was used as the positive control and peptidylprolyl isomerase B (*Ppib)* was used as the reference gene. The 10nM concentration of $E_2$ induced expression of *Pgr* (Fig. 5A), *Nppc* (Fig. 5E) and *Greb1* (Fig. 5H) compared to the reference.

The transcript levels of *Pgr, Nppc,* and *Greb1* in 4-OP treated Ishikawa cells were significantly elevated in both the 100nM and 1000 nM treatments (Fig. 5A) compared to vehicle treated cells. 4-OP concentrations of 1000 nM showed similar or greater upregulation than the 10 nM $E_2$ treatment. *Pgr* and *Nppc* in fluoranthene treated cells was upregulated by 10nM treatments and a statistically significant increase in mRNA expression was seen in the 1000 nM treatments (Fig. 5B). Significant downregulation was measured in dibenz(a,h)anthracene treated cells at both 100nm and 1000nm treatments (Fig. 5D). $E_2$ treatment at 10 nM demonstrated significant upregulation in *Nppc* expression when compared to the control (Fig. 5E). Following exposure, significant *Nppc* upregulation was demonstrated in 4-OP treatments at both 100 nM and 1000 nM at (Fig. 7E). Insignificant upregulation of *Nppc* mRNA expression was seen in both 10nM and 100 nM concentration treatments of fluoranthene (Fig. 5F), and similarly with chrysene (Fig. 5G). Dibenz(a,h)anthracene treatment resulted in an insignificant downregulation of mRNA *Nppc* expression (Fig. 5H). Chrysene showed almost no difference in mRNA expression compared to the control across all three genes (Fig. 5K), while

dibenz(a,h)anthracene showed consistent downregulation in mRNA expression. Significant reduction was seen in *Pgr* expression following dibenz(a,h)anthracene treatments.

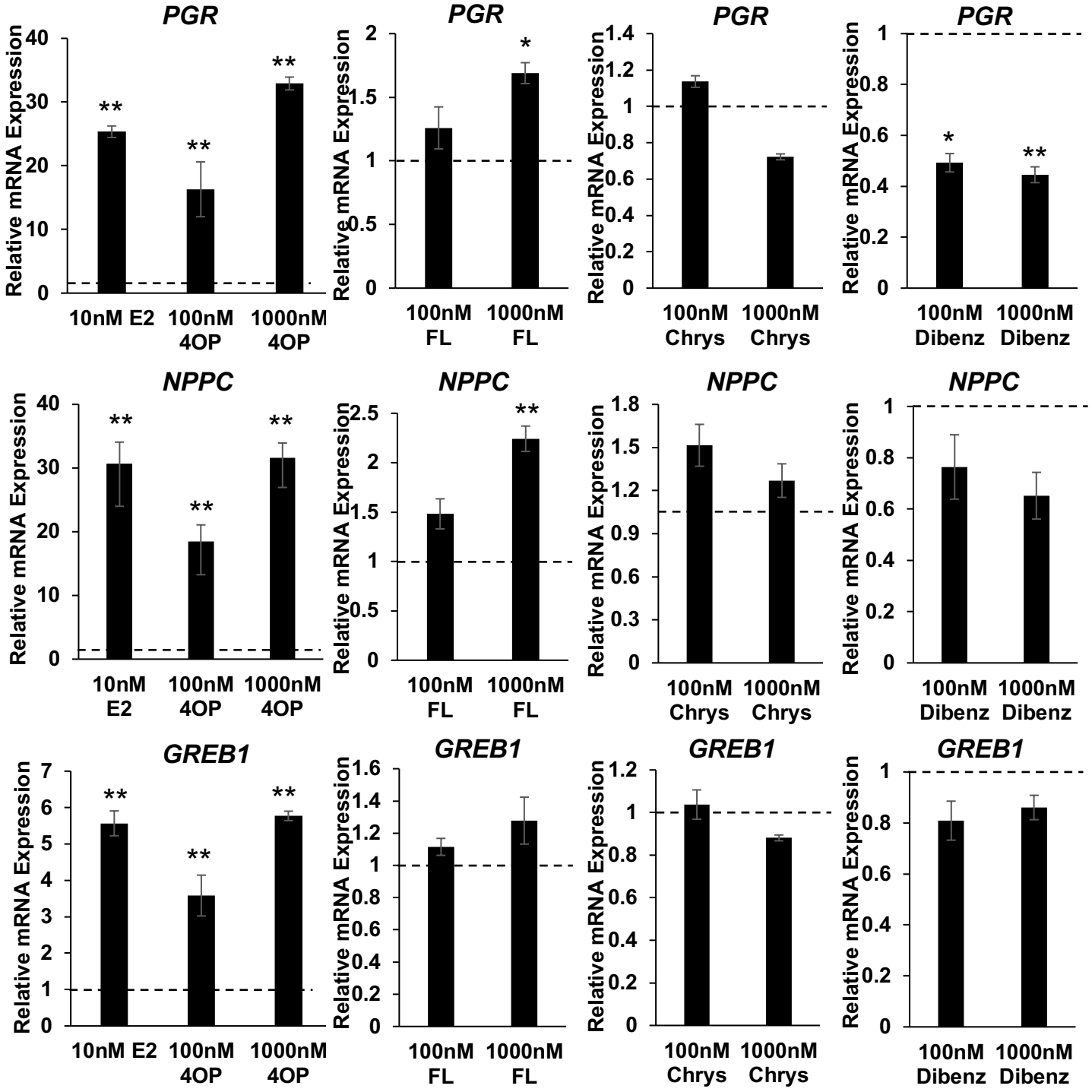


**Figure 5 (A-K).** mRNA expression in the Ishikawa cell line of *Pgr*, *Nppc,* and *Greb1* compared to control gene *Ppib* in fold-units. $E_2$ treatment was given in 10 uM concentrations. Ishikawa cells were 100 nM and 1000 nM concentrations of 4-OP, fluoranthene, chrysene and dibenz(a,h)anthracene. For all qRT-PCR experiments, mRNA levels were normalized to *PPIB* and set relative to 0 nmol/L or Veh. Genetic expression is graphed as the ± SEM of 4 independent experiments. * $p < .05$ and ** $p < .01$ as determined by ANOVA.

### 3.4 | ESR1 and ESR2 mRNA expression, ERα absorption in EDC-treated Ishikawa cells.

To assess whether Ishikawa cells are receptive to hormone disruption by the prioritized EDCs, expression of estrogen receptors ESR1 and ESR2 in addition to ERα protein was determined via qRT-PCR. Western blot analysis was utilized to confirm expression level of ERα after 6 h treatment compared to vehicle. Previous genetic research has demonstrated similarity of function in genetic regulation in regard to ESR1 between endogenic and exogenic estrogens, such as Bisphenol-A.[24] mRNA expression for $E_2$ treated Ishikawa cells was significantly lower compared to the vehicle expression levels for ESR1 (Fig. 8A). Similarly, 4-OP, fluoranthene, and dibenz(a,h)anthracene all demonstrated significant levels of downregulation compared to the vehicle (Fig. 8A). Chrysene did not demonstrate a significant relative difference in mRNA expression compared to the control.

$E_2$ treatment with supplementary western blot analysis shows a decrease in ERα protein level.[25] ERα levels were significantly downregulated in the 4-OP treatment group, with confirmation of activity within dibenz(a,h)anthracene, fluoranthene and chrysene treatments. (Fig. 8B). Fluoranthene significantly upregulated ESR2 expression, while dibenz(a,h)anthracene treatment resulted in statistically significant downregulation of ESR2 expression compared to the vehicle.

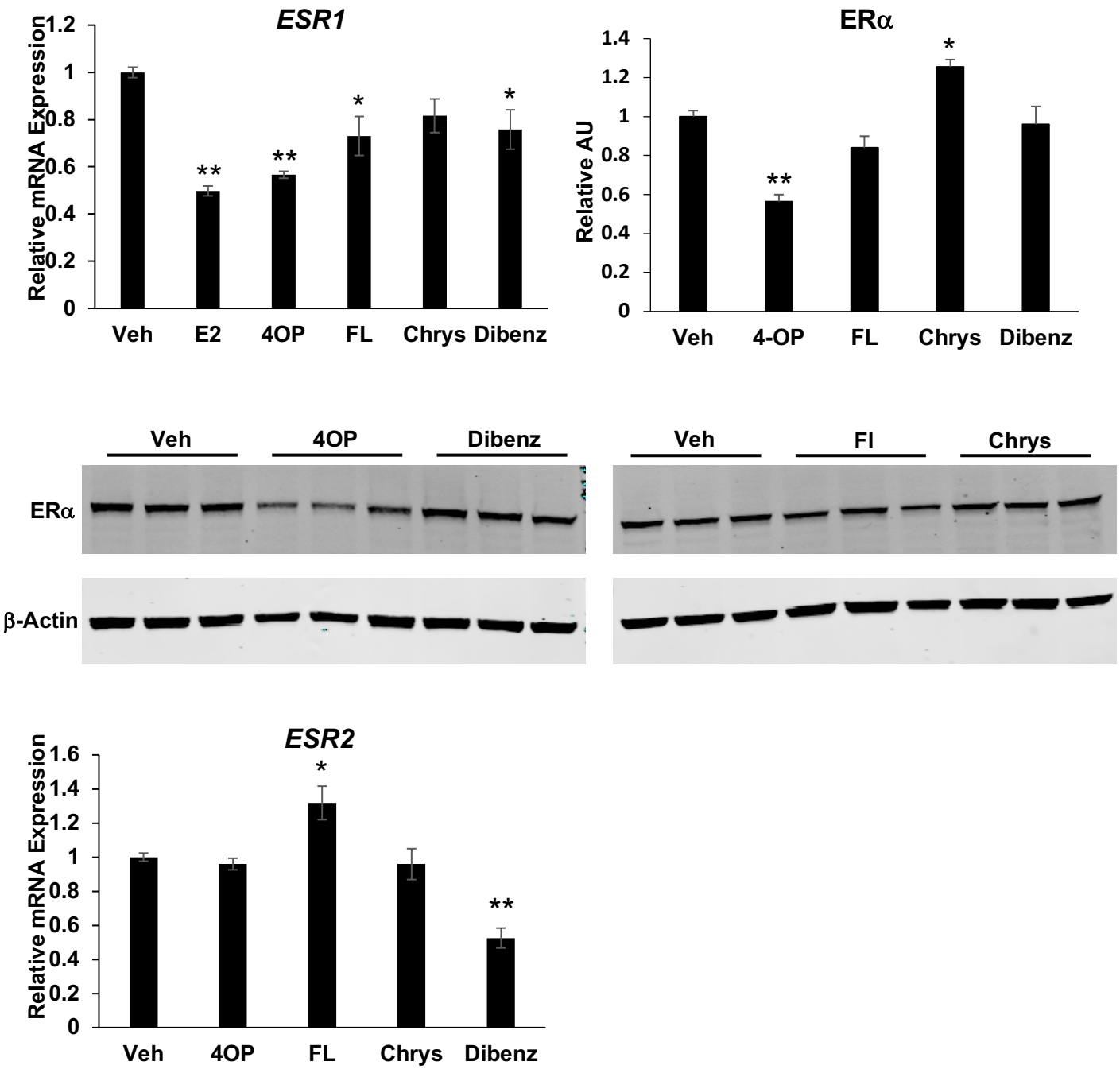



**Figure 6.** (a) ESR1 and (b) ERα mRNA expression under vehicle, estradiol, 4-OP, fluoranthene, chrysene and dibenz(a,h)anthracene treatment groups relative to the *Ppib* reference treatment, with supplemental (c) western blot analysis. (d) ESR2 expression relative to the *Ppib* reference group, showing expression as an outcome of vehicle, 4-OP, fluoranthene, chrysene and dibenz(a,h)anthracene treatments. Genetic expression is graphed as the ± SEM of 4 independent experiments. * $p < .05$ and ** $p < .01$ as determined by ANOVA.

### 3.5 | Endogenous simulation of *Pgr, Greb1* and *Nppc* expression in EDC-treated Ishikawa cells.

Endogenous conditions were simulated by treating Ishikawa cells with both the EDC and $E_2$, observing mRNA expression from three genes. *Pgr* expression as a result of 4-OP was antagonized by $E_2$ treatment. Expression of *Pgr* due to treatment of fluoranthene (Fig.7D), chrysene (Fig. 7G) and dibenz(a,h)anthracene (Fig. 7J) were agonized by the presence of $E_2$ in treatment conditions. While the 4-OP + E2 treatment antagonized both individual expressions of 4-OP and $E_2$ in *Greb1* (Fig.7B), we saw that the addition of $E_2$ in other treatment groups had an agonist effect. The sum expression of *Greb1* in $E_2$ + fluoranthene, chrysene or dibenz(a,h)anthracene treatments was greater than that of solely the EDC. Similarly, expression of *Nppc* was upregulated in by estradiol for fluoranthene, chrysene and dibenz(a,h)anthracene treatments. Expression of *Nppc* in Ishikawa cells was downregulated from the singular 4-OP treatment group to the $E_2$ supplementation.

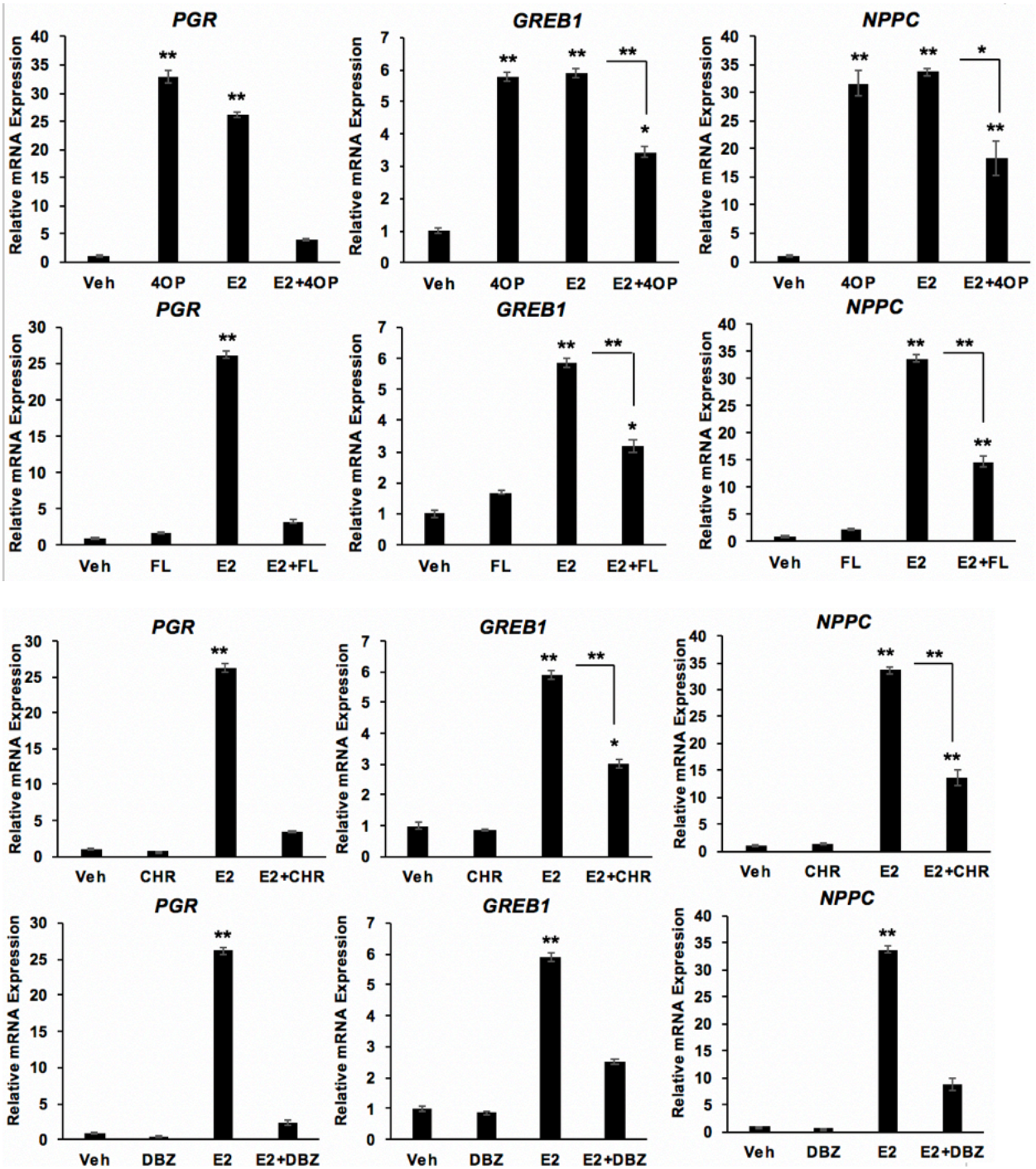



**Figure 7 (A-L).** Comparison of EDC, E2 and combined (E2 + EDC) effects on Pgr, Greb1 and Nppc expression in Ishikawa cells. Expression is graphed as the ± SEM of 4 independent experiments. * $p < .05$ and ** $p < .01$ as determined by ANOVA.

### 3.6 | LDH leakage with CRCM dose treatments across Ishikawa, HepG2 and MCF-7 cells at both 6 and 24 hrs.

Ishikawa cells showed a steady decline in LDH release complimentary to diminishing concentrations across both temperature groups for 6 hr assays, while 24 hr assays showed less of a dose-response relationship. At 24 hr exposures, 100%, 50% and 25% groups all reached 30x fold compared to the vehicle across both temperature groups. 12.5% concentration at 37 C reached 25x fold in LDH release compared to vehicle.

HepG2 cell treatments showed a dose-response release of LDH at the 6 hr time interval. This relationship was less visible in the 24 hr treatment group. MCF-7 cells were the least affected comparatively, with heated 6 hr treatments showing the greatest cytotoxic outcome. Cytotoxicity was measured in all treatment groups at both time intervals for all cell types.

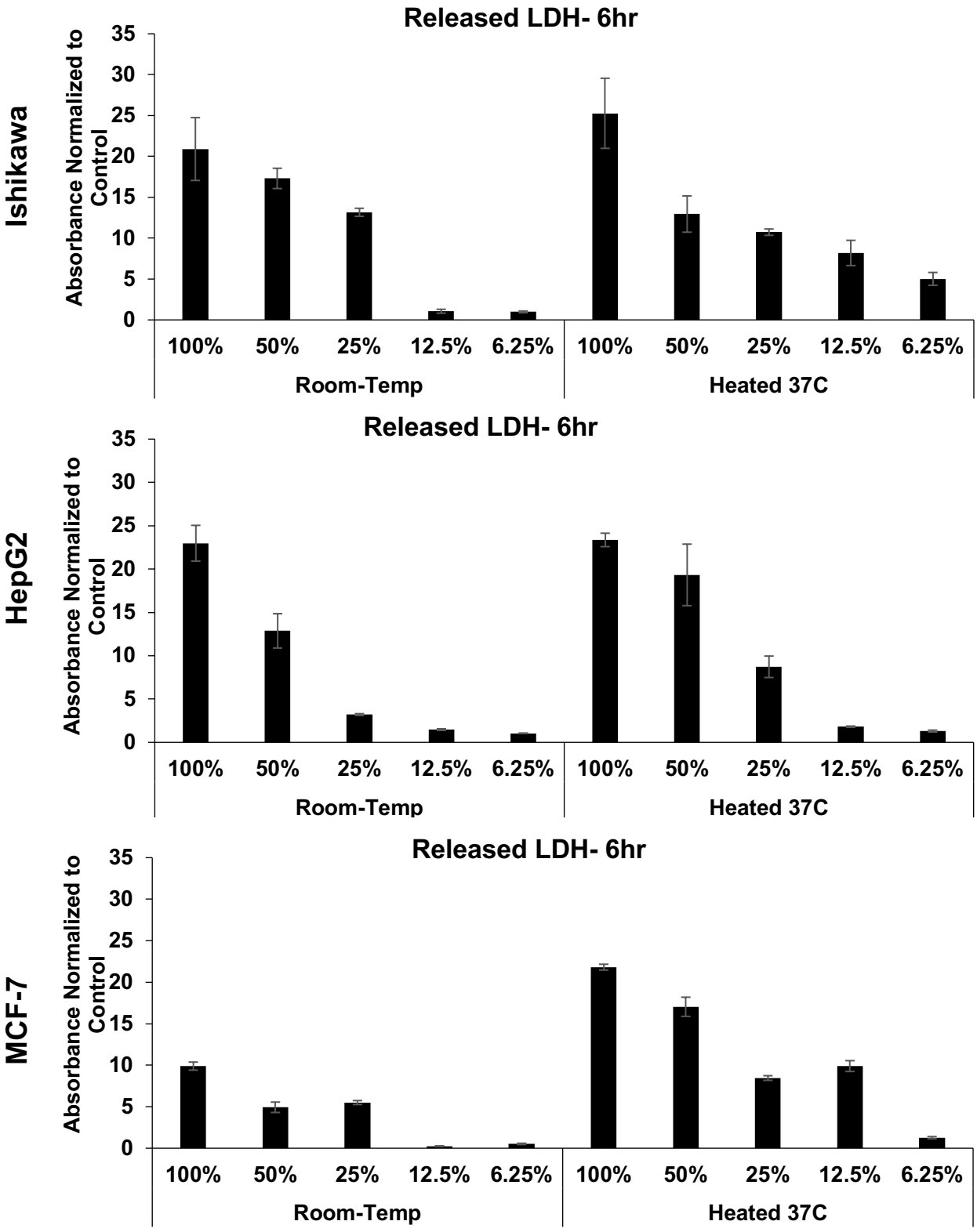



**Figure 8.** Released LDH in Ishikawa, HepG2 and MCF-7 6 hr cell treatments of room temperature and heated 37 C mediums at 100, 50, 25, 12.5 and 6.25% concentrations.

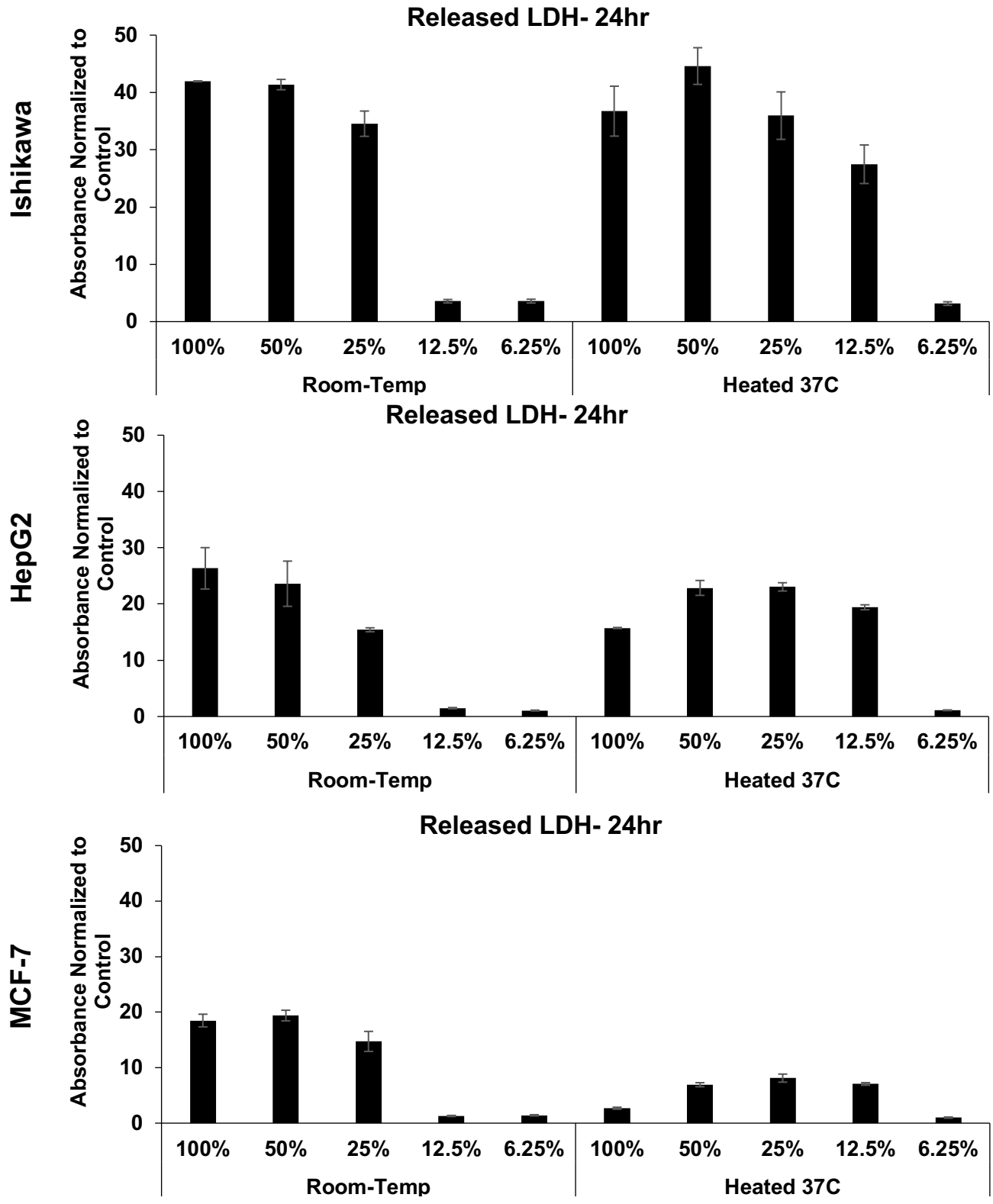



**Figure 9.** Released LDH in Ishikawa, HepG2 and MCF-7 24 hr cell treatments of room temperature and heated 37 C mediums at 100, 50, 25, 12.5 and 6.25% concentrations.

### 3.7 | CRCM upregulate *Greb1* mRNA expression in Ishikawa cells under 37 C treatment.

Among all four medium treatment types, only the 37 C prepared 24-hr incubation with 12.5% concentration treatment showed significant upregulation in *Greb1* expression. Other treatment groups showed slight downregulation but no significant alterations in mRNA expression.

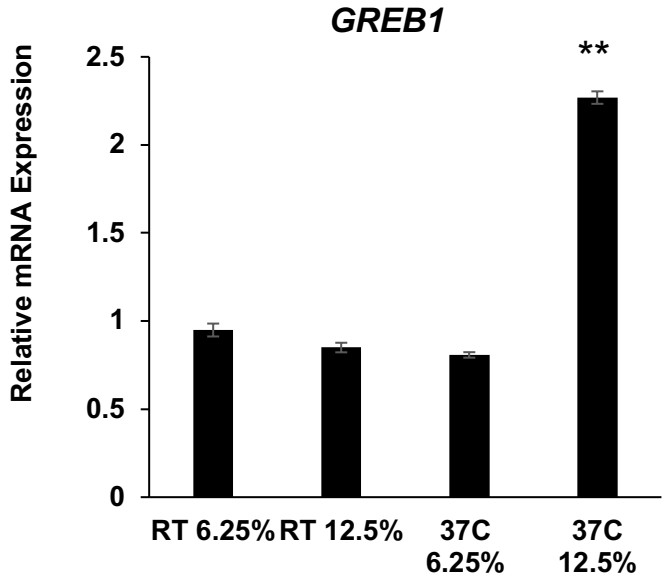



**Figure 10.** *GREB1* expression in Ishikawa cells after treatments with varying CRCMs. Treatment groups of were classified as room temperature or heated to 37 C for 24 hr. Each treatment type was further diluted to 6.25% and 12.5% concentrations. *Greb1* expression is graphed as the ± SEM of 4 independent experiments. * $p < .05$ and ** $p < .01$ as determined by ANOVA.

## 4 | Discussion

Overall, the data collected in this study elucidate how we might juxtapose emerging QSAR models with more traditional forms of research and the black box around complex mixtures.

LDH release assays demonstrated that CRCM exerts cytotoxic effects on all three cells lines tested in this study (Ishikawa, HepG2, and MCF-7). Previous work by the National Toxicity Program (NTP) demonstrated cytotoxicity in HaCaT, HPL-1D and FHs-74-Int cell types,

representative of skin, lung epithelial, and small intestinal cells *in vitro.*[26] HepG2 cells of the liver represent a portion of the digestive system, which could be directly in contact with crumb rubber particles ingested by individuals. Unintentional inhalation and ingestion of crumb rubber particles are cited by the NTP as potential exposure pathways. Utilizing the CRCM in LDH studies also showed the stark difference in cytotoxicity profiles of mixtures versus that of individual chemicals, the most common approach used today (Fig. 4).

Increased expression of *Greb1* as an outcome of CRCM exposure was found only in the 37 C treatment at 12.5% concentration (Fig 10). *In vitro* studies mentioned earlier also included heating treatments of 60 C. We did not treat the crumb rubber to temperatures above 37 C in this research, but the potential to further increase temperature and thus leachability of chemicals exist. With this in mind, further cytotoxicity and gene interaction is a potential outcome. Additionally, previous work by the NTP have not looked at beyond cytotoxic outcomes. Therefore, this data is the first insight into reproductive mechanisms by which crumb rubber has the potential to mediate reproductive effects.

Related to the chemical prioritization step conducted in this study, the NTP's also attempted to select specific chemicals involved in the vulcanization of crumb rubber but labeled this selection as "untargeted". The selection did not involve any QSAR analyzation, unlike the methods used in the endocrine profiling portion of this study. Here we see that the EDCs chosen for further *in vitro* testing in our study all have significant potential to contribute to the overall cytotoxicity and gene expression fluxes of CRCM. Although we are unsure as to whether the chemicals previously identified in the crumb rubber study are also present in "Al's Crumb Rubber", chemicals tested in the study were purposefully chosen as not having substantial scientific literature on the EDC potential. Therefore, these findings also contribute to the overall literature on endocrine effects of fluoranthene, dibenz(a,h)anthracene and chrysene in particular, outlining effects on target genes and the estrogen receptor pathway. While insignificant findings for chrysene were in line with one previous study,[27] our study also shows

significant upregulation of ERα and significant upregulation of *Greb1* and *Nppc* with supplemental E2, indicative of estrogenic behavior. We found zero relevant papers on fluoranthene activity. The findings of this study indicate significant upregulation of *Pgr and Nppc* in fluoranthene treatments. Endogenous treatment with E2 + fluoranthene demonstrated significant increases in *Greb1* and *Nppc*, suggesting estrogenic potential. 4-OP estrogenic activity was confirmed across all analyses. Significant interference was seen in ESR1 and ESR2 mRNA expression for dibenz(a,h)anthrancene in addition to downregulation of *PGR*, showing potential for estrogenic interaction despite a previous study claiming antagonist activity.[28] Our findings on chrysene were more in line with predictions produced by the CoMPARA and CERAPP models (Table 2).

## 5 | Conclusions

Further discussion is needed to determine the implications of these results being that methods used in this study need to be further extrapolated into exposure models. Based on the findings of this study, crumb rubber is a potential mediating factor for harmful outcomes in both the male and female reproduction systems.

## 6 | Supplemental Tables

**Table S1: Antibodies**

| Antibody ID | Antibody | Source | Catalog Number | Purpose |
|---|---|---|---|---|
| AB_2223041 | Anti-Actin Antibody, clone C4 | Millipore, Temecula, MA | MAB1501 | Western blotting (1:3,000) |
| AB_2617128 | Estrogen Receptor alpha (D8H8) Rabbit mAb | Cell Signaling Technology Danvers, MA | 86445 | Western blotting (1:1,000) |
| AB_621843 | Goat Anti-Rabbit IgG, IRDye® 800CW | LI-COR Biosciences, Lincoln, NE | 926-32211 | Western blotting (1:3,000) |

| | | | | |
|---|---|---|---|---|
| | Conjugated secondary antibody | | | |
| AB_10956588 | Goat anti-mouse IRDye 680RD Goat anti-Mouse IgG (H + L), 0.5 mg secondary antibody | LI-COR Biosciences, Lincoln, NE | 926-68070 | Western blotting (1:3,000) |

**Table S2: TaqMan Real-Time PCR Primers**

| **Primer** | **Gene** | **Description** |
|---|---|---|
| Hs00168719 | *PPIB* | Peptidylprolyl isomerase B |
| Hs00536409 | *GREB1* | Growth regulation estrogen receptor binding 1 |
| Hs01556702 | *PGR* | Progesterone receptor |
| Hs00360930 | *NPPC* | Natriuretic peptide C |
| Hs01046816 | *ESR1* | Estrogen receptor 1 |
| Hs01100353 | *ESR2* | Estrogen receptor 2 |

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