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THE EFFECTS OF PHTHALATE METABOLITES ON HEPATIC NUCLEAR RECEPTOR ACTIVITY

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ABSTRACT

CAR2, PXR, PPARα, and PPARγ are nuclear receptors found in human hepatocytes and other cells that bind xenobiotics and within the nucleus regulate the transcription of a variety of genes, including those involved in biotransformation. Included among the xenobiotic receptor activators are the phthalates, chemicals used pervasively as plasticizers that are of toxicological concern. My thesis research used a series of transactivation, mammalian two hybrid and real-time PCR assays to compare the activation of these xenobiotic receptors by several phthalates. Phthalates are chemicals that have an aromatic ring base with two ortho alkyl or alkyl aryl groups. The phthalates addressed here include the most commonly used moieties, di-2-ethylhexyl phthalate (DEHP) and di-isononyl phthalate (DiNP), and phthalate metabolites, mono-2-ethylhexyl phthalate (MEHP), mono-isononyl phthalate (MiNP), and monobenzyl phthalate (MBzP). DEHP was shown to activate the human CAR2 splice variant selectively, and also PXR at higher doses, consistent with previously published findings. DiNP similarly activated CAR2 and PXR. The monophthalate derivatives of DEHP and DiNP are the primary metabolites generated in mammalian systems and have not been rigorously evaluated for their receptor activation potential. The main hypothesis of this research project was that these phthalate metabolites could also function as potent CAR2 activators. The results demonstrated that MEHP does in fact potently activate CAR2, PXR, and to a lesser extent PPARα. MiNP was also found to activate CAR2, and to slightly activate PPARα, while PXR exhibited as much as a 25-fold increase in receptor activity in the presence of MiNP. Therefore, the monophthalate metabolites examined in this study were indeed relatively potent activators of the human

xenobiotic receptors, CAR2 and PXR. These results are novel and demonstrate that even the primary major metabolites of the di-phthalates retain biological activity.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Introduction

Xenobiotics and Metabolism in Humans

All organisms, including humans, regularly come into contact with a multitude of organic and synthetic substances that exist within the environment around them and must be prepared to deal with these chemicals as they are contacted. Any such chemical found within an environment that would not ordinarily be expected to exist within a living organism is referred to as a xenobiotic. Xenobiotics include an enormous array of different compounds and may include naturally occurring chemicals like toxins from plants, man-made pollutants, or helpful synthetic chemicals like medicines. Xenobiotics may enter an organism by inhalation, ingestion, or other intentional or unintentional channels, and subsequently undergo absorption and potentially biotransformation in order to facilitate their elimination.

Biotransformation is the process through which xenobiotics or any other chemicals are enzymatically altered in order to facilitate their excretion. Most xenobiotics absorbed into tissues are lipophilic and are biotransformed into more water soluble metabolites to allow for passage through the kidneys or intestines; a process which also frequently lowers the reactivity of the xenobiotic. [1] Many cell tissues can conduct biotransformation to some extent, but the liver is the major biotransformation organ in vertebrates. [1] There are four major categories of reactions that increase water solubility of xenobiotics: hydrolysis, reduction, oxidation, and conjugation; the last of which creates the most significant change. [1] The first three types are generally considered Phase I of biotransformation, while conjugation is considered Phase II. Phase I serves to change

a molecule's polarity only a small amount, but adds additional functional groups, making the molecule more accessible to other enzymes. Cytochromes P450 are regarded as Phase I metabolic enzymes. Phase II conjugates the now more polar molecules with glutathione or other large polar molecules making the molecule significantly larger and more water soluble and thus easier to excrete. Phase I biotransformation is occasionally skipped with molecules that can be conjugated immediately. [2] While these metabolic processes work with the intent of inactivating and removing xenobiotics, making a molecule more hydrophilic can also make it more biologically active. These newly biologically reactive metabolites may be toxic or carcinogenic to the animal metabolizing them. [3] The large number of xenobiotics that exist are often recognized by protein sensors in the cell, a xenosensing process carried out by a network of specialized xenobiotic receptors that in turn function to regulate and often enhance the biotransformation processes that orchestrate the elimination of xenobiotics from the body.

Xenobiotic Receptors

As indicated, xenobiotic receptors are a group of nuclear receptors that are characterized by their binding of a broad set of xenobiotic ligands and subsequent nuclear localization. They serve as transcription factors for genes whose products are involved in xenobiotic metabolism as well as other important physiological functions of the cell. Three such receptors of critical importance are the Constitutive Androstane Receptor (CAR), the Pregnane X Receptor (PXR), and the Persoxisome Proliferator-Activated Receptors (PPARs), all of which are members of the nuclear receptor superfamily. [4] Each of these nuclear xenobiotic receptors, when activated, is capable of binding DNA in the nucleus to alter transcription of genes involved in xenobiotic

metabolism, transport and other physiological functions. [3,5] Some of the key metabolic genes activated by these receptors include CYP2B6 and CYP3A4 in the case of CAR2 and PXR; PDK4, ACOX1, and CPT2 for PPARα; and ACOX1 for PPARγ. [6–9]

Xenobiotic receptors and their associated biotransformative processes are not always active; in the absence of a xenobiotic ligand, most receptors sit in a low activity or an inactive state. [3] However, when a ligand enters and binds a receptor, the receptors are swift to activate a biotransformative response in order to enzymatically modify and enhance elimination of the chemicals. [3] Xenobiotic receptors contrast somewhat with most other receptors in that they exhibit relatively promiscuous binding affinities instead of recognizing a highly specific set of only a few ligands. [3] This provides the ability to respond to the enormous number of xenobiotics that could potentially be absorbed and participate in toxic effects. Additionally, the already broad repertoire of nuclear receptors can be expanded further by the formation of splice variants derived from the wild type genes.

Splicing involves the cutting and rearrangement of segments of the mRNA during the process of transcription. In wild type cases, splicing involves the removal of all intron DNA and the reconnecting of the remaining exon DNA. Splicing reactions are conducted by an enzyme complex called the spliceosome which is a collection of enzymes on a scaffold that hold, cut, and reconnect the segments of DNA. [10] However, splicing may not occur the same way every time during mRNA processing, and pieces of or even entire exons or introns may be included or left out, resulting in different mRNA and protein products. Alterative splicing can have a variety of effects based on the location and size of the change; an alteration to bases that will code for a structural part of the protein may not result in any change in function whatsoever, though, even a small change to an active site can drastically change the binding affinity or activity of a protein,

and may even inactivate it completely. Splicing mRNA in different ways provides the direct benefit of expanding the proteome of an organism without need to create more genes within the DNA. CAR relies on splice variants to cover a larger set of ligands, and while its two major splice variants, CAR2 and CAR3, have only small insertions, these, nevertheless, confer significant changes in the resulting specificity and associated biological activities of the receptor proteins.

Xenobiotic Receptor Activity

CAR and PXR are somewhat redundant transcriptional activators that bind a wide variety of ligands and activate unique, but overlapping sets of genes involved primarily in biotransformation and metabolism. [11]

CAR (NR1I3), when inactive, resides in the cytoplasm of liver hepatic cells, tethered by a set of proteins that include Cytoplasmic CAR Retention Protein (CCRP) and Heat Shock Protein 90 (HSP90). [12,13] Once released, by ligand binding or indirect activation, CAR will translocate to the nucleus where it will heterodimerize with Retinoid X Receptor alpha (RXRα) and bind to one or more co-activators. This complex will bind to one of a few xenobiotic response elements (XREM) to activate gene transcription. [12] This process is summarized in Figure 1.

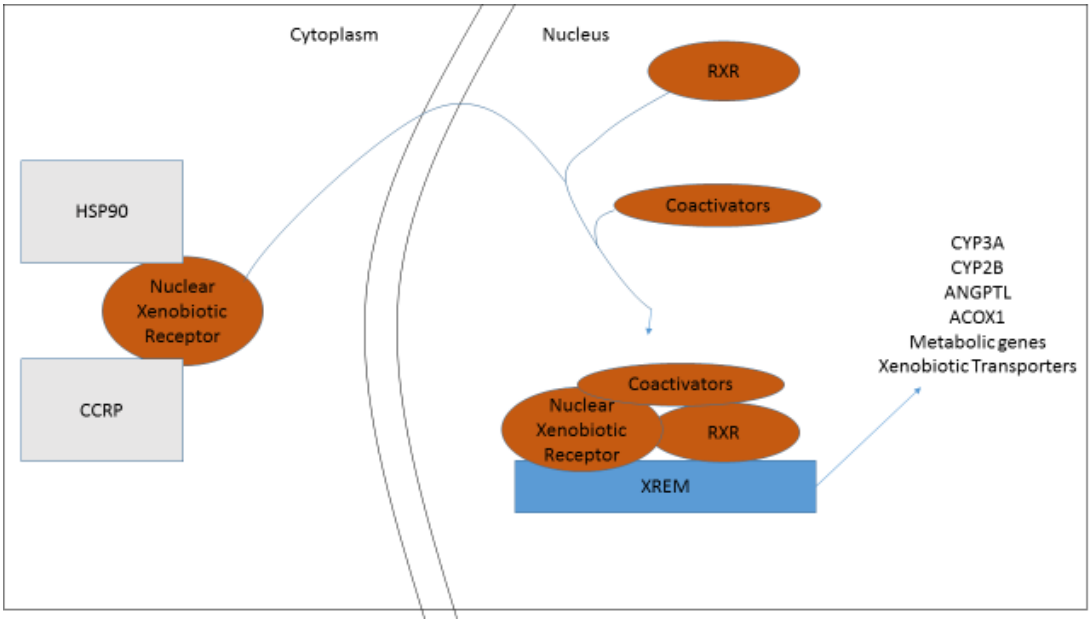

Figure 1: An Overview of Nuclear Receptor Activation and Nuclear Translocation.

Human CAR has a large number of splice variants that are similarly structured to it, but only three, CAR1, CAR2, and CAR3, are considered to have a notable biological effect. CAR1, the most common splice variant, is active to some extent without a ligand and will spontaneously dissociate from the tethering proteins and translocate to the nucleus, while all other splice variants require ligand binding to translocate. [11,14] CAR3 has a 15 base pair insertion at the beginning of its 8th exon that results in a 5 amino acid insertion, but is activated by nearly the same set of ligands as CAR1. [15,16] However, this insertion abolishes the constitutive activity of CAR3, resulting in a ligand-activated receptor. [15,16] CAR2 has a 12 base pair insertion at the beginning of its 7th exon resulting in a 4 amino acid insertion that also abolishes the constitutive activity. [15,17] This insertion allows for binding a different set of ligands, while still activating a similar set of genes as CAR1 and CAR3, expanding the set of potential xenobiotics that can be recognized. [15,17] When activated, all forms of CAR are involved in the activation or inhibition of

expression of many cytochromes P450 along with other metabolic genes and some transporters.[18] One commonly used and studied phthalate, DEHP only activates CAR2 and thus CAR2 will receive more focus in the present study. [14]

PXR acts in much the same way as CAR; it is retained in the cytoplasm by CCRP and HSP90, until activation when it translocates to the nucleus where it binds with RXRα and co-activators and activates gene transcription. [19] CAR and PXR activate the transcription of CYP3A4 which degrades 50-60% of current clinical drugs and the CYP2B family which degrades 25-30% of other clinical drugs, thus accounting for up to 90% of clinical drugs. [3]

PPAR has three different major forms: alpha, beta/delta, and gamma. Each isotype has a different set of ligands and target genes, but all are common in liver tissue and serve the same general purpose of modulating metabolism, especially when considering lipids. [20] All isotypes of PPAR act in a similar manner to CAR and PXR as they follow the same process of activation: translocation to the nucleus, heterodimerization with RXRα and co-factors, and binding DNA to activate transcription. [21] PPARα, like CAR1 will translocate in the absence of a bound ligand. [21] Activated PPAR-RXRα complexes bind one of many peroxisome proliferator response elements (PPRE) to activate or repress the transcription of metabolic genes. [21]

Xenobiotic Receptors and Phthalates

Phthalates are a class of xenobiotics that are of high interest to the field of toxicology due to their ubiquity. Phthalates are used in a great deal of different industrial and consumer products that include PVC, adhesives, lotions, flooring, and medical devices, but are not chemically bound to most of the products they are included in, thus allowing them to leech out into the

environment where humans may contact them through ingestion, inhalation, skin absorption or other modes of entry. [22] Exposure to phthalates in high concentrations has been linked with changes in male reproductive development, making them a notable public health concern. [23]

All of the nuclear receptors indicated bind and activate the metabolism of various phthalates. Phthalates are ester derivatives of phthalic acid (Figure 2) with two alkyl or alkyl aryl groups. [24] Phthalates that are absorbed are in most cases quickly metabolized and excreted, frequently as monophthalate esters. [25] Many different phthalates are used in industrial settings, and many cosmetics, plastics, and solvents contain phthalates to some degree. [22] DEHP and DiNP in particular are produced and utilized commercially in massive scale. MEHP and MiNP represent the primary biological metabolites of DEHP and DiNP, respectively. MBzP is a primary metabolite of benzyl butyl phthalate, and often detected in biological samples within the human population.

It has previously been established that DEHP and DiNP are selective and potent activators of human CAR splice variant, CAR2, and that PPAR is activated by DEHP, MEHP, DiNP, and MiNP. [14,26,27] These phthalates, in addition to the monophthalate MBzP, are used here as a sample of the phthalates one may encounter commonly. Thus, the phthalates of interest in the present study, di-2-ethylhexyl phthalate (DEHP), mono-2-ethylhexyl phthalate (MEHP), di-isononyl phthalate (DiNP), mono-isononyl phthalate (MiNP), and monobenzyl phthalate (MBzP), are displayed in Figure 2.

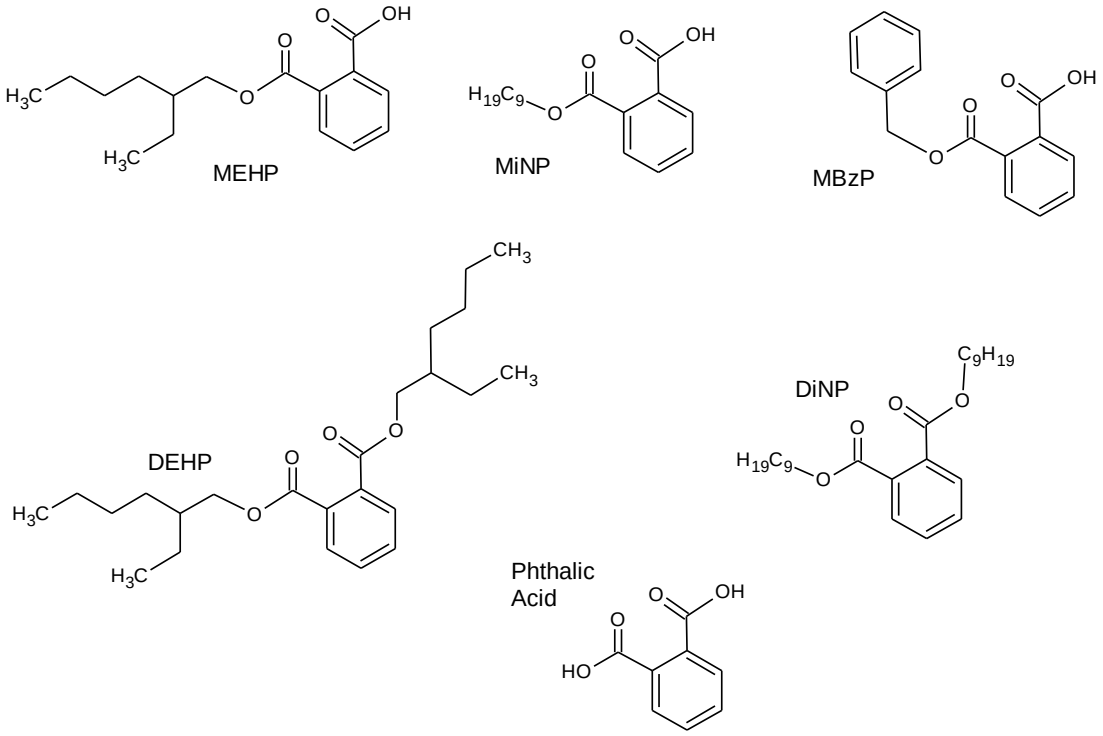

Figure 2: Phthalate Structures. The phthalates included are di-2-ethylhexyl phthalate (DEHP), mono-2-ethylhexyl phthalate (MEHP), di-isononyl phthalate (DiNP), mono-isononyl phthalate (MiNP), and monobenzyl phthalate (MBzP).

Experimental Background and Hypothesis

The US Centers for Disease Control regularly releases a report with statistical data on the exposure of various chemicals to humans living in the United States. The most recent report from February of 2015 found a geometric mean concentration of 11.0µg/mL MBzP, 3.43µg/mL MEHP, and no detectable level of MiNP in the urine of tested persons, among many other phthalate metabolites as indicators of phthalate exposure. [28] Phthalates like DEHP have been identified as potential carcinogens and as endocrine disruptors and the regular exposure to a large number of phthalates daily makes understanding the risks of regular phthalate exposure all the

more important. [29] The report presented here will further characterize the potential for risk to humans caused by exposure to common phthalates following their metabolism.

Research involving the activation of xenobiotic receptors including CAR, PXR, and PPAR has established that the presence of diphthalates in living systems swiftly induces processes to clear the phthalates from the host system. [30] It is further known that the major way that this clearance occurs is through biotransformation in which the target chemicals are made larger and more polar for ease of excretion. [25] While the metabolic response to the diphthalates is understood fairly well, the response of the same receptors to the metabolic products of these reactions is not well elucidated. As it is unreasonable to attempt to analyze all of the potential breakdown products of the diphthalates, this research project focuses on select but major metabolic products resulting from the breakdown of the diphthalates of interest.

To further characterize the activation of the xenobiotic receptors CAR2, PXR, PPARα, and PPARγ, a series of transactivation assays will be performed. Responses obtained from the diphthalate, DEHP, will be compared to MEHP, and DiNP will be compared with MiNP in order to characterize their respective activation profiles for their receptors of interest. A set of mammalian-two-hybrid assays will also be completed to quantify the interaction of the receptors of interest with the co-activator SCR1, in the presence of RXRα. These assays will only use the ligand binding domains of the receptors, as described in the methods section. MEHP, MiNP, and MBzP will serve as representative monophthalates for the activation of CAR2, PXR, and the alpha and gamma forms of PPAR.

All of the transactivation and mammalian two hybrid assays were performed in COS-1 cells transformed with plasmids containing the human genes of interest. COS-1 cells, immortalized *Cercopithecus aethiops* kidney fibroblasts, were used in the experiments as they

are primate-derived cells, but lack in their own endogenous expression of the xenobiotic receptors systems under study in these investigations. [31] COS-1 cells are CV-1 cells transformed with an SV40 plasmid expressing large T antigen which immortalizes the cells. To further show that the results obtained with the COS-1 cell models are representative of what occurs in human tissues, a set of primary hepatocytes obtained from donated human liver tissues via The Liver Tissue Procurement and Distribution System at the University of Pittsburgh Medical Center were assessed for changes in RNA involved in the expression of genes known to be associated with CAR, PXR, and PPAR activation. A quantitative real-time polymerase chain reaction (qPCR) will be performed on complementary DNA (cDNA) generated from the extract of the human hepatocytes.

I hypothesize that the monophthalates will activate the xenobiotic receptors in a similar manner to that of the diphthalates, but will do so with less potency as they have already undergone metabolism. Considering the comparison of monophthalates, I expect that MEHP and MiNP, which are the primary initial metabolites of the diphthalates, will be more potent activators of complex transcriptional complex formation than MBzP. Of note, MBzP has not been a well-studied phthalate, so the data obtained in these experiments will contribute to its comparative biology. I also hypothesize that the results obtained in the human liver hepatocytes will be concordant with those observed in the transactivation assays conducted in the COS-1 mammalian cell line.

Materials and Methods

Chemicals

DMSO (CAS #67-68-5), DEHP (CAS #117-81-7), DiNP Isomer Mixture (CAS #68515-48-0), WY-14643 (WY; CAS #50892-23-4), Troglitazone (TG; CAS #97322-87-7), and T0901317 (T0; CAS #293754-55-9) were purchased from Sigma-Aldrich (St Louis, MO). MEHP (CAS #4376-20-9), MBzP (CAS #2528-16-7), and MiNP (EDF-014) were purchased from AccuStandard (New Haven, CT). CITCO (CAS #338404-52-7) was purchased from BIOMOL International which is now Enzo Life Sciences, Inc. (Farmingdale, NY). All chemicals used were of 98% purity or greater with the exception of DiNP which exists as a mixture of C_9 isomers.

Cell Culture and Transfections

COS-1 cells were obtained from ATCC and stored in liquid nitrogen upon arrival. When needed, COS-1 cells were thawed at 37°C and were plated. The plating and incubation conditions as well as the media composition for COS-1 cell maintenance were all described previously. [16] The media used in the present study differs from the reference in that it also contains 1mM sodium pyruvate, the sodium bicarbonate concentration was reduced to 0.075%, the L-glutamine was not added, the media was made 1% Gibco Minimum Essential Media with Non-Essential Amino Acids, and the final concentrations of antibiotics were altered to be 100

units/mL penicillin G and 100μg/mL streptomycin. All media components were purchased from Thermo Fisher Scientific (Waltham, MA), except for the FBS which was purchased from Atlanta Biologicals (Norcross, GA).

Separate maintenance and experimental mediums were made and differed from each other in that the experimental medium made use of FBS treated with dextran coated charcoal, while the culture maintenance medium used untreated FBS. COS-1 cells were selected for this experiment based on their lack of expression of CAR when transfected with an empty vector. [26] COS-1 cells, when not in use, were incubated in a Sanyo Scientific CO_2 incubator at 37°C and an atmosphere of 5% CO_2. The cells were grown to confluency and then separated from one another through the use of a solution of 0.25% trypsin in Ethylenediaminetetraacetic acid (EDTA). Following separation, the cells were transfected.

Transfection methods for the COS-1 cells in both the transactivation and mammalian two hybrid assays were equivalent and have been presented previously [16], with the following changes: the 48 well plates were filled with cells in an approximate confluency of 50% based on observation under a light microscope, and the amounts and types of DNA constructs as well as the treatments used were altered as described individually below. All treatments were performed in triplicate and all luciferase experiments were repeated at least once. All treatment chemicals were diluted in DMSO. CITCO, T0, WY, and WY or TG served as positive controls for CAR2, PXR, PPARα, and PPARγ respectively. The final control concentrations for the transactivation assays were: 3μM CITCO, 0.1μM T0, 50μM WY, and 50μM TG. The mammalian two hybrid assays used the same control concentrations except PPARγ which received 50μM WY. Transfections incubated for 24 hours prior to treatment.

Two samples of human hepatocytes were acquired from The Liver Tissue Procurement and Distribution System in Pittsburgh and were maintained before treatment as described previously. [32] The background information for each of the donors can be seen in Table 1. The culture conditions for treatment of the hepatocytes has been reported previously. [33] The maintenance media was replaced on the cell cultures once a day for three days before a treatment media was placed on the cells. The treatments and concentrations used are as shown in the labels of the qPCR graphs in the results section. The cells were incubated with treatment media for 24 hours before the cells were washed with PBS (pH 7.4) and the RNA extraction and cDNA generation procedures were performed.

Table 1: Human Hepatocyte Donor Information. All data provided by the Liver Tissue Procurement and Distribution System in Pittsburgh and the personal notes of the lab member that received the samples.

Received	Number	Age	Sex	Source	Condition	NOTES
09-Apr-15	1	71	M	Resection	neuroendocrine tumor	Prior Chemoembolization
14-May-15	2	62	F	Resection	Potential HepatoCellular Carcinoma	No Prior Chemoembolization

Plasmids

The majority of the plasmid constructs used in the present experiment were generated and reported previously or were purchased from an established company. pTracer-RXRα [34], pTracer-CAR2, 2B6-XREM-PBREM, 3A4-XREM, pM-CAR2 LBD, pVP16-SRC1-RID, pcDNA 3.1-RXRa LBD [17], PPRE [35], and pM-PXR LBD [36] were reported previously in published works. The pRL-CMV *Renilla* reporter construct was purchased from Promega (Madison, WI), and the pFR-

Luc construct was purchased from Stratagene (now Agilent Technologies; Santa Clara, CA). pTracer-PXR, pTracer-PPARα, pTracer-PPARγ were made previously by members of the Omiecinski Lab through sub-cloning procedures. The primers used to create the pTracer-PXR, pTracer-PPARα constructs are displayed in Table 2; pTracer-PPARγ was verified to have the correct insertion through sequencing.

Table 2: Primer Sequences for Previously Created pTracer Constructs.

Construct	Restriction Site	Sequence
pTracer-PXR	Forward (EcoR1)	5'-GATCGAATTCGACATGGAGGTGAGACCCAAAGAAAG-3'
	Reverse (EcoRV)	5'-GATCGATATCTCAGCTACCTGTGATGCCGAACAAC-3'
pTracer-PPARα	Forward (Nhel)	5'-GGATCCGCTAGCCGGGTCATGGTGGACACGGAAAGCCCACTCTG-3'
	Reverse (Kpnl)	5'-GGATCCGGTACCTCAGTACATGTCCCTGTAGATCTCCTGC-3'

Transactivation Assay

A transactivation assay was used to demonstrate the activation of the nuclear xenobiotic receptors CAR, PXR, PPARα, and PPARγ in response to DEHP, MEHP, DiNP, and MiNP. The diphthalate and monophthalate metabolite pairs were compared in their responses. Transactivation assays add several plasmid constructs by transfection to a model cell system, here COS-1 cells, to enable modeling of a target system of interest, in this case a human hepatocyte. For this set of assays four different plasmids were transfected, one containing the full sequence of one of the xenobiotic receptors of interest, another with RXRα, a third with a DNA binding site for the xenobiotic receptor linked to a luciferase reporter gene, and a *Renilla* background reporter construct. When inside a cell, the xenobiotic receptor will be transcribed

and then translated into a protein which will interact with RXRα and subsequently bind the reporter plasmid to induce the production of firefly luciferase if a proper activating ligand is present. Thus, only when a sufficient amount of a ligand that activates the nuclear receptor of interest is present will a firefly luciferase signal be provided. The *Renilla* luciferase background construct is fully active at all times regardless of the presence of ligands to provide an expression baseline. The intensity of fluorescence for firefly luciferase is recorded and then chemically inactivated while *Renilla* luciferase is induced and then recorded. The *Renilla* luciferase is used to normalize the firefly luciferase values to allow for a comparison between the treatments and controls.

The luciferase reporter transfection assay procedure was previously detailed. [17] The constructs used, however, were somewhat different, listed here on a per well basis: 25ng of the full sequence of the target gene of interest in pTracer-CMV2 plasmid, 25ng of pcDNA 3.1 with RXRα inserted, 10ng of *Renilla* background reporter, and 100ng of 2B6-XREM-PBREM, PPRE, or 3A4-XREM for CAR2, PPAR, or PXR respectively. All treatments performed on the COS-1 cells are indicated by their labels within the figures in the following sections. All luciferase data were gathered through the use of a Promega Dual-Luciferase Reporter Assay System (Madison, WI) and a Turner BioSystems Veritas Microplate Luminoeter (Sunnyvale, CA). Figure 3 displays an example, using the CAR2 xenobiotic receptor, of what the proteins involved in a transactivation assay are understood to do.

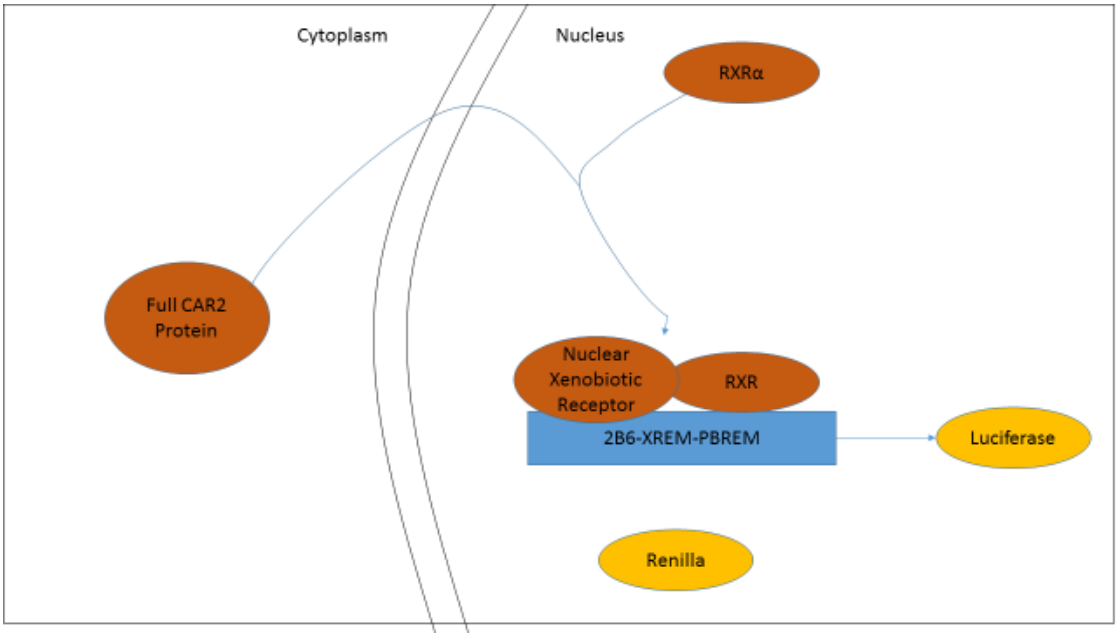

Figure 3: CAR2 as an Example of the Transactivation Assay. CAR2 is produced, binds a ligand and translocates to the nucleus where it binds with RXRα. The heterodimer then binds the reporter construct activating the production of firefly luciferase.

Plasmid Sub-Cloning for Mammalian Two Hybrid

A pM plasmid containing the PPARγ ligand binding domain (LBD) and the first 90 bases of the hinge region as an insert was created for use in the mammalian two hybrid assay. The human PPARγ sequence of interest was amplified from p-Tracer PPARγ via PCR that made use of Bioline Accuzyme DNA Polymerase (Taunton, MA). The primer sequence used to amplify the PPARγ gene sequence is displayed in Table 3. The PPARγ sequence of interest was isolated and concentrated by agarose gel electrophoresis and gel extraction using Denville Scientific Agarose HS (Holliston, MA) for the gel and following the manufacturer's instructions for the illustra GFX PCR DNA and gel Band Purification Kit (GE Healthcare, Piscataway, NJ), with the exception of the addition of a 3 minute spin following the wash step and a second elution step that moved the eluted DNA solution back to the top of the column. The concentration and quality

of the extracted DNA was determined with the use of a Thermo Scientific NanoDrop 2000 UV-Vis Spectrophotometer (Waltham, MA).

Table 3: PPARy LBD Primer Sequences for Sub-Cloning and colony PCR

Construct	Primer	Sequence
pM-PPARγ	Forward (BamHI)	5'-TGAGGATCCGTCAGTACTGTCGGTTTCAG-3'
	Reverse (HindIII)	5'-CTGCAAGCTTCTAGTACAAGTCCTTGTAG-3'

The purified PPARγ fragments and empty pM plasmid provided in the Clontech Matchmaker Mammalian Assay Kit 2 (Mountain View, CA) were sequentially digested by BamHI and HindIII-High Fidelity restriction enzymes from New England BioLabs Inc. (Ipswich, MA) following the provided instructions. A purification of the digested DNA fragments was performed after each digestion using the same supplies listed above, the first being a solution purification, and the second being a gel purification. The digested pM vector and PPARγ fragment were ligated together through the use of the New England BioLabs Inc. Quick Ligation Kit (Ipswich, MA) following the manufacturer's instructions for a 1:3 and a 1:5 molar ratio of insert to vector.

The ligated DNA was transformed into Bioline α-Select Chemically Competent Cells (Taunton, MA) using the recommended procedure, save for the use of 25 µL instead of 50 µL of competent cells. The transformed bacteria were plated on Fluka Analytical agar media (Sigma-Aldrich, Buchs SG, Switzerland) containing 0.05mg of ampicillin per mL of media. Cells were left to incubate for 24 hours at 37℃ and were subsequently stored at 4℃. A colony PCR was performed using the primers originally used to amplify PPARγ LBD to test for the presence of the intended insert; the portion of the colony not used in the PCR was added to LB broth media

containing 0.05mg of ampicillin per mL of media to grow up into a larger culture. The plasmids of a culture found by colony PCR to have the proper insert were extracted through the use of an Omega Bio-Tek EZNA plasmid DNA mini kit II (Norcross, GA) and a sample of the resulting plasmid solution was found to contain the correct insert in the correct reading frame by sequencing in the Penn State Nucleic Acid Facility (University Park, PA). The primers used for sub-cloning PCR and colony PCR were purchased from Integrated DNA Technologies (Coralville, IA).

Mammalian Two Hybrid

A mammalian two hybrid assay was performed using COS-1 cells as a model system to determine which of a set of monophthalates would induce the LBD of the xenobiotic receptors CAR2, PXR, PPARα, and PPARγ to interact with the receptor interaction domain (RID) of the co-activator Steroid Receptor Co-Activator 1 (SCR1) in the presence of the LBD of heterodimer partner Retinoid X Receptor alpha (RXRα). The mammalian two hybrid assay used a system of five transfected constructs to create an assayable system similar to human liver cells. Unlike the transactivation assay, a standardized reporting system was used that is based on the Clontech Matchmaker Mammalian Assay Kit 2 (Mountain View, CA). This system used only the parts of the proteins of interest that interact with one another, while all DNA binding, activation, and reporting components are part of a standardized system.

The mammalian two hybrid system used here contains two key constructs, one in a pM vector and a second in a pVP16 vector, and one supporting construct in a pcDNA 3.1 vector. The protein that results from the pM vector, contains a GAL4 DNA binding domain that is capable of

binding the pFR-Luc reporter and a portion of a protein of interest that is capable of binding to other related proteins. The protein from the pVP16 plasmid, contains a GAL4 activation domain for the reporter plasmid and the RID of SRC1, a co-activator of CAR2, PXR, and the PPARs. The supporting construct contains the LBD of RXRα to allow for heterodimerization with the LDB of the protein of interest in the pM construct. RXRα LBD is included as each of the xenobiotic receptors of interest requires binding to RXRα before the binding of co-activators like SRC1 can occur. The LBD of the nuclear receptor will interact with SRC1 and RXRα, only when ligand is present.

When all of the constructs are generated and active, the DNA binding domain of the pM construct will bind the GAL4 site on pFR-Luc. The constructs including parts of SRC1 and RXRα can then interact with the LBD attached to the pM construct. If a stable complex is made that includes at minimum the pM vector protein and the pVP16 vector protein, the activation domain from the pVP16 construct protein will activate the production of firefly luciferase and produce an assayable signal. This process is depicted in Figure 4.

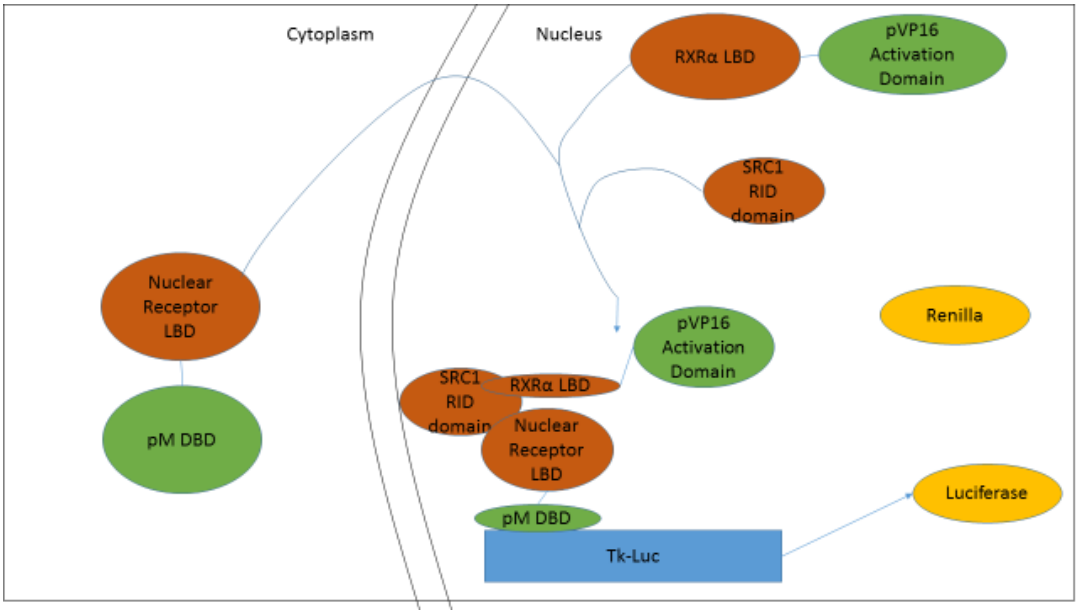

Figure 4: An Overview of the Mammalian Two Hybrid Assay.

The Promega Dual-Luciferase Reporter Assay System (Madison, WI) was used to generate and collect data here as well as in the transactivation assay; as such, the *Renilla* background reporter was used in this assay as well. The firefly luciferase reporter for this assay, however, is pFR-Luc which contains GAL4 DNA binding sites for general use with the standardized protein motifs of this assay. A Turner BioSystems Veritas Microplate Luminoeter (Sunnyvale, CA) was used to perform the assay data collection.

The process for conducting the mammalian two hybrid assay has been described previously. [17] The assays were conducted using for each well: 20ng of the ligand binding domain (LBD) of the gene of interest in pM for CAR2 and PXR or included the last 90 base pairs of the hinge region in addition to the LBD for PPARα and PPARγ, 20ng of the SRC1 RID domain in pVP16, 10ng of RXRα LBD in pcDNA 3.1 plasmid, 100ng of pFR-Luc reporter, and 10ng of

Renilla baseline reporter. The same assay kits and machinery were used in this assay as were used in the transactivation assay.

Human Primary Hepatocyte RNA Isolation and cDNA Reverse Transcription

Two samples of human hepatocyte preparations were provided by The Liver Tissue Procurement and Distribution System from the University of Pittsburgh Medical Center. The information for each donor is listed in Table 1. Following 3 days of cell maintenance as described in the cell cultures section above, 600µL of TRIzol Reagent was added to homogenize the hepatocytes. The RNA was then extracted from the samples following the manufacturer's instructions for the Thermo Fisher Scientific TRIzol reagent (Waltham, MA). The quality, purity, and concentration of the extracted RNA were determined using a Thermo Scientific NanoDrop 2000 UV-Vis Spectrophotometer (Waltham, MA). The extracted RNA was converted to stable cDNA through use of the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Waltham, MA) with a starting mass of 2µg of RNA for each treated tissue sample. The cDNA reaction held the reaction mixture at 25°C for 10 min., followed by 37°C for 2 hours, and finally 85°C for 5 min. Stable cDNA was stored at -20°C. The cDNA from each extraction was used in a series of quantitate real time PCR (qPCR) analysis tests.

Human Primary Hepatocyte qPCR Analysis

A set of qPCR experiments were performed with human primary liver tissue to determine which of a set of common metabolic genes was activated to help clear the presence of the monophthalates MEHP and MiNP and thus by interpretation which xenobiotic receptors were

activated. The genes of interest were: Cytochrome P450 family 2 subfamily B polypeptide 6 (CYP2B6), Cytochrome P450 family 3 subfamily A polypeptide 4 (CYP3A4), acyl-CoA oxidase 1 (ACOX1), Angiopoietin-like protein 4 (ANGPTL4), Carnitine palmitoyltransferase 2 (CPT2), and pyruvate dehydrogenase kinase isozyme 4 (PDK4). The expression of these genes has been linked to the activity of one or more of the xenobiotic receptors of interest for the present experiment. The expression of CYP2B6 and CPY3A4 are expressed in response to CAR and PXR activity [7], ACOX1 is associated with PPARα and PPARγ activation [8,9], ANGPTL4 activity is associated with PPARβ activation [37], CPT2 is associated with activation of PPARα [8], and PDK4 is associated with PPARα and PPARβ activity [8,6].

Quantitative PCR involves extracting a whole set of RNA from a primary tissue sample and using a reverse transcriptase polymerase chain reaction (rtPCR) to convert mRNAs into more stable cDNA. The cDNA is then used as a template for a series of PCR reactions with primers that allow the production of only one target gene per reaction. The DNA binding dye SYBR green is included with the reaction to bind and display the presence of stable double-stranded DNA, with a stronger signal indicating more DNA has been produced. Roughly 30 reaction cycles are conducted so that the increase in double-stranded DNA can be shown out to the limit of the available resources in the reaction to create new DNA. The PCR process is depicted in Figure 5.

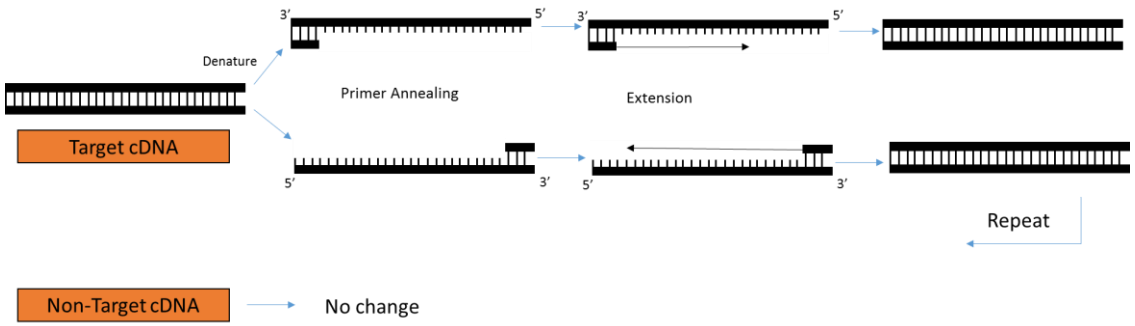

Figure 5: General Polymerase Chain Reaction (PCR) Mechanism. SYBR green binds to doubled stranded DNA and fluoresces to provide the signal measured in the assay.

The resulting sigmoidal curve of fluorescence from the reaction is used to determine the relative amounts of RNA that were in the primary tissue samples. The relative RNA levels are used to determine if a gene was up or down regulated in response to the presence of a phthalate. The specific amount of DNA in a sample is calculated through the use of a standard gene that is expressed at the same level regardless of treatment; for the present experiment glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference.

Each qPCR reaction was performed with a technical replicate within a pair of wells of a 96 well plate and used a Quanta Biosciences PerfeCTa SYBR Green SuperMix (Gaithersburg, MD) according to the recommended protocol, but split into 13µL reactions. Each reaction was created and split into 13µ aliquots to allow for a technical replicate; the un-split reactions included: 15µL of PerfeCTa SYBR Green SuperMix, 3µL of template cDNA at 20ng/µL, 1µL each of a 5µM forward and reverse primer, and 10µL of sterile water. The primers used to detect the presence of gene targets of interest are as displayed in Table 4. These primers were tested for their efficiency using a pooled sample containing cDNA of the positive controls, negative controls, and the highest treatment concentration for each phthalate serially diluted in tenfold steps into 6 samples. These samples were used to calculate efficiency of the primers and

determine the relative fluorescence unit (RFU) values at which target genes would be analyzed. All primers were found to be roughly 100% efficient and the calculations were treated as such. The assay itself was performed with a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Hercules, CA) set for a 12µL reaction with the following cycle: 45℃ for 5 min, 95℃ for 3 min, 95℃ for 15 sec, 60℃ for 1 min, repeat from 95℃ for 15 sec 39 times, and the cycle ended with a melt curve that ran from 65℃ to 95℃ increasing by 0.5℃ every 5 seconds. The efficiencies were determined and data was collected using the Bio-Rad CFX Manager program.

Table 4: Primers Sequences Used for qPCR Analysis. The CYP and ACOX primers were created in-house while all others were obtained from The Harvard Primer Bank.

qPCR Primer Name	Sequence (5'-3')
Hs_Acox1_352-371_FP	GAG CAG CAG GAG CGC TTC TT
Mm_Acox1_435-414_RP	AGT TCC ATG ACC CAT CTC TGT C
Human ANGPTL4_FP	GTC CAC CGA CCT CCC GTT A
Human ANGPTL4_RP	CCT CAT GGT CTA GGT GCT TGT
Human CPT2_FP	CAT ACA AGC TAC ATT TCG GGA CC
Human CPT2_RP	AGC CCG GAG TGT CTT CAG AA
Human PDK4_FP	GGA AGC ATT GAT CCT AAC TGT GA
Human PDK4_RP	GGT GAG AAG GAA CAT ACA CGA TG
Human CYP2B6_FP	AGA CGC CTT CAA TCC TGA C
Human CYP2B6_RP	GCG GAT TTG TCT TGG TGA AGG
Human CPY3A4_FP	GGC CCA CAC CTC TGC CTT
Human CPY3A4_RP	AAG CCC CAC ACT TTT CCA TAC TT

Statistics

All statistical analysis was performed in GraphPad Prism 6 and Excel. For the transactivation and mammalian two hybrid assays, the firefly luciferase values were normalized to the *Renilla* luciferase values for the three technical replicates in each test. These values were used to create a log agonist vs response curve, which was used to calculate the EC_{50} values. For

the qPCR fluorescence data, the delta-delta Ct values were calculated for each technical replicate in reference to the GAPDH control. These values were used to conduct a one-way ANOVA with a 95% confidence interval which compared the sample fluorescence values to the fluorescence values of the DMSO negative control.

Results

Transactivation Assay

A series of transactivation assays were performed in order to compare commonly used phthalates and one of their major metabolites' ability to activate a set of xenobiotic receptors. The activity of CAR2, PXR, PPARα, and PPARγ were each assayed in the presence of DEHP, MEHP, DiNP, or MiNP and the activation of each receptor was compared between the diphthalate and its corresponding monophthalate metabolite. The luciferase data were normalized against a *Renilla* luciferase background and is displayed in Figures 6 and 7. The EC_{50} values for the entire set of experiments are indicated in Table 5. Monophthalates are indicated on the graphs by blue lines, while black lines represent diphthalates.

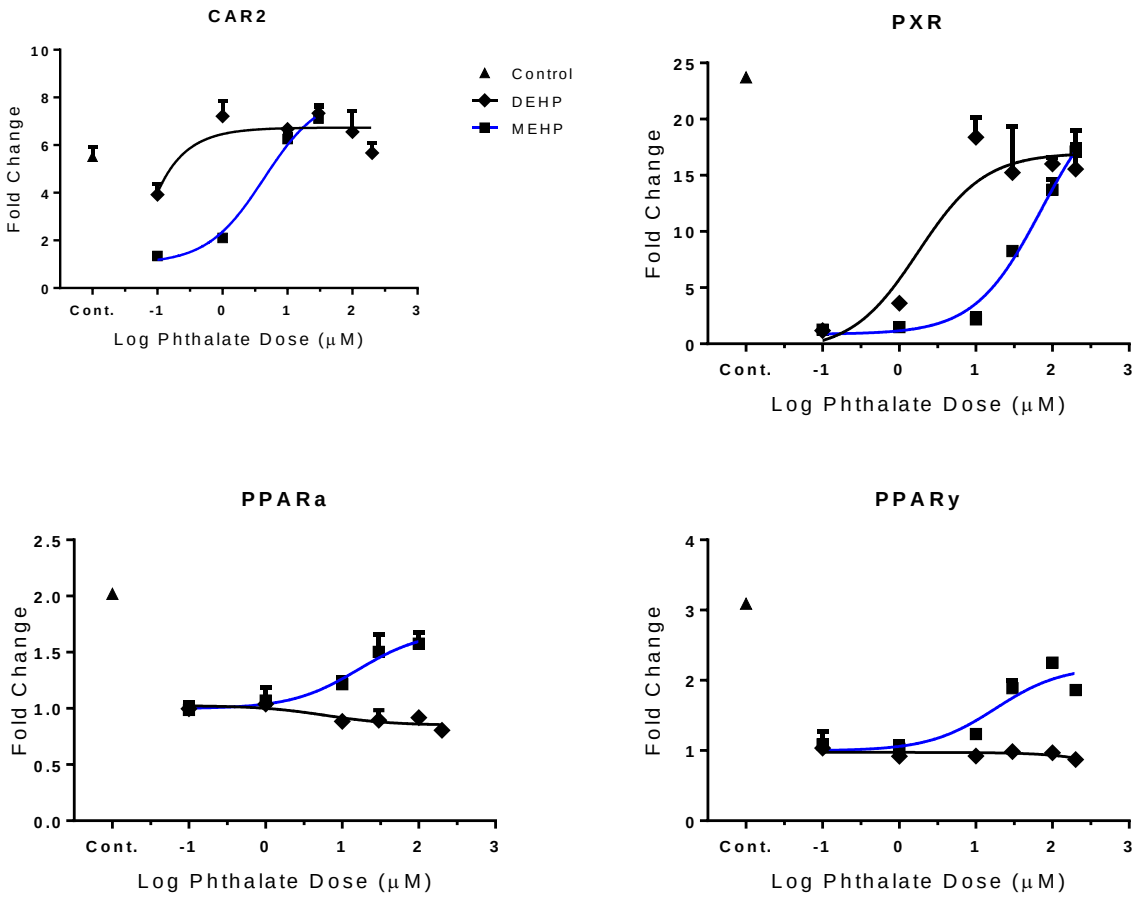

Figure 6: Activation of the 2B6-XREM-PBREM, PPRE, or 3A4-XREM Reporter Constructs by CAR2, PPARα and PPARγ, or PXR Respectively, in Response to Treatment with DEHP or MEHP.

MEHP induced activation of CAR2 and PXR; while the PPAR receptors, conversely, had less than a 2 fold change between the lowest and highest concentration, indicating little difference in activity. DEHP activated PXR and required a lesser concentration for median activation than MEHP. The activity of both PPAR genes, however, was not significantly altered by the presence of DEHP. The activity of CAR2 in DEHP implied that the concentrations used in the present experiment has saturated the system and only the lowest concentration approached a level of activation under the maximal amount. MEHP had a slightly higher maximal activation of both CAR2 and PXR as compared to DEHP.

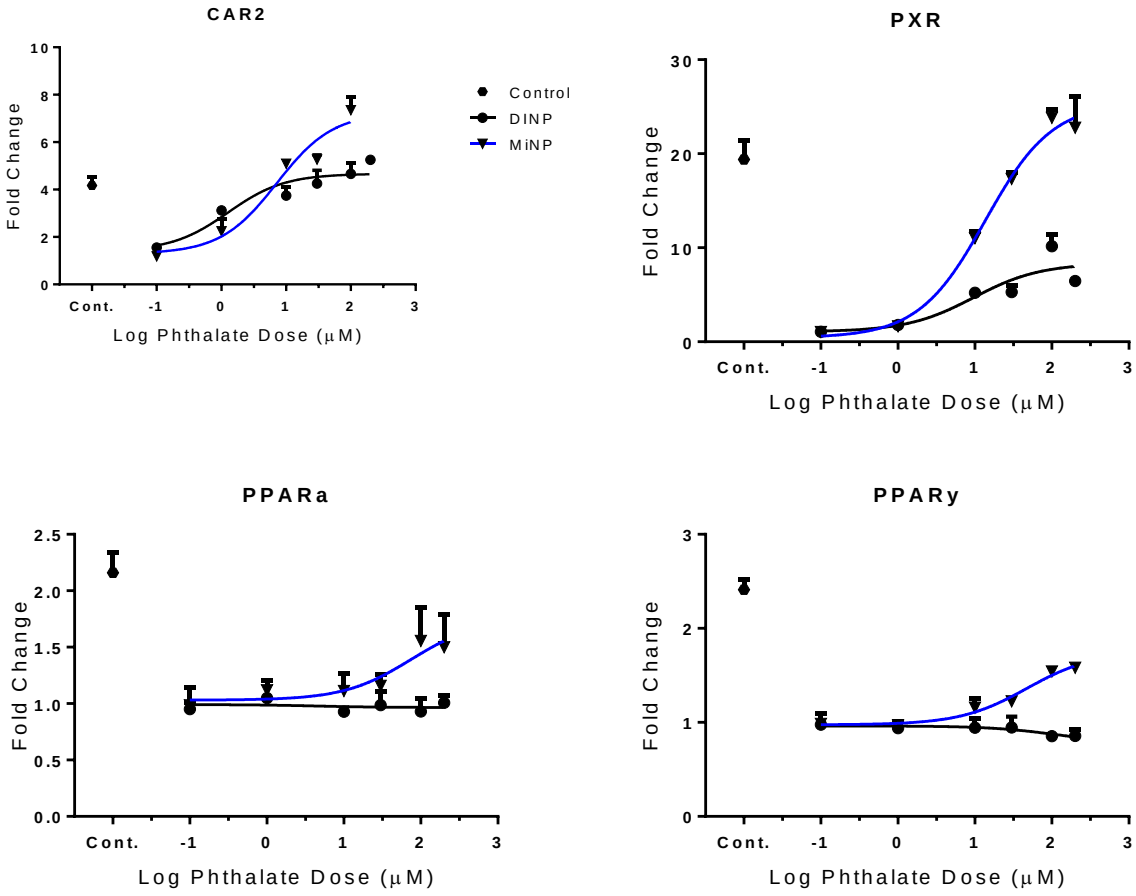

Figure 7: Activation of the 2B6-XREM-PBREM, PPRE, or 3A4-XREM Reporter Constructs by CAR2, PPARα and PPARγ, or PXR Respectively, in Response to Treatment with DiNP or MiNP.

DiNP induced a significant increase in activity of CAR2 and PXR, but had much less of an effect on the PPAR receptors, which did not noticeably respond even at the highest concentration. MiNP had a similar effect, activating CAR2 and PXR, and changing very little of the PPAR activity. MiNP induced a higher maximal activation of both CAR2 and PXR as compared to DiNP, and resulted in a much higher maximal activation of PXR capping at around a 25 fold increase.

Table 5: EC_{50} Values for Transactivation Assays. All values are shown in µM concentration.

Phthalate	CAR2	PXR	PPARα	PPARγ
MEHP	4.32	70.88	16.15	17.99
DEHP	N/A	1.762	6.091	N/A
MiNP	7.157	13.7	1.869	47.26
DiNP	0.1033	9.62	4.604	110

DEHP induced PPARα and PXR at 50% of maximal activation at much lower concentrations than that required by MEHP; CAR2 also fit this profile, but the interruption of the sigmoidal curve prevented the calculation of an EC_{50} value. The data collected here did not allow for this comparison with PPARγ as there was not enough information to make an interpretation on DEHP. CAR2 and PXR were both activated at 50 percent maximum by a lower concentration of DiNP. PPARα and PPARγ were activated at lower concentrations by MiNP than by DiNP, but it is of note that neither of these receptors appears to have been activated by DiNP at all.

These studies show that DEHP and DiNP, as well as their metabolites, MEHP and MiNP are potent activators of CAR2 and PXR. In contrast, only the monophthalates are capable of activating PPARs.

Mammalian Two Hybrid

Mammalian two hybrid assays were performed to measure the interaction of the LBD of the xenobiotic receptors of interest and the RID of SCR1 in the presence of the RXRα LBD. This series of experiments measured the initiation of the events immediately preceding xenobiotic receptor activation of transcription in the presence of the monophthalates MEHP, MiNP, and MBzP. The interaction between SCR-1 and the receptor indicates that the chemical tested binds to the active site of the nuclear receptor. The dose response curves resulting from these data can

be seen in figure 8, while the EC_{50} values are shown in Table 6.

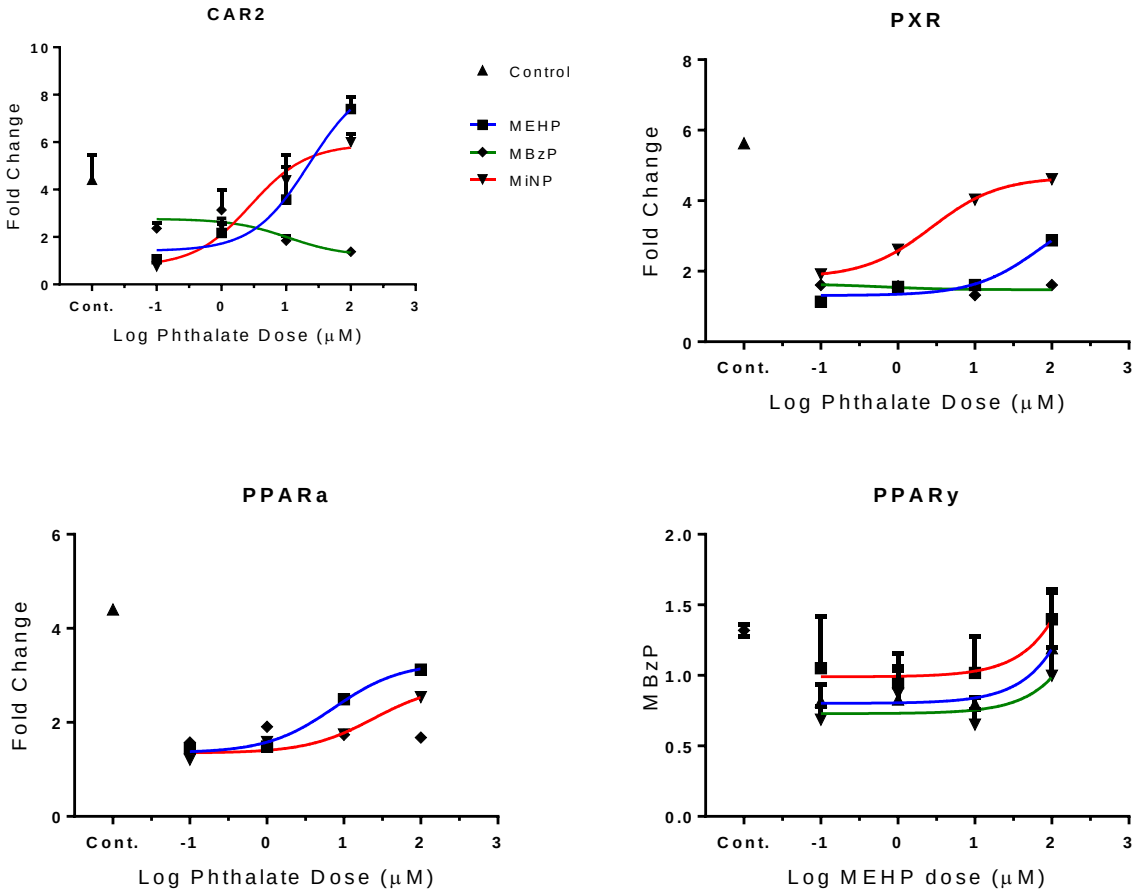

Figure 8: Activation of the pFR-Luc Reporter Construct by CAR2, PPARα, PPARγ, or PXR, in Response to Treatment with MEHP, MiNP, or MBzP.

CAR2 responded significantly to both MEHP and MiNP as was seen in the transactivation assays above, but the response to MBzP, while initially above a twofold change, decreased with increasing concentration. PXR similarly responded to MiNP, but had a far smaller fold change response than the transactivation assay at comparable concentrations of substrate. PXR was slightly activated by MEHP, but only at the highest tested concentration while MBzP did not induce a response whatsoever. PPARα displayed a similar response to that seen in the transactivation assays as both MEHP and MiNP induced a small change. MBzP did

not activate PPARα. PPARγ appeared to be slightly induced in response to all three monophthalates, but none of the data approached a twofold change.

Table 6: Mammalian Two Hybrid EC_{50} Data. All values are shown in µM concentration.

Phthalate	CAR2	PXR	PPARα	PPARγ
MEHP	22.44	72.20	7.45	N/A
MiNP	2.81	2.73	24.35	N/A
MBzP	10.69	0.62	N/A	N/A

CAR 2 and PXR followed a similar pattern of half maximal activation, with MiNP being a more potent activator of the receptors than MEHP. EC_{50} values were calculable for MBzP, but the minor change with concentration for CAR2 and the lack of any response from PXR implies that this data may be irrelevant. MEHP was a more potent activator of PPARα than MiNP, but both monophthalates produced only small responses from the receptor, even at the highest concentrations tested. MBzP did not induce a response from PPARα whatsoever at the concentrations tested. PPARγ only began to show a response to the monophthalates at the highest concentration tested according to Figure 8; this response, however, was not capable of producing EC_{50} value data.

These studies confirmed that the monophthalates MEHP and MiNP are potent activators of CAR2 and PXR, with little or no activity with PPARs.

Human Hepatocyte qPCR

Human hepatocytes from two donors were treated with one of a set of phthalates or other known agonist controls before RNA was extracted from the treated tissue and converted to cDNA. The cDNA was used in qPCR assays to determine the change in xenobiotic receptor

activity as compared to baseline production of GAPDH. One-way ANOVAs were performed on three total collections of samples from two donors and a representative sample of this data is show below in Figure 9, while all remaining data are presented in the Appendix.

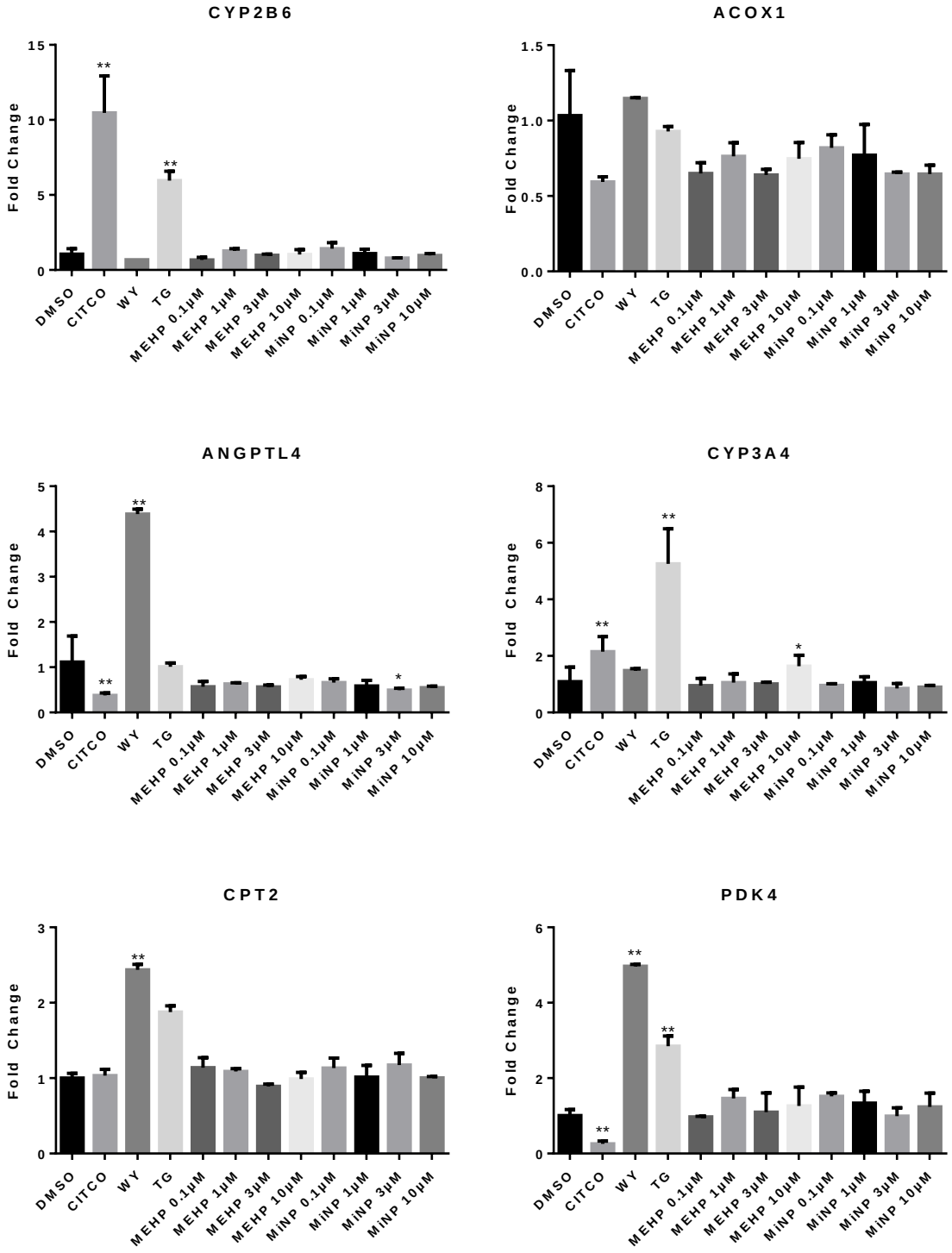

Figure 9: Donor 1 Technical Replicates 1 and 2 Combined Data for a Quantitative Real Time PCR Assay. Only target genes that are both statistically significant and have a minimum 2-fold change are marked as significant with "" marks. Genes with "*" have an error bar that crosses a 2-fold significance cutoff.**

The data presented in Figure 9 represents all of the data collected from donor one, and is representative of the data collected overall. The expression of CYP2B6 and CYP3A4 is associated with the activation of CAR2 and PXR receptors. CYP2B6 expression was activated by exposure to CITCO and TG to an extent well over the 2-fold cutoff. This suggests that either CAR2 or PXR was activated or that both were. WY did not have a notable effect on CYP2B6 activation in donor 1, but donor 2 (Shown in Appendix 1) had significant activation. Data collected on CYP3A4 suggests that TG and WY activate the receptors associated with its expression. CITCO only activated CYP3A4 in the second technical replicate for donor 1, but is shown as significant in the combined data. Also, only in donor 1, technical replicate 2 was a target phthalate activation observed, with MEHP at a concentration of 10μM reaching a greater than 2 fold change.

ACOX1 is activated by PPARα and PPARγ activity. Here, none of the chemicals tested resulted in a 2-fold change in expression. The first technical replicate for donor 1 did have a few genes inhibited to the point of a 2-fold reduction, but these results were not observed in the second technical replicate or in the second donor.

CPT2, an indicator of PPARα activation, was activated by the presence of WY only in donor 1, but not in donor 2 and none of the other tested chemicals had a notable effect. PDK4, which is expressed in response to both PPARα and PPARβ, was inhibited when the tested cells were in the presence of CITCO and activated in the presence of WY. TG was also activated, but only in donor 1. None of the tested phthalates had an effect at the tested concentrations.

ANGPTL4, a gene associated with PPARβ provided mixed results. CITCO was inhibitory to ANGPTL4 in both the second donor and the first technical replicate of the first donor, but not the second technical replicate. WY on the other hand was activated only in the

first donor and not in the second. Finally, both the first replicate of the first donor and the data for the second donor showed a significant inhibitions of ANGPTL4 in response to all of the tested phthalates all concentrations, but this inhibition was entirely absent in the second technical replicate for donor 1.

While much of the data for the qPCR assays may have been statistically significant, only data that was statistically significant and showed a 2 fold or more change beyond the control was considered biologically significant and was marked as such. It should be noted that the tissue samples used in the qPCR assays came from two different individuals with very different backgrounds and medical histories, and as such much of the inconsistency in the data may be attributed to these differences.

Discussion

The present study addresses the activation of the human xenobiotic receptors CAR2, PXR, PPARα, and PPARγ in response to the phthalates and phthalate metabolism products DEHP, MEHP, DiNP, MiNP, and MBzP.

CAR2

CAR2 was activated by DEHP, MEHP, DiNP, and MiNP in the transactivation assay experiments. Results from both transactivation assays indicated that the diphthalate was a more potent activator of CAR2 than its monophthalate metabolite. Perhaps this result occurred because the smaller, more hydrophilic MEHP molecule is difficult to bind a low concentrations. DEHP reached a plateau in luciferase expression at a lower concentration than the minimum used in this study, which resulted in the lack of a calculable EC_{50}. This information, however, has been reported previously as 0.211μM [14] which supports the above interpretations.

DEHP was previously found, in similar experiments, to be an activator of CAR2, but that study also reported only a minimal activation by MEHP at 10μM, which, here, approached the maximal level of activation achieved by DEHP.[14] The difference may result from the use of a larger number of cells added to the wells at the start of the experiments in the present assay, which could mask the effect of toxicity on the cells at high phthalate concentrations.

While CAR2 activity reached half maximal activation at lower concentrations in response to the diphthalates than the monophthalates it is also of note that in both cases the monophthalates also had a higher efficacy. This may have been a consequence of the potency of the diphthalates. At high enough concentrations, the phthalates can be toxic to the cells, as was

found but not displayed in the data for the CAR2 transactivation assays. High concentrations of the diphthalates may have inhibited the ability of the COS-1 cells to act and produce luciferase, thus plateauing the curve early. Alternatively, since the monophthalates are somewhat smaller than the diphthalates, this may open an opportunity for better binding in the flexible active site of the CAR2 receptor.

MEHP and MiNP were demonstrated to be activators of CAR2 in the mammalian two hybrid assay, while MBzP resulted in a slight decrease in activity at the tested concentrations. The mammalian two hybrid data demonstrated that CAR2 was activated by MEHP and MiNP at similar levels to those found in the transactivation assay. The luciferase values for MBzP response started and ended low and observations did not reveal overt toxicity to COS-1 cells, thus the system is not likely saturated and it is unlikely that MBzP induces CAR2 to bind its heterodimer partner or co-activators.

The experiments conducted in human liver tissue demonstrated no significant activation of CAR2 in either CYP2B6 or CYP3A4 in the presence of MEHP or MiNP in contrast with the transactivation and mammalian two hybrid assays. This may have been a consequence of the cell plating conditions used in the assay. The COS-1 cells were plated sparsely within a sterile flask, whereas the hepatocytes were plated on collagen and covered with Matrigel. The more protective plating conditions used to keep the hepatocytes viable could have also reduced the availability of the phthalates to the cells. If these conditions do reduce the amount of the phthalates that enter the cells, then the exposure level and thus the need for metabolism within the cells is lessened, resulting in a lower activation of the xenobiotic receptors and a lower activation of the associated metabolic genes. These effects may be seen further in that only the highest concentration of MEHP was capable of producing a measurable effect in the CYP3A4 expression. CYP2B6 and

CYP3A4 were, however, activated by exposure to CITCO and TG. One alterative is that the phthalates did enter the hepatocytes, but the metabolic enzymes already present in the cells were enough to reduce their concentration such that it was to low too activate CAR2.

PXR

PXR, like CAR2, was activated by both DEHP and MEHP with DEHP having a far lower EC_{50} and MEHP reaching a higher level of maximal activation. CAR2 and PXR's frequent association with one another as similar receptors makes this result reasonable. The transactivation assay involving DiNP and MiNP also showed DiNP as the more potent activator of the pair, but where there was only a small increase in efficacy caused by MiNP in CAR2, PXR increased from a roughly 8 fold activation above baseline in response to DiNP to a roughly 25 fold difference in activation when exposed to MiNP. It appears in this case that PXR is activated vastly more strongly by MiNP than by DiNP or any of the other phthalates tested. This marked rise in reactivity toward the metabolite may imply a better fit in the PXR active site from the smaller molecule. Alternatively, this result could indicate that MiNP is highly toxic to human hepatocytes as a swift clearance may be important for such as molecule. It is also possible that DiNP is less antagonistic than expected as the DiNP solution used contained a mixture of isomers, only some of which may activate PXR. When compared with a similar study from the literature, DiNP was found, much as it was here, to induce a dose based response to only a moderate degree. [26]

The mammalian two hybrid assay data were similar to the data derived in the transactivation assays, but with far lower overall fold changes. These assays are indirect

measurements of activation, so a different fold change may result than what would be observed in living tissue. Additionally, the interacting domains were attached to yeast GAL4 domains which are not native to the tested interacting domains and as such, the interacting domains may have needed to take on sterically unfavorable positions for interaction, thus reducing the overall amount of interaction. Finally, concerning the activation of the production of CYP2B6 and CYP3A4, it is not possible to tell based on the results of this test whether CAR2 or PXR or both were activated to produce the observed result. Determining whether the transactivation or mammalian two hybrid data were more accurate in its representation of PXR activity and why the CYP genes were not activated in the qPCR assays will require additional experiments.

PPARα

PPARα exhibited very little activation in the transactivation assays, with no results achieving above a 2 fold activation. A previous study described activation of human PPARα by MEHP and MBzP in a similar transactivation assay and reported a significant activation, though only just above a 2 fold change. [38] Both the Hurst and Waxman study and the data presented here displayed an initial rise in activity between 1μM and 10μM concentrations, but the data from the present study had a lower fold change. [38] Since the referenced study only observed a 2 fold change, slight alteration to the constructs used in the present study may yield similar results. [38] The results from the mammalian two hybrid assay for PPARα showed a rise above 2 fold in both MiNP and MEHP, but only slightly so and near the maximum concentrations used, while MBzP did not ever rise above the baseline.

For PPARα, the human hepatocyte data was more supportive of the data seen in the transactivation assays, as ACOX1, CPT2, and PDK4 all lacked biologically significant activation by the tested phthalates. However, this result makes it difficult to tell the difference between a lack of activation and a lack of availability of the molecule as described above. Considering the controls, the general agonist WY activated expression of CPT2 and PDK4. TG was also agonistic, but only for PDK4 expression. CITCO seemed to down-regulate PDK4 expression. While a 2 fold change did not occur, ACOX1 also approached a 2 fold reduction in activity in response to CITCO. This may be an indicator that CITCO was activating some other metabolic system and as a result, down-regulated PPARα; the CAR2 or PXR receptor systems seem reasonable candidates for this based on their response to CITCO in qPCR assays.

PPARγ

PPARγ was slightly, but not significantly activated by MEHP and MiNP in the transactivation assays; no points of data reached a 2 fold change. The diphthalates exhibited no notable activation at the concentrations tested. The same result was seen in the mammalian two hybrid assay and in the lack of activation of ACOX1 in the human liver samples. Despite this apparent lack of activation, it should be noted that the firefly luciferase values observed for the DMSO baseline control in the PPARγ transactivation assays and mammalian two hybrid assay were at or above the luciferase levels seen at maximal activation in the other constructs. This implies that PPARγ may have been maximally activated in all instances of these two assays. This result appears to makes sense in that PPARγ is known for its modulation of lipids. [11] Lipids existed in excess in media the cells were grown in for these assays, thus the phthalates did not

likely have an opportunity to have an effect on PPARγ activity as the lipids present in the media for the COS-1 cells had already activated it maximally. The lack of activation in the qPCR assays of ACOX1, which PPARγ regulates, can likely be attributed to the lack of availability of the phthalates as mentioned above. No real conclusions can be drawn from these data until these assays are repeated in a system devoid of known ligands for PPARγ.

Conclusions

The transactivation assays, mammalian two hybrid assays, and human hepatocyte qPCR assays further characterized the xenobiotic receptor response to a common set of phthalates. CAR2 was activated by DEHP, MEHP, MiNP, and DiNP in both transactivation and mammalian two hybrid assays, but not in the qPCR assay, while MBzP did not activate CAR2 noticeably. DEHP is an established activator of CAR2 and the results found here may support the conclusion that MEHP, DiNP, and MiNP are as well, at sufficient concentrations. MBzP was not supported as an activator of CAR2 in the present study. PXR was also activated by DEHP, MEHP, DiNP, and MiNP, but not MBzP. However, these interpretations were made with in vitro data and further studies in human or mouse systems will have to be conducted to demonstrate the concentrations or conditions required for CAR2 or PXR activation in animals, including humans.

The activation of PPARα in response to monophthalates MEHP and MiNP was similar to what is reported in the literature, while DiNP, DEHP, and MBzP all failed to activate this xenobiotic receptor. Few interpretations can be made about PPARγ activation from the data obtained in the present study due to the receptor's response to the lipids found in the media in which the assays were conducted.

Since the culturing system used for the hepatocytes mimics conditions found in the body the lack of activation may be an indicator that higher concentrations of phthalates than were used here are required for significant activation. The transactivation assay results supported established data in most cases, but the mammalian two hybrid assays needed to be repeated many times in different conditions to get a consistent result, including changing which proteins domains were placed in which of the constructs. This, in addition to receiving different results when the inserts were exchanged implies that many of the potential construct setups may not be representative of what is occurring in vivo.

Several conclusions can be drawn from the data collected in the present report. First, much of the transactivation assay data that has already been reported in other studies were supported by the data collected here, further implying that our current understanding of the xenobiotic receptors of interest is accurate. Second, the large response of PXR to MiNP exposure 25 fold over the standard could imply that PXR is well suited to binding the smaller, more polar MiNP metabolite than the expected environmental target DiNP. Additionally, the magnitude of the response could be an indication that hepatocytes have developed to remove MiNP quickly, a response that may imply toxicity. This could be tested further with the use of an in vivo model looking at both application of DiNP as a precursor to MiNP and the application of MiNP itself. Finally, the lack of a response from the human hepatocytes where transactivation assay produced a significant signal could indicate that transactivation and similar assays may overstate the potential risk of phthalates as they have been excluded from cells or immediately metabolized to an inactive form here. This ambiguity could be resolved with an additional experiment that compared transcriptional changes in liver cell cultures with and without a Matrigel matrix.

Further proof may be provided in separating the lever cells from the support matrix and attempting to extract the phthalates from the matrix.

A key point to consider in the results of this set of experiments is the contrast it highlights with existing data collected in mice. Human CAR2 has been previously reported to be activated by both DEHP and DiNP; [26] this was reinforced here as well as showing that MEHP and MiNP activate CAR2. Mice, however, have been previously shown to lack the CAR2 isoform. [14] Additionally, mouse PPARα and PPARγ has previously been reported to react to both MEHP and MBzP but at lower concentrations than the human PPAR genes, [38] while human PPAR was not activated by any of the phthalates tested here. Thus, mice may not serve as an accurate model for human response to phthalate exposure.

Appendix A

Additional Graphs

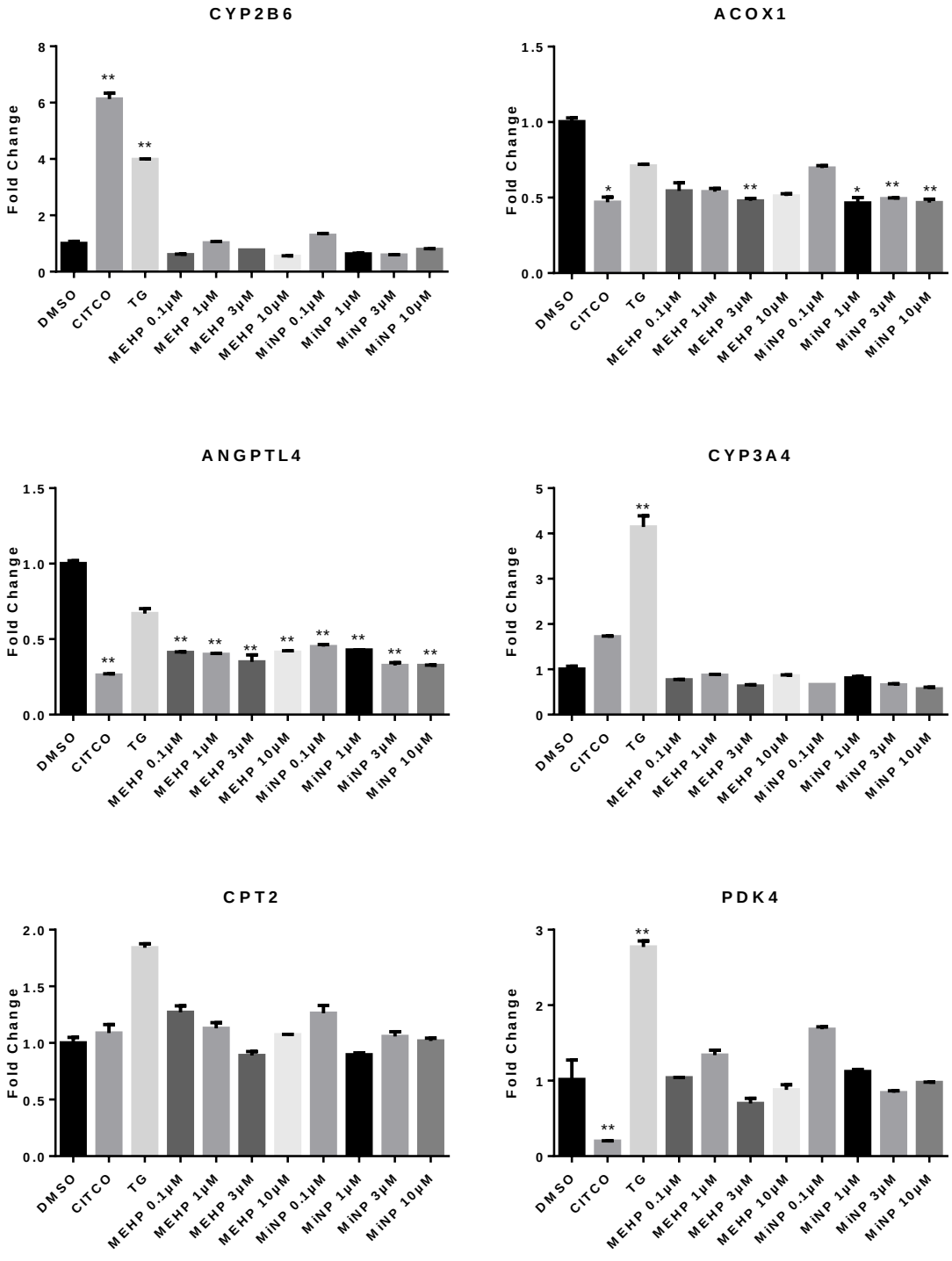

Figure 10: Human Hepatocyte qPCR Histogram for Technical Replicate 1 of Donor 1 Marked for Biological Significance

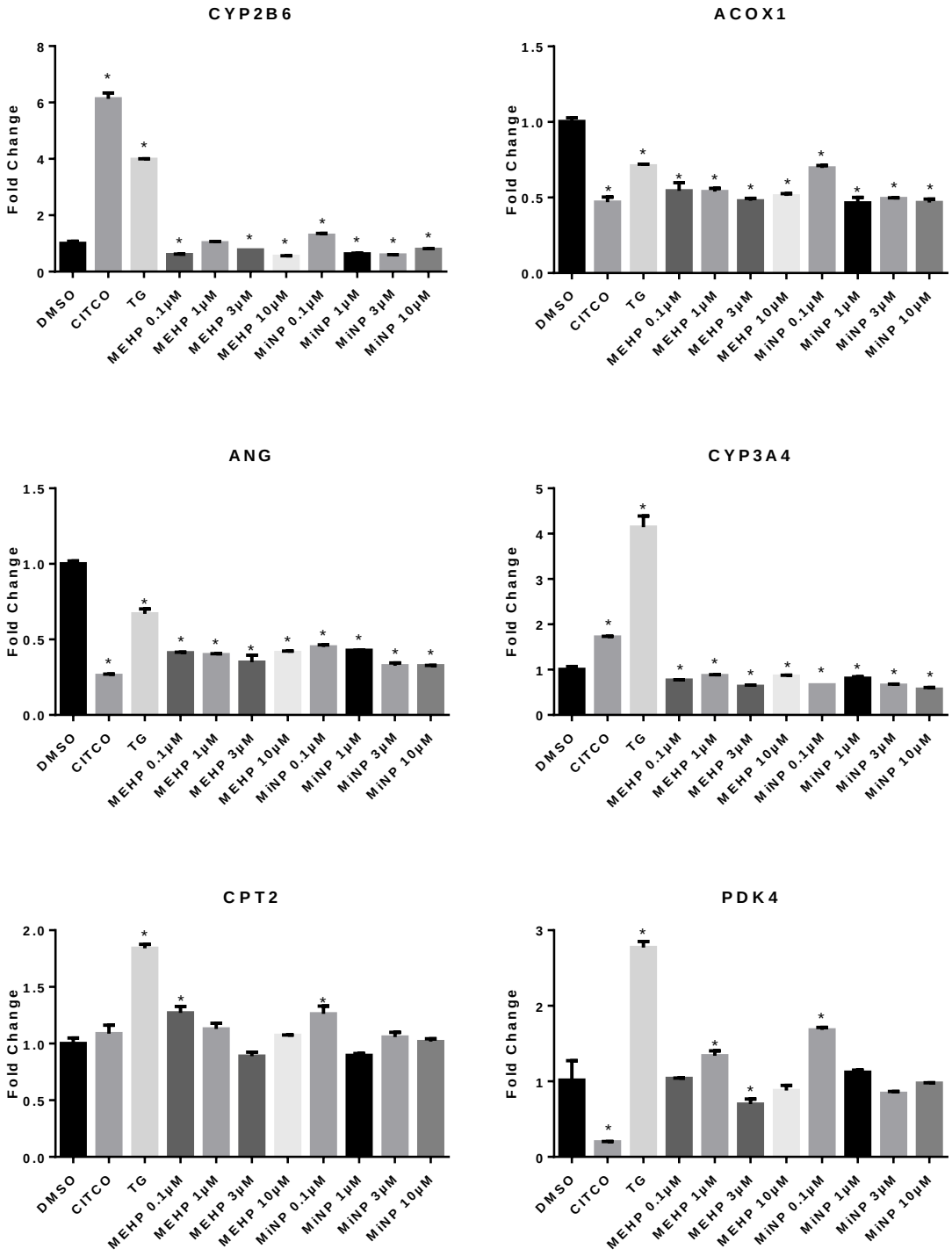

Figure 11: Human Hepatocyte qPCR Histogram for Technical Replicate 1 of Donor 1 Marked for Statistical Significance

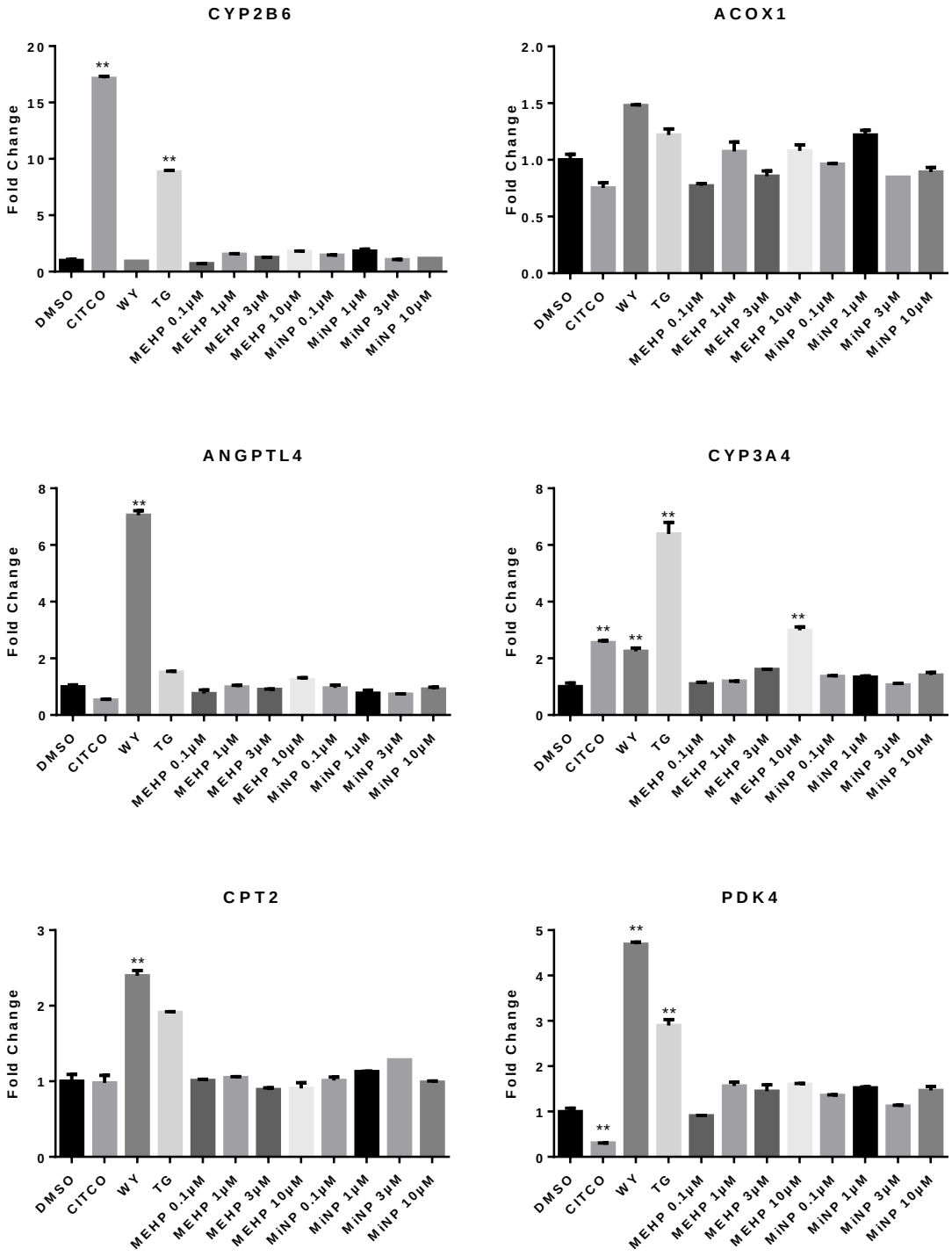

Figure 12: Human Hepatocyte qPCR Histogram for Technical Replicate 2 of Donor 1 Marked for Biological Significance

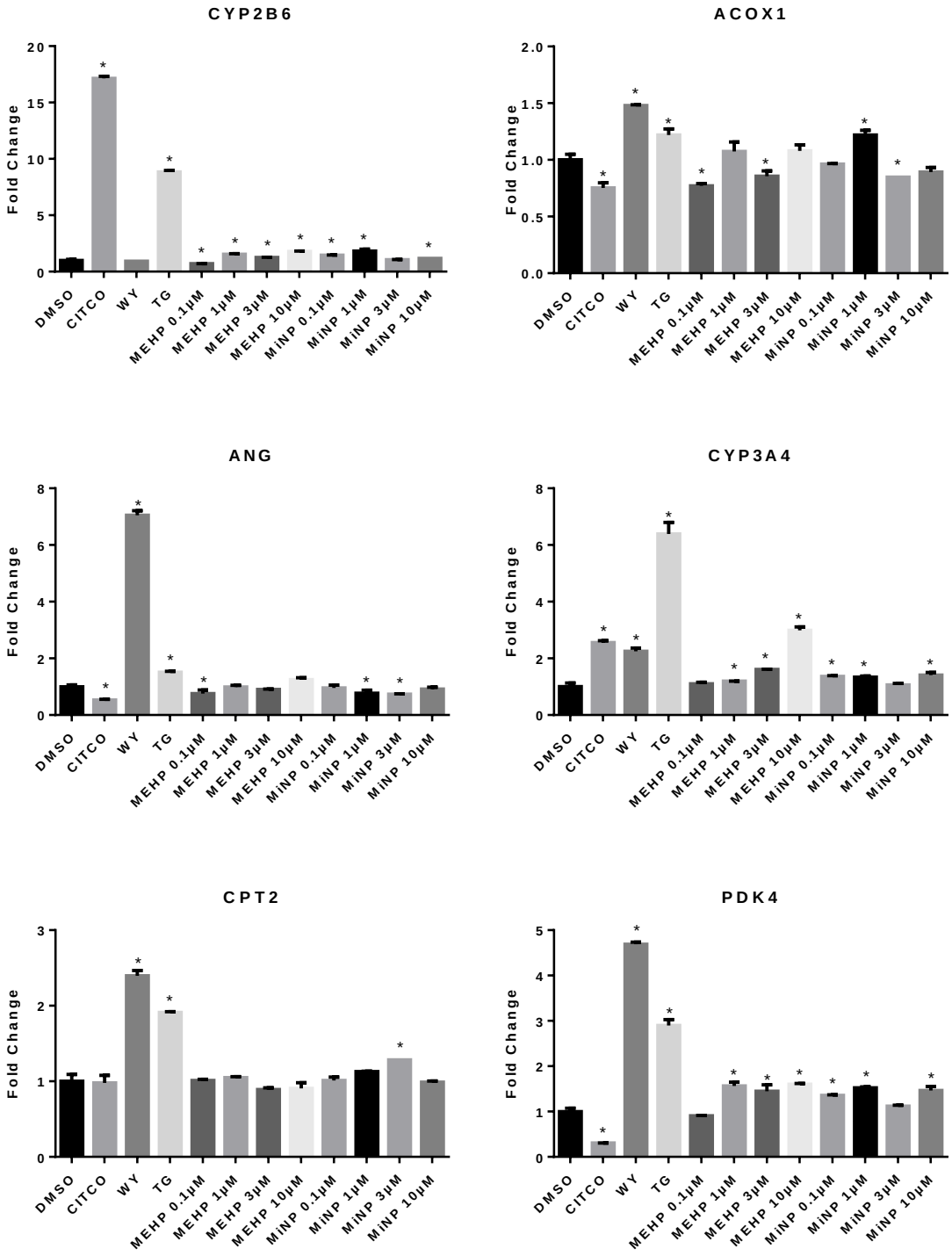

Figure 13: Human Hepatocyte qPCR Histogram for Technical Replicate 2 of Donor 1 Marked for Statistical Significance

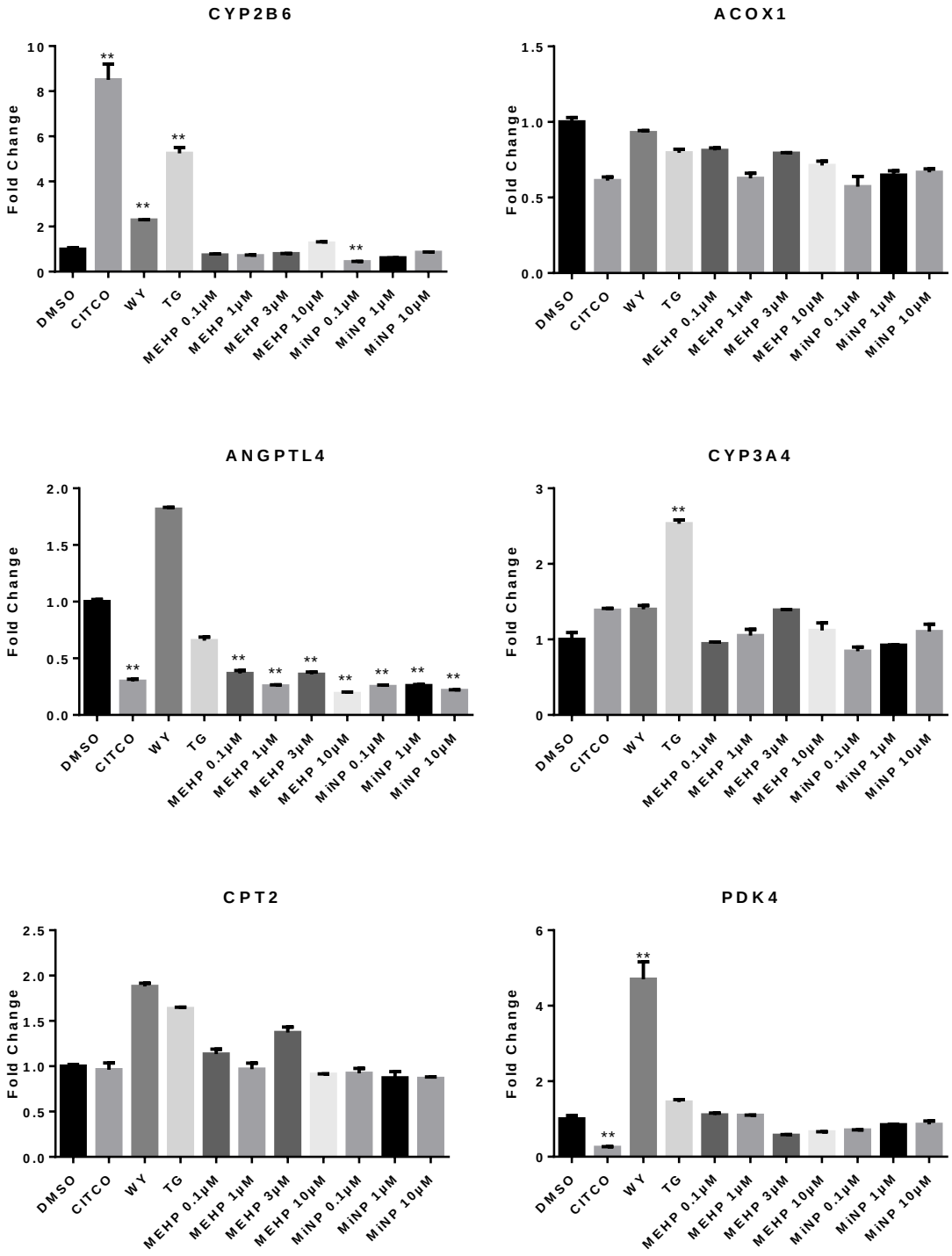

Figure 14: Human Hepatocyte qPCR Histogram for Donor 2 Marked for Biological Significance

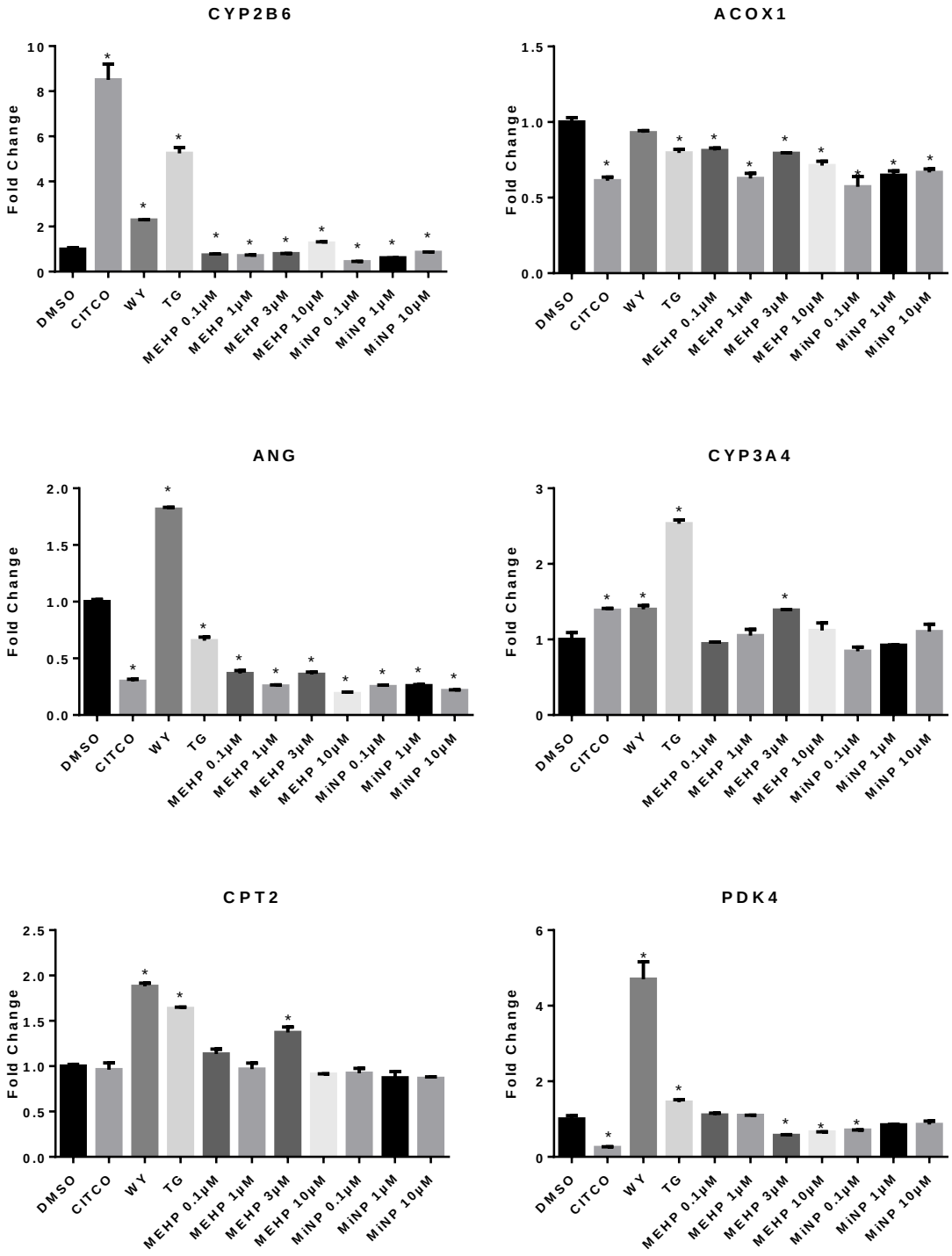

Figure 15: Human Hepatocyte qPCR Histogram for Donor 2 Marked for Statistical Significance

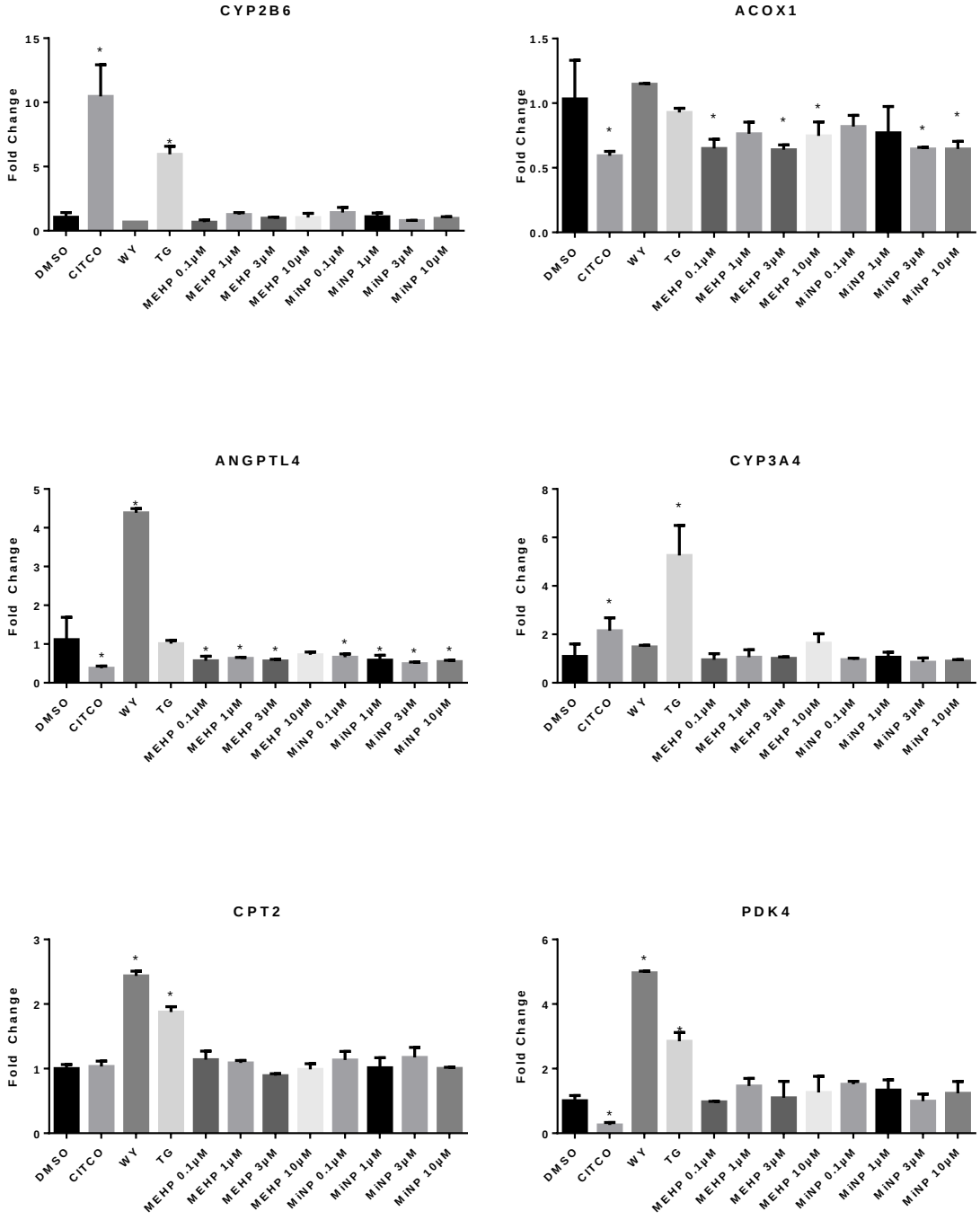

Figure 16: Human Hepatocyte qPCR Histogram for the Combined Data from Technical Replicates 1 and 2 of Donor 1 Marked for Biological Significance

BIBLIOGRAPHY

(1) Curtis D. Klaassen, and John B. Watkins III (Eds.). (2010) Casarett and Doull's Essentials of Toxicology Second. The McGraw-Hill Companies, Inc.
(2) Klaassen, C. D. (Ed.). (1996) Biotransformation of Xenobiotics, in *Casarett and Doull's Toxicology The Basic Science of Poisons* Fifth., pp 113–186. McGraw-Hill, Inc.
(3) Ma, Q. (2008) Xenobiotic-Activated Receptors: From Transcription to Drug Metabolism to Disease. *Chem. Res. Toxicol. 21*, 1651–1671.
(4) Honkakoski, P., and Negishi, M. (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J 347*, 321–337.
(5) Germain, P., Staels, B., Dacquet, C., Spedding, M., and Laudet, V. (2006) Overview of Nomenclature of Nuclear Receptors. *Pharmacol. Rev. 58*, 685–704.
(6) Degenhardt, T., Saramäki, A., Malinen, M., Rieck, M., Väisänen, S., Huotari, A., Herzig, K.-H., Müller, R., and Carlberg, C. (2007) Three Members of the Human Pyruvate Dehydrogenase Kinase Gene Family Are Direct Targets of the Peroxisome Proliferator-activated Receptor β/δ. *J. Mol. Biol. 372*, 341–355.
(7) Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T., and Kliewer, S. A. (2002) Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol. Pharmacol. 62*, 638–646.
(8) Mandard, S., Müller, M., and Kersten, S. (2004) Peroxisome proliferator-activated receptor a target genes. *Cell. Mol. Life Sci. CMLS 61*, 393–416.
(9) Rogue, A., Lambert, C., Jossé, R., Antherieu, S., Spire, C., Claude, N., and Guillouzo, A. (2011) Comparative Gene Expression Profiles Induced by PPARγ and PPARα/γ Agonists in Human Hepatocytes. *PLoS ONE* (Agoulnik, I., Ed.) *6*, e18816.
(10) Oltean, S., and Bates, D. O. (2014) Hallmarks of alternative splicing in cancer. *Oncogene 33*, 5311–5318.
(11) Omiecinski, C. J., Vanden Heuvel, J. P., Perdew, G. H., and Peters, J. M. (2011) Xenobiotic Metabolism, Disposition, and Regulation by Receptors: From Biochemical Phenomenon to Predictors of Major Toxicities. *Toxicol. Sci. 120*, S49–S75.
(12) Tolson, A. H., and Wang, H. (2010) Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv. Drug Deliv. Rev. 62*, 1238–1249.
(13) Kobayashi, K., Sueyoshi, T., Inoue, K., Moore, R., and Negishi, M. (2003) Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. *Mol. Pharmacol. 64*, 1069–1075.
(14) DeKeyser, J. G., Stagliano, M. C., Auerbach, S. S., Prabhu, K. S., Jones, A. D., and Omiecinski, C. J. (2009) Di(2-ethylhexyl) phthalate Is a Highly Potent Agonist for the Human Constitutive Androstane Receptor Splice Variant CAR2. *Mol. Pharmacol. 75*, 1005–1013.
(15) Lamba, J. K. (2004) Expression of Constitutive Androstane Receptor Splice Variants in Human Tissues and Their Functional Consequences. *J. Pharmacol. Exp. Ther. 311*, 811–821.

(16) Auerbach, S. S., Stoner, M., Su, S., and Omiecinski, C. J. (2005) Retinoid X Receptor- -Dependent Transactivation by a Naturally Occurring Structural Variant of Human Constitutive Androstane Receptor (NR1I3). *Mol. Pharmacol. 68*, 1239–1253.
(17) Auerbach, S. S., DeKeyser, J. G., Stoner, M. A., and Omiecinski, C. J. (2007) CAR2 Displays Unique Ligand Binding and RXR Heterodimerization Characteristics. *Drug Metab. Dispos. 35*, 428–439.
(18) Ueda, A., Hamadeh, H. K., Webb, H. K., Yamamoto, Y., Sueyoshi, T., Afshari, C. A., Lehmann, J. M., and Negishi, M. (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol. Pharmacol. 61*, 1–6.
(19) Kliewer, S. A., Goodwin, B., and Willson, T. M. (2002) The Nuclear Pregnane X Receptor: A Key Regulator of Xenobiotic Metabolism. *Endocr. Rev. 23*, 687–702.
(20) Desvergne, B., Feige, J. N., and Casals-Casas, C. (2009) PPAR-mediated activity of phthalates: A link to the obesity epidemic? *Mol. Cell. Endocrinol. 304*, 43–48.
(21) Abbott, B. D. (2009) Review of the expression of peroxisome proliferator-activated receptors alpha (PPARα), beta (PPARβ), and gamma (PPARγ) in rodent and human development. *Reprod. Toxicol. 27*, 246–257.
(22) Centers for Disease Control and Prevention. (2009) National report on human exposure to environmental chemicals. Department of Health and Human Services.
(23) Pak, V. M., McCauley, L. A., and Pinto-Martin, J. (2011) Phthalate Exposures and Human Health Concerns A Review and Implications for Practice. *AAOHN J. 59*, 228–233.
(24) Wittassek, M., Koch, H. M., Angerer, J., and Brüning, T. (2011) Assessing exposure to phthalates - The human biomonitoring approach. *Mol. Nutr. Food Res. 55*, 7–31.
(25) Anderson, W. A. C., Castle, L., Scotter, M. J., Massey, R. C., and Springall, C. (2001) A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Addit. Contam. 18*, 1068–1074.
(26) DeKeyser, J. G., Laurenzana, E. M., Peterson, E. C., Chen, T., and Omiecinski, C. J. (2011) Selective Phthalate Activation of Naturally Occurring Human Constitutive Androstane Receptor Splice Variants and the Pregnane X Receptor. *Toxicol. Sci. 120*, 381–391.
(27) Bility, M. T., Thompson, J. T., McKee, R. H., and David, R. M. (2004) Activation of Mouse and Human Peroxisome Proliferator-Activated Receptors (PPARs) by Phthalate Monoesters. *Toxicol. Sci. 82*, 170–182.
(28) The US Centers for Disease Control. (2015) CDC Fourth National Report on Human Exposure to Environmental Chemicals.
(29) U.S. Department of Health and Human Services. 13th Report on Carcinogens.
(30) Sueyoshi, T., and Negishi, M. (2001) Phenobarbital Response Elements of Cytochrome P450 Genes and Nuclear Receptors. *Annu. Rev. Pharmacol. Toxicol. 41*, 123–143.
(31) ATCC. (2014) COS-1 ATCC ® CRL-1650™ Cercopithecus aethiops kidney. *ATCC*.
(32) Page, J. L., Johnson, M. C., Olsavsky, K. M., Strom, S. C., Zarbl, H., and Omiecinski, C. J. (2007) Gene Expression Profiling of Extracellular Matrix as an Effector of Human Hepatocyte Phenotype in Primary Cell Culture. *Toxicol. Sci. 97*, 384–397.
(33) Goyak, K. M. O., Johnson, M. C., Strom, S. C., and Omiecinski, C. J. (2008) Expression profiling of interindividual variability following xenobiotic exposures in primary human hepatocyte cultures. *Toxicol. Appl. Pharmacol. 231*, 216–224.
(34) Auerbach, S. S. (2003) Alternatively spliced isoforms of the human constitutive androstane receptor. *Nucleic Acids Res. 31*, 3194–3207.

(35) Stoner, M. A., Auerbach, S. S., Zamule, S. M., Strom, S. C., and Omiecinski, C. J. (2007) Transactivation of a DR-1 PPRE by a human constitutive androstane receptor variant expressed from internal protein translation start sites. *Nucleic Acids Res. 35*, 2177–2190.
(36) Vanden Heuvel, J. P., Thompson, J. T., Frame, S. R., and Gillies, P. J. (2006) Differential Activation of Nuclear Receptors by Perfluorinated Fatty Acid Analogs and Natural Fatty Acids: A Comparison of Human, Mouse, and Rat Peroxisome Proliferator-Activated Receptor-alpha, -beta, and -gamma, Liver X Receptor-beta, and Retinoid X Receptor-alpha. *Toxicol. Sci. 92*, 476–489.
(37) Georgiadi, A., Lichtenstein, L., Degenhardt, T., Boekschoten, M. V., van Bilsen, M., Desvergne, B., Muller, M., and Kersten, S. (2010) Induction of Cardiac Angptl4 by Dietary Fatty Acids Is Mediated by Peroxisome Proliferator-Activated Receptor beta/delta and Protects Against Fatty Acid-Induced Oxidative Stress. *Circ. Res. 106*, 1712–1721.
(38) Hurst, C. H., and Waxman, D. J. (2003) Activation of PPAR and PPAR by Environmental Phthalate Monoesters. *Toxicol. Sci. 74*, 297–308.

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Related coursework:

- Honors Molecular and Cell Biology
- Metabolic Biochemistry
- Human Immunology and Virology
- Technical Writing
- Organic Chemistry
- Techniques in DNA and Protein Analysis

Undergraduate Research at Penn State

With Curt Omiecinski, PhD **August 2013 to present**
Studied human liver metabolic changes in response to plasticizer exposure

- Designed and optimized gene cloning procedure
- Performed and analyzed transactivation and mammalian two hybrid assays
- Collaborated on the writing of a journal article

With Dawn Luthe PhD **May to July 2012**
Compared expression of insect resistance protein to actual insect resistance in maize

- Extracted RNA from maize leaf and root samples
- Researched defense systems of related maize strains
- Collaborated with researchers performing similar work

Skills

Gene Cloning Design and Implementation
Phenol and Kit DNA/RNA Extraction
Reverse Transcription and Real Time PCR
Luciferase Assay Design and Analysis
Aseptic Technique
GraphPad Prism 6
Spectrophotometer Quantification
Gel Electrophoresis

Leadership Experience

Lead Example Small Business Startup for Entrepreneurship Class

- Organized team direction and efforts through semester long project
- Presented product idea and business plan to other entrepreneurial students

President of the Penn State Harrisburg Anime Association

- Organized trips to 3 conferences
- Grew club attendance from 10 to 30 members

Awards and Activities

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