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Los Angeles

**RNAi High Throughput Screenings Facilitate the Identification of Proteins
Necessary for TCDD-induced CYP1A1 Enzymatic Activity and Aid in the
Discovery of a Novel Role for Sin3A in AHR-Mediated Gene Expression**

**A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular Toxicology**

by

Parrisa Sherry Solaimani

2012

ABSTRACT OF THE DISSERTATION

RNAi High Throughput Screenings Facilitate the Identification of Proteins Necessary for TCDD-induced CYP1A1 Enzymatic Activity and Aid in the Discovery of a Novel Role for Sin3A in AHR-Mediated Gene Expression

by

Parrisa Sherry Solaimani

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2012

Professor Oliver Hankinson, Chair

The aryl hydrocarbon receptor (AHR) pathway is activated upon exposure to environmental pollutants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (B[a]P), which leads to the induced expression of several genes. Toxicity of AHR occurs through the bioactivation of procarcinogens and eicosanoids by its target genes. In this study we identified several proteins that modulate the induction of the AHR target gene *Cyp1a1*. For this purpose we optimized procedures for the use of an RNAi high throughput screening, as well as for the construction and use of endoribonuclease-prepared siRNA (esiRNA) to validate the screening results. Expression of PDC, CD9, TMEM5, and Sin3A were found to be necessary for the induction of CYP1A1 whereas expression of Rbm5, ARMC8, PDC,

CD9, TMEM5, Sin3A, Rab40C, Rad50, and Ube2i were necessary for both AHR expression and CYP1A1 induction. Additional studies were performed on the transcription factor candidate Sin3A, from which we found that Sin3A physically associates with the 5'-flanking regulatory regions of *CYP1A1* in both human and mouse cell lines, and may potentially act as a coactivator for *CYP1A1* induction. These studies established an essential role for Sin3A in the AHR-mediated induction of gene expression. We next examined the role of eicosanoids in AHR-dependent TCDD toxicity. CYP1A1, and other cytochrome P450s such as CYP2S1, can metabolize arachidonic acid into a variety of bioactive eicosanoids which play a significant role in the inflammatory response. From these studies we found that TCDD increased the levels of eicosanoids likely generated by cytochrome P450s in several tissues. Furthermore, these changes correlated with an increase in CYP1A1, CYP1B1, and CYP1A2 mRNA expression and were observed in wildtype mice but not *AhR* null mice. This demonstrated that these effects are mediated through AHR. Lastly, we explored dexamethasone-mediated regulation of CYP2S1. An initial screening looking for inhibitors of CYP2S1 revealed that dexamethasone, a glucocorticoid receptor (GR) ligand used to treat inflammatory diseases, represses its expression in multiple cell lines. We further found that dexamethasone regulates CYP2S1 via the GR and this occurs through the recruitment of histone deacetylases to the CYP2S1 promoter and enhancer.

The dissertation of Parrisa Sherry Solaimani is approved.

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DEDICATION

This dissertation is dedicated to my parents, Hedayat Solaimani and Susan Reed. The drive and motivation which have facilitated all my successes are attributable to my mother, who put forth so much effort during her life to influence my path and ensure I overcome adversity. The success of my efforts, however, are attributable to my father, who has sacrificed more than any parent should and has provided more continued support than most parents would.

TABLE OF CONTENTS

Abstract of the Dissertation	ii
Committee Page.....	iv
Dedication Page.....	v
Table of Contents.....	vi
List of Figures.....	ix
List of Tables.....	xii
List of Supplementary Materials.....	xiv
Acknowledgments.....	xv
Vita.....	xvii
Publications.....	xviii
Presentations.....	xix
Chapter 1: Introduction to the Dissertation	1
References	18

Chapter 2:	Development and Validation of an RNAi High Throughput Screening (HTS) Facilitates the Identification of Proteins Necessary for the AHR-Dependent Induction of Cyp1a1 by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin.....	25
	A. Abstract.....	26
	B. Introduction.....	28
	C. Materials and Methods.....	31
	D. Results	37
	E. Discussion	46
	F. Tables and Figures	52
	G. References.....	71
Chapter 3:	A Novel Role for Sin3A in the Induction of Cyp1a1 Expression by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin.....	75
	A. Abstract.....	76
	B. Introduction	78
	C. Materials and Methods.....	81
	D. Results	87
	E. Discussion.....	91
	F. Tables and Figures	96
	G. References	104
Chapter 4:	2,3,7,8-Tetrachlorodibenzo-p-dioxin Treatment Alters Eicosanoid Levels in Several Organs of the Mouse in an Aryl Hydrocarbon Receptor-dependent Fashion	107
	A. Abstract	108

	B. Introduction.....	108
	C. Materials and Methods.....	109
	D. Results.....	111
	E. Discussion.....	113
	F. References.....	115
	G. Supplementary Tables and Figures.....	117
Chapter 5:	CYP2S1 is negatively Regulated by Corticosteroids in Human Cell Lines	130
	A. Abstract	131
	B. Introduction.....	131
	C. Materials and Methods.....	132
	D. Results	132
	E. Discussion	134
	F. References	134
	G. Supplementary Tables and Figures.....	136
Chapter 6:	Conclusion to the Dissertation	139

LIST OF FIGURES

Figure 2.1: Methods for the Production of esiRNA.....	52
Figure 2.2: GST-RNaseIII Overexpression and Purification.....	53
Figure 2.3: Flowchart for the RNAi High Throughput Screening Workflow	54
Figure 2.4: RNAi High Throughput Screening Methods Validation	55
Figure 2.5: Quality Verification of PCR Products and esiRNA.....	56
Figure 2.6: esiRNA Screening Methods Validation.....	57
Figure 2.7: Transcript Levels of siRNA targets, CYP1A1, ALDH3A1, and NQO1 after siRNA Mediated Knockdown and TCDD Induction	58
Figure 2.8: Rab40C and CryGD are Necessary for TCDD-induced CYP1A1 Protein Expression and AHR Protein Expression.....	60
Figure 2.9: ARMC8, Sin3A, and TMEM5 are Necessary for TCDD-induced CYP1A1 Protein Expression but not for AHR Protein Expression.....	61
Figure 2.10: Workflow for EROD Rescue Methods	62
Figure 2.11: AHR Overexpression Rescues EROD Activity in the presence of siARMC8, Rad50, Rab40C, Rbm5, and CRYGD.....	63
Figure 2.12: Induction of Luciferase Activity by the pGL- <i>CYP1A1</i> Reporter Construct in the Presence of siRNA	64
Figure 2.13: Overexpression of ARMC8 or Rbm5 in the Presence of their Respective siRNA Rescues EROD Activity and Validates siRNA Results	65
Figure 3.1: Transcript levels of Sin3A, CYP1A1, ALDH3A1, NQO1, AHR, and ARNT in TCDD-induced Hepa-1 cells Transfected with siSin3A	75

Figure 3.2: Sin3A is Necessary for TCDD-induced CYP1A1 Protein Expression but not for AHR Protein Expression.....	76
Figure 3.3: Overexpression of AHR does not Rescue the Effects of siSin3A on TCDD-induced CYP1A1 EROD Activity.....	78
Figure 3.4: Sin3A Recruitment to the <i>CYP1A1</i> Promoter in TCDD-induced Hepa-1, HepG2, and MCF-7 cells.	79
Figure 3.5: Sin3A is Necessary for the Induction of the pGL- <i>CYP1A1</i> Luciferase Reporter Gene	81
Figure 1: Metabolism of Arachadonic Acid (and Linoleic Acid) by the Cyclooxygenase, Lipoxygenase and Cytochrome P450 Pathways.....	109
Figure 2: “Heat Map” Summarizing the Results from the Three Experiments Measuring Eicosanoids Levels.....	112
Figure 3: Effect of TCDD on Cyp1a1, Cyp1a2, Cyp1b1, and Pla2g12a mRNA Levels in Liver, Spleen, Lung and Heart of Male Wild-type Mice.....	113
Supplementary Figure 1: Effect of TCDD on PLa2g4a, Ephx2 and Ptgs2 mRNA Levels in Male Wild-type Mice Treated with TCDD or Vehicle	129
Figure 1: Dexamethasone Represses CYP2S1.....	132
Figure 2: A Variety of Corticosteroids Repress CYP2S1 mRNA Expression.....	133
Figure 3: Knockdown of Glucocorticoid Receptor Inhibits Repression of CYP2S1 by DEX.....	133
Figure 4: DEX does not Decrease the CYP2S1 mRNA Half-life.....	133

Figure 5: Trichostatin A Reverses Repression of CYP2S1 mRNA Expression by DEX.....	134
Supplementary Figure 1: DEX Induces Expression of GILZ.....	135
Supplementary Figure 2: DEX Represses CYP2S1 in AsPC 1 Cells.....	136
Supplementary Figure 3: Trichostatin A Reverses Repression of CYP2S1 mRNA Expression by DEX in AsPC1 Cells.....	137

LIST OF TABLES

Table 2.1: EROD and qPCR Data Summary for esiRNA Validated Hits from the High Throughput Screening.....	66
Table 2.2: RNAi High Throughput Screening Results from Known CYP1A1 Transcriptional Regulators.....	67
Table 2.3: Data Summary Addressing the Potential that the Effects of the Indicated siRNA on CYP1A1 Expression is Mediated through AHR	68
Table 2.4: GeneGo© Pathway Maps Relevant to the RNAi Screening Hits	69
Table 3.1: Sin3A is Necessary for CYP1A1 EROD Activity.....	42
Supplementary Table S-1: LC Gradient.....	117
Supplementary Table S-2: Optimized Mass Spectrometric Parameters.....	118
Supplementary Table S-3: Optimized Mass Transitions, Collision Energy for Each Compound, and Internal Standards used to normalize each Compound Measurement.....	119
Supplementary Table S-4: Inter-assay Validation. Comparison between Technical Replicates using %CV.....	120
Supplementary Table S-5: Intra-assay Validation. Comparison of the same Samples that was Measured on two Different Days based on % CV Values.....	121
Supplementary Table S-6: Real Time Primer Sequences.....	122
Supplementary Table S-7A: Data for Experiment 1.....	123
Supplementary Table S-7B: N/A.....	124
Supplementary Table S-7C: N/A.....	125

Supplementary Table S-7D: N/A.....	126
Supplementary Table S-7E: N/A.....	127
Supplementary Table 8: Data for Experiment 2.....	128

LIST OF SUPPLEMENTARY MATERIALS

Supplementary Table 2.1: RSA Analyzed Raw Data Results from the Druggable Genome RNAi High Throughput Screening

Supplementary Table 2.2: Table of the Top 93 Hits from the RNAi High Throughput Screening with both Justifications for Omitting Hits, and a Summary of the Data for Pursued Hits

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Chapter 2 represents a manuscript that will be submitted for publication. Authors that contributed to this work also include Parrisa Solaimani, Robert Damoiseaux and Oliver Hankinson. Chapter 3 also represents a manuscript that will also be submitted for publication. Authors that contributed to this work include Parrisa Solaimani and Oliver Hankinson. Chapter 4 is a reprint of: Bui P, Solaimani

P, Wu X, Hankinson O. 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment alters eicosanoid levels in several organs of the mouse in an aryl hydrocarbon receptor-dependent fashion. *Toxicol Appl Pharmacol*, 2012, 259(2):143-51. Parrisa Solaimani performed the mouse tissue RNA extraction, cDNA synthesis, and quantitative PCR analysis as well as assisted in the writing of the paper. Chapter 5 is a reprint of: Bebenek IG, Solaimani P, Bui P, and Hankinson O. CYP2S1 is negatively regulated by corticosteroids in human cell lines. *Toxicol Letters*, 2012, 209(1):30-4. Parrisa Solaimani contributed to the experimental design, lab work and writing of the paper.

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PUBLICATIONS

- Bui P, **Solaimani P**, Wu X, Hankinson O. 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment alters eicosanoid levels in several organs of the mouse in an aryl hydrocarbon receptor-dependent fashion. *Toxicol Appl Pharmacol*, 2012, 259(2):143-51.
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PRESENTATIONS

Solaimani P.S., Damoiseaux R. and Hankinson O. Identification of Proteins Involved in AHR-Mediated Cyp1a1 Induction Through RNAi High-Throughput Screenings. *Toxicologist*, 2012, 126(1):336.

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Chapter 1

Introduction to the Dissertation

Cytochrome P450s

The cytochrome P450's are an ancient and diverse superfamily of enzymes which are found in most organisms including animals, plants, and microorganisms (Hrycay and Bandiera 2009). These enzymes are potent oxidants that are known as monooxygenases for their ability to insert one of the two oxygens from O₂ into their substrate, while the second oxygen is reduced to a water molecule with the help of the electron donor NAD(P)H (Meunier et al. 2004). In this manner CYPs are involved in the phase I metabolism of xenobiotics and endogenous substrates, which enables lipophilic compounds to become more polar and thereby facilitates their excretion by the body. Since the polar metabolites can serve as reactive intermediates before being further metabolized by phase II conjugating enzymes, this can lead to either metabolic detoxification or bioactivation (Muruganandan and Sinal 2008). Xenobiotics metabolized by CYPs include drugs, carcinogens, polycyclic aromatics, arylamines, dioxins, polychlorinated biphenyls, anesthetics, solvents, pesticides and plant products; while endogenous substrates metabolized by CYPs include amino acids, steroid hormones, cholesterol, bile acids, prostaglandins, fatty acids, leukotrienes, vitamins, and lipid or steroid hydroperoxides (Nelson et al. 2004). There are 18 families of P450's in both mouse and human with 105 *Cyp* genes in the mouse genome and 58 *CYP* genes in the human genome (Hrycay and Bandiera 2009). *CYP* nomenclature is based on amino acid similarity of the enzymes. A 40% similarity means the *CYP*'s are in the same family, while greater than 55% similarity means that they are in the same subfamily (Martignoni et al. 2006). Within the cell, CYP enzymes are mostly confined to the endoplasmic reticulum (90%), with the remainder residing in the

inner mitochondrial membrane (Seliskar and Rozman 2007). Within tissues, CYP enzymes are expressed almost everywhere. The liver contains the greatest number of individual P450's, however, and along with the intestinal epithelial are the predominant sites of drug metabolism (Martignoni et al. 2006).

TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin)

TCDD is a halogenated aromatic hydrocarbon (HAH) that is a byproduct of combustion processes found in forest fires, volcanos, and chemical plants (White and Birnbaum 2009). It is known as one of the most toxic chemicals known to man but since TCDD is highly resistant to metabolic degradation its mechanism of toxicity is not through bioactivation, rather pathogenesis is caused by the chemical itself (Mimura et al. 1997; Rifkind 2006). Due to this resistance to biodegradation as well as its adsorption to sediment, the EPA predicts that TCDD may have a removal half-life of over 50 years in the environment. In the adult human the average half- life of TCDD is approximately 8 years regardless of exposure method, but since TCDD has lipophilic properties this appears to be somewhat dependent on weight and body fat composition (Miniero et al. 2001). The combination of a long half- life in the environment and its lipophilic properties permit TCDD to bioaccumulate, for example in fish and meat, and this can lead to unacceptable levels of exposure to humans through environmental sources (Frakes et al. 1993).

There have been many documented cases of TCDD exposure in isolated human populations including in Italy from a chemical plant explosion, in Vietnam from Agent Orange herbicide contamination, and in Ukraine from the poisoning of their president Victor Yushenko. Follow up studies from cases like these reveal

that adverse health effects of TCDD include cardiovascular disease, diabetes, cancer, immunological and reproductive diseases, altered metabolism and growth signaling, and abnormalities of the skin, teeth, and nails including chloracne (White and Birnbaum 2009). Both exposures in Vietnam and Italy led to an increase in all cancers, specifically lung, rectal, non-Hodgkin's lymphoma, myeloid leukemia, soft-tissue sarcoma, and chronic lymphocytic leukemia (Bertazzi et al. 2001; White and Birnbaum 2009).

The CYP1 Family

The CYP1 family contains 3 members consisting of CYP1A1, CYP1A2, and CYP1B1, whose function is predominately in the metabolism of xenobiotics (Rifkind 2006). As indicated by the absence of a phenotype in the knockout mice under 'normal' conditions, it appears CYP1 enzymes play no significant role in reproduction or physiological functions of mice, but their metabolism of xenobiotics and environmental toxicants makes the mechanism of regulation of these enzymes of particular interest to toxicologists (Dalton et al. 2000). The *CYP1* family has been studied as the hallmark of aryl hydrocarbon receptor (AhR)-mediated gene induction, particularly *CYP1A1*. AhR is a ligand activated transcription factor and although AhR binds numerous ligands, the most well-known of these include TCDD and benzo[a]pyrene (B[a]P). TCDD in particular is the most potent inducer of *CYP1A* (Mimura and Fujii-Kuriyama 2003) with mRNA induction levels over 100-fold as measured by qPCR (Hankinson 2005). B[a]P is a polycyclic aromatic hydrocarbon (PAH) which humans are environmentally exposed to from tobacco smoke, automobile exhaust, industrial by-products, and charbroiled foods (Ma and

Lu 2007). It has been well recognized that the mechanism of CYP1 toxicity is through the bioactivation of these PAH's, such as 7,12-dimethylbenz[a]-anthracene (DMBA) and B[a]P, to bay region epoxide metabolites which are highly reactive with DNA. Subsequent mutations that alter gene expression and cell cycle progression can ultimately lead to tumors and cancer (Androutsopoulos et al. 2009). This theory was tested by Buters et al in 2002, where they compared the presence of DNA adducts and tumors in *Cyp1b1* wildtype and knockout mice that were treated with DMBA. DNA adducts were found in all tissues that they examined in only the *Cyp1b1* wildtype mice, implicating that *CYP* expression is necessary for PAH-mediated toxicity (Buters et al. 2002).

***CYP1A1* Across Species**

There are 40 pairs of orthologous mouse-human *CYP* genes encoding enzymes with analogous amino acid sequences. Of those, 84% are evolutionarily conserved in their expression profiles (Hrycak and Bandiera 2009). *Cyp1a1* and *1a2* represent two such orthologous genes. The genomic structure of mouse *Cyp1a1* and *Cyp1a2* loci is similar to that of the human, particularly the 1.5kb region upstream from *1A1*, which is highly conserved. This makes the mouse a good model for studying the regulation of *CYP1A1* transcription (Nukaya and Bradfield 2009). Both human and mouse *CYP1A1* and *CYP1A2* are oppositely orientated and share a 5' flanking region with no open reading frames between them. Human *CYP1A1* and *1A2* genes are located on chromosome 15 and are separated by a 23.3kb segment. There are a total of 13 XRE's between *CYP1A1* and *1A2*, 7 of which are located within 1.3kb upstream from the *1A1* transcriptional

start site (Corchero et al. 2001). The mouse *Cyp1a1* and *Cyp1a2* genes are located on chromosome 9 and are separated by a smaller 13.9kb segment. There are a total of 8 XRE's between *Cyp1a1* and *1a2*, and analogous to humans, 7 XRE's are located less than 1.4kb upstream from the *1a1* transcriptional start site (Nukaya and Bradfield 2009). The cluster of XRE's and head to head orientations are also conserved among cattle, dogs and rats (Nukaya and Bradfield 2009). When sequence conservation is defined as more than 70% sequence identity over 50bp in length, the 1.5kb upstream region immediately proximal to the mouse *Cyp1a1* gene shares 55% sequence conservation with that of the human *CYP1A1* gene. It has been reported that two XREs in mouse (-488 and 981 from *Cyp1a1*) are fully conserved in the position and sequence with the cluster of human XREs (497 and -980 from *CYP1A1*), and all of them show AhR binding activity in vitro (Nukaya and Bradfield 2009). This suggests that these XRE sites could be universal AhR regulatory elements for *CYP1A1*. In terms of catalytic activity, both mouse and human CYP1A1 can catalyze the 3-hydroxylation of benzo[a]pyrene, as well as the O-deethylation of ethoxyresorufin (EROD); the latter which is used as a marker for catalytic activity of CYP1A1 (Androutsopoulos et al. 2009). With respect to its sequence, expression, and function CYP1A1 exhibits a high degree of interspecies conservation which provides evidence of the importance of this enzyme in mammalian biology (Muruganandan and Sinal 2008).

***CYP1A1* Regulation**

Upon exposure to and binding of ligands such as PAH's or HAH's, AhR dissociates from its chaperone proteins (hsp90, XAP2) and cochaperone protein

(p23) (Flaveny et al. 2010; Monostory et al. 2009). This dissociation permits AhR to enter the nucleus, where it can form a heterodimer with the Aryl hydrocarbon nuclear translocator protein (ARNT) and subsequently bind xenobiotic response element (XRE) core consensus sequences (5'-TNGCGTG-3') within the DNA (Monostory et al. 2009; Nukaya and Bradfield 2009). AhR and ARNT are both members of the bHLH-PAS family of transcription factors (basic Helix-loop-Helix and Per-ARNT-Sim) which is important for DNA binding and dimerization (Hankinson 2005). Once in the nucleus, the AhR/ARNT complex activates transcription by binding to at least one XRE from the cluster of XREs located 1.5kb upstream from the *CYP1A1* transcriptional start site. This mechanism is true for a number of target genes encoding for drug metabolizing enzymes such as CYP1A1, CYP1A2, and CYP1B1 (Whitlock 1999). These XREs are randomly arranged to allow for at least one heterodimer to bind, but it's unclear if multiple complexes can bind concurrently (Hankinson 2005; Nukaya and Bradfield 2009). Nucleosomal disruption over the promoter region (-60 to -200bp) of *CYP1A1* by the transcriptional activation domain (TAD) of AhR, is key in order to relieve repression over the promoter region and allow for the binding of transcription factors (Hankinson 2005). Chromatin remodeling is initiated by BRG-1 (a component of the SWI/SNF ATP-dependent chromatin-modeling complex), but various co-activator proteins displaying HAT (histone acetyltransferase) activity have been shown to associate with the enhancer region of *CYP1A1* in a ligand dependent manner (Wang and Hankinson 2002). These include CBP/p300, SRC-1 (steroid receptor coactivator 1), NCoA-2 (or SRC-2), and p/CIP (Beischlag et al. 2002; Taylor et al. 2009; Wang and Hankinson 2002). Acetylation of histones on their N-

terminal tails by these coactivators reduces histone-DNA affinity and nucleosome-nucleosome interactions. The Med220 and CDK8 subunits of the TRAP/DRIP/ARC/Mediator complex then associate with the *CYP1A1* enhancer and may function to convey regulatory signals from the enhancer to the promoter (Taylor et al. 2009; Wang et al. 2004). General transcription factors can then bind (Sp1, PolIII, TBP, TFIIB, TFIIF), as well as other more specific transcription factors which have been shown to associate with the promoter region including Rb (retinoblastoma protein), Mybbp1a (Myb binding protein 1a), ubiquitin-like protein Nedd8, BRCA1 (breast cancer 1), CoCoA (Coiled-coil coactivator), GAC63 (GRIP1-associated coactivator 63), and promyelocytic leukemia (PML) nuclear bodies (Akabane et al. 2007; Antenos et al. 2002; Beischlag et al. 2002; Chen et al. 2006; Elferink et al. 2001; Jones et al. 2002; Kang et al. 2006; Kim and Stallcup 2004; Kobayashi et al. 1996; Kobayashi et al. 1997; Kumar et al. 1999; Kumar and Perdew 1999; Puga et al. 2000; Rowlands et al. 1996; Swanson and Yang 1998; Taylor et al. 2009; Tojo et al. 2002; Wang and Hankinson 2002; Wang et al. 2004). These have been examined in mouse and human, however, not all were examined in both species or on the endogenous gene. In the field, it is generally assumed that there are more co-activators that are yet to be discovered and that further investigation is needed for a complete list of factors necessary for *CYP1A1* regulation (Androutsopoulos et al. 2009; Fujii-Kuriyama and Kawajiri 2010; Hankinson 2005). In addition, very few studies have focused on proteins involved in chromatin modification and epigenetic mechanisms as a means for regulation of these genes (Beedanagari et al. 2010; Deb and Bandiera 2009).

Negative regulation of *CYP1A1* is achieved by a feedback modulator of the AhR/Arnt pathway called AhRR (Aryl Hydrocarbon Receptor Repressor). AhRR is inducible in an AhR dependent manner, and can bind AhR and XRE's. Through this mechanism, it is thought to repress *CYP1A1* expression through competitive inhibition in both mouse and human (Akabane et al. 2007; Mimura and Fujii-Kuriyama 2003; Monostory et al. 2009). *CYP1A1* expression can also be inhibited by AhR receptor phosphorylation. Within the bHLH region of AhR there is a nuclear localization signal (NLS) which is masked by hsp90 in the absence of ligand binding. Binding of ligand exposes the NLS for nuclear localization, but entry into the nucleus in the presence of ligand can be inhibited by the phosphorylation of the NLS in both human and mouse (Ikuta et al. 2004).

CYP1A1 and Cancer

The overexpression of CYP1A1 in certain cancers makes it an important anti-cancer target to study. CYP1A1 in particular has been found to be overexpressed in human breast cancer (Brockdorff et al. 2000), esophageal carcinomas (Murray et al. 1994), urinary bladder tumors (Murray et al. 1995), and human lung adenocarcinomas; the later where CYP1A1 was suggested to be used as a prognostic and/or diagnostic tool for patients (Oyama et al. 2007). CYP1A1 mediated carcinogenesis due to DNA-reactive metabolites is particularly evident in tissues that are directly exposed to the procarcinogens. This is apparent from the presence of tumors on the tongue, esophagus and stomach in mice orally exposed to PAH's, as well as from the presence of tumors on the lungs of smokers who inhale B[a]P (Shi et al. 2010). Expression of CYP1A1, however, has been found to

be increased several tissues including in the lung (Thum et al. 2006), liver (Chang et al. 2003), placenta (Hakkola et al. 1997), and brain (Miksys and Tyndale 2006) of human cigarette smokers. Despite the ability of CYP1A1 to metabolically activate PAH's CYP1A1 is the most important CYP in detoxification of B[a]P, particularly in the intestine, and without it B[a]P will accumulate in the blood of treated mice causing a long term exposure, immunosuppression, and death (Shi et al. 2010).

CYP2S1

The human CYP2S1 gene is located on chromosome 19q in a cluster of CYP2 family members which share 47–49% sequence homology to CYP2S1, including CYP2A6, CYP2A13, CYP2B6, and CYP2F1 (Saarikoski et al. 2005a). Unlike many other cytochrome P450s, CYP2S1 is expressed at low levels in the liver but at high levels in extrahepatic tissue such as in the lung, skin, and colon (Saarikoski et al. 2005b). Identification of the CYP2S1 gene was achieved through a homology search aimed at discovering novel TCDD inducible genes (Rylander et al. 2001), as well as in our laboratory as a TCDD-inducible gene in mouse Hepa-1 hepatoma cells (Rivera et al. 2002). Despite the fact that TCDD and PAHs are strong inducers of *CYP1* family members, members of families 2 through 4 are not generally inducible in a similar manner which makes CYP2S1 unique in this regard (Hankinson 1995). In addition to TCDD, however, CYP2S1 has also been shown to be inducible by hypoxia via Hypoxia Inducible Factor I (Hif-1) and by all-trans-retinoic acid (Rivera et al. 2007; Smith et al. 2003). Further evidence of its induction by PAHs in humans is demonstrated by the elevated expression of CYP2S1 detected in smokers (Thum et al. 2006), and based on the findings in our laboratory that CYP2S1

catalyzes the oxidation of BaP-7,8-diol, it may play important roles in B[a]P-induced carcinogenesis (Bui et al. 2009). This is consistent with the observations that CYP2S1 expression has been found at high levels in many tumors of epithelial origin and has been associated with a poor prognosis in certain colorectal cancers (Kumarakulasingham et al. 2005).

The Aryl Hydrocarbon Receptor (AhR)

TCDD has been shown to induce a number of genes responsible for xenobiotic metabolism, previously named the AhR battery. In addition to the induction of genes in the *CYP1* family and CYP2S1, NAD(P)H: quinone oxidoreductase 1 (*NQO1*), UDP-glucuronosyltransferase 1a6 (*UGT1a6*), glutathione-S-transferase a1 (*GSTa1*), and aldehyde dehydrogenase 3a1 (*ALDH3a1*) have all been shown to be inducible by TCDD through an AhR-mediated pathway (Nebert et al. 2000). This has been demonstrated specifically in MCF-7 and Hepa-1 cells for *ALDH3* (Hsu et al. 2007; Takimoto et al. 1992). The aldehyde dehydrogenases are a family of NAD(P)⁺-dependent enzymes that catalyze the irreversible oxidation of many aldehydes to carboxylic acids (Xie et al. 1996) but the mechanism of their induction has not been examined as thoroughly as the *CYP*'s (Yeager et al. 2009). Previous studies have shown that the liganded AhR will form a heterodimer with ARNT and bind XREs 3kb upstream of the *ALDH3* transcriptional start site to initiate transcription in a manner similar to what is observed with *CYP1A1* (Boesch et al. 1999). This suggests that proteins involved in the regulation of *CYP1A1* may also be involved in the regulation of other AhR-inducible genes, but further investigation is needed.

In addition to its role in xenobiotic metabolism, AhR has been shown to have a plethora of physiological roles which is further supported by the presence of over 200 differentially expressed genes in AhR knockout mice (Mitchell and Elferink 2009). In the cell, AhR is necessary for G1 cell cycle progression, cell proliferation, differentiation, apoptosis, cell adhesion, and matrix metabolism (Kung et al. 2009; Puga et al. 2000). Physiological roles for AhR include teratogenesis, vascular development, inflammation, and other immune system problems (Fujii-Kuriyama and Kawajiri 2010; Kawajiri and Fujii-Kuriyama 2007). These endogenous roles for AhR have only recently been realized, and further investigation is needed for a full understanding of how AhR itself is regulated in the involvement of these various processes.

Eicosanoids

CYP enzymes are not only responsible for metabolizing xenobiotics, but also endogenous compounds such as steroid hormones and eicosanoids (Nebert and Karp 2008). The metabolism of omega-3 or omega-6 essential fatty acids by members of the CYP1, CYP2, CYP3, or CYP4 families leads to the synthesis of eicosanoids, although mutating any individual *CYP* does not result in physiological defects or lethality implicating that there may be some redundancy in their metabolism (Nebert and Russell 2002). In addition to eicosanoid synthesis, cytochrome P450's are also involved in eicosanoid degradation and the mechanisms by which CYP's bioactivate or degrade eicosanoids include monooxygenation, ω - and ω -1 hydroxylation, epoxidation, one electron oxidation, peroxidation, and isomerization (Capdevila et al. 2000). There are over 150 known eicosanoids in

mammals which belong to several classes including prostaglandins, prostacyclins, thromboxanes, leukotrienes, epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, hydroperoxyeicosatetraenoic acids , and lipoxins (Nebert and Karp 2008). These eicosanoids serve as signaling molecules, particularly for inflammatory responses and the central nervous system. Their effects on physiological responses, however, are vast including pro-inflammatory effects, anti-inflammatory effects, vasodilation, vasoconstriction, bronchiolar constriction and dilation, the recruitment of hematopoietic cells, and cancer (Nebert and Karp 2008). Several functions of eicosanoids are remarkably analogous to those of AhR, and from this observation it has been suggested that the toxicities of AhR could be mediated through *CYP1* induction and subsequent synthesis of eicosanoids (Nebert and Karp 2008). Although the role of the CYP1 subfamily in the synthesis and degradation of these signaling molecules has been established, the role of eicosanoids in TCDD mediated toxicity has not been previously explored.

Swi-independent 3a (Sin3a)

Sin3, or Swi-independent 3, was first identified in 1987 from a genetic screen in yeast as a negative regulator of the Hop (HO) endonuclease, which is responsible for mating type switching through its actions to cleave ds DNA (Sternberg et al. 1987). Since then Sin3a has been identified as a transcriptional regulator that is critical for several signaling pathways and associated biological processes such that the knockout mice exhibit an embryonic lethal phenotype. In addition to embryonic development Sin3a plays a role in DNA repair, chromatin modifications, gene transcription, and mitochondrial metabolism (Dannenberget al. 2005).

As a scaffolding protein Sin3a associates with several different proteins and thereby exerts its effects in specialized multi-subunit complexes. The structural motifs of Sin3a that serve as protein-interaction domains include 4 paired α -helices (PAH), a histone interaction domain (HID), and a highly conserved region (HCR) (Grzenda et al. 2009). Through the association of its HID motif with HDAC1/HDAC2 Sin3a is known to exhibit its main histone deacetylase enzymatic activity (Silverstein and Ekwall 2005). Histone deacetylation generally leads to repression of gene expression, and as such Sin3a has been predominantly identified as a co-repressor. Recent studies, however, provide accumulating evidence that Sin3a also plays a role in transcriptional activation including upregulation of *GAM3/ADR6*, *TMEM71*, and *MAPK Hog1* target genes (De Nadal et al. 2004; Ellison-Zelski and Alarid 2010; Yoshimoto et al. 1992; Ythier et al. 2010). In addition to HDAC activity, Sin3a has been shown to interact with several enzymatic proteins that are capable of histone methylation, DNA methylation, chromatin remodeling, and N-acetyl-glucosamine transferase activity (Ellison-Zelski and Alarid 2010). The structure and function of Sin3a in these complexes is not clearly understood, however, and remains an active area of research (Grzenda et al. 2009).

RNAi and endoribonuclease-prepared siRNA (esiRNA)

RNA interference is a mechanism whereby gene expression is inhibited at the transcription level by double stranded RNA. This is a biologically conserved mechanism that was first discovered in response to an injection of dsRNA into *C. elegans*, which lead to specific silencing of genes homologous to the dsRNA (Fire et al. 1998). This response has been described as a defense mechanism against viral

infections where the long dsRNA is digested by the enzyme Dicer (an endoribonuclease in the RNaseIII family) to small interfering RNA (siRNA) which are approximately 21bp in length (Ortiz-Quintero 2009). These siRNAs are incorporated into the RNA induced silencing complex (RISC), which can then match mRNA sequences in the cytoplasm for sequence specific targeted degradation. RISC cleaves the matching mRNAs such that the fragments are subject to degradation and are prevented from translating into functional proteins (Macrae et al. 2006). In mammalian cells, the dsRNA dependent protein kinase (PKR) binds dsRNA larger than 30 base pairs, non-specifically, and elicits an interferon (IFN)-induced antiviral pathway which shuts down general transcription and can lead to apoptosis (Henschel et al. 2004; Stark et al. 1998). Since dsRNA shorter than 30 base pairs does not elicit this IFN response, synthesized siRNA approximately 21bp in length are commonly transfected into mammalian cells to overcome this problem. RNAi has now emerged as a valuable tool to look at lack of function effects of particular gene targets and is used in a variety of scientific research.

High throughput screenings have recently gained popularity due to their ability to provide an abundant acquisition of data, but a major hurdle in large RNAi screenings is the presence of off-target effects and the resulting plethora of false positives that will need to be sorted through (Kittler et al. 2007). In addition, synthetic siRNA to different sequences within a gene has varied inhibitory ability so each mRNA must be screened for efficiency (Yang et al. 2002). Endoribonuclease-prepared siRNAs (esiRNAs) are another resource for RNAi experiments which offer a cost-effective alternative to current siRNAs available. In this method, esiRNAs are prepared through an RNaseIII digestion of an in vitro transcribed 400-600bp

fragment of dsRNA. This generates a pool of siRNAs, which enables efficient and specific silencing of a target mRNA, and therefore abolishes the need to identify effective silencers for each mRNA (Kittler et al. 2004). In addition, esiRNAs have been shown to be just as effective if not more effective than synthesized siRNAs yet have a 12-fold reduction in off-target effects (Kittler et al. 2007). For effective siRNA mediated sequence specific silencing, certain properties of the siRNA must be fulfilled including sequence length, GC-content and nucleotide composition (Henschel et al. 2004). DEQOR (design and quality control of siRNAs for RNAi) is a website that is used to determine the optimal region of the target transcriptome which satisfies these requirements for esiRNA construction, as well as ensuring minimal cross-silencing. This is accomplished by BLASTing the proposed sequence against the species entire genome in 21bp fragments, while sliding 1 nucleotide over at a time (Henschel et al. 2004). The region with the least amount of cross silencers, and optimal region for siRNA mediated sequence specific silencing gets the highest score. RIDDLE (RNAi by DEQOR-designed lookup of esiRNAs) is a website that takes this a step further and takes the 400-600bp fragment with the highest score and subjects it to a Primer design website, Primer 3 (Kittler et al. 2007). Previous use of esiRNAs in high throughput screenings has led to the discovery of genes that induce cell cycle arrest upon silencing (Kittler et al. 2007), as well as to the identification of chromatin proteins that regulate embryonic stem cell identity (Fazzio et al. 2008). These studies demonstrate that esiRNAs are a useful tool for performing RNAi high throughput screenings.

Conclusions

In this study, we investigated the AhR-mediated induction of *Cyp1a1* expression with the use of an RNAi high throughput screening. These methods have not been explored for this purpose and Chapter 2 summarizes the optimization of these methods as well as our results. The production and use of esiRNA was also implemented for subsequent data validation. In Chapter 3 of the dissertation we examine a promising candidate hit from our screening further and aim to determine if the transcriptional regulator, Sin3a, is necessary for the TCDD mediated induction of *Cyp1a1*. Sin3a has not previously been identified as a regulator of AhR-mediated gene induction, and the role of Sin3a as an activator of gene expression has not been widely explored. We next looked downstream of *Cyp* expression in Chapter 4 to determine the role of eicosanoids in AhR-dependent TCDD toxicity. CYP1A1 and other cytochromes P450s can metabolize arachidonic acid into a variety of bioactive eicosanoids which can play a significant role in the inflammatory response. CYP2S1 is one such example, thus in Chapter 5 we explored the negative regulation of CYP2S1 by various anti-inflammatory corticosteroids. The results from these studies provide valuable insight into the regulation of cytochrome P450's as well as the resulting toxicity of their induction.

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Chapter 2

Development and Validation of an RNAi High Throughput Screening (HTS) Facilitates the Identification of Proteins Necessary for the AHR-Dependent Induction of CYP1A1 by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

ABSTRACT

The aryl hydrocarbon receptor (AHR) has been shown to have a plethora of physiological roles, and upon dysregulation, carcinogenesis can occur. One such target gene of AHR encodes the xenobiotic metabolizing enzyme CYP1A1, which can be induced by the environmental contaminants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (B[a]P) via the AHR. With the use of high throughput technologies we set out to identify proteins that modulate the AHR-dependent induction of *Cyp1a1*. For this purpose we optimized procedures for an RNAi high throughput screening, and also for the production of endoribonuclease-prepared siRNAs (esiRNA). An siRNA library targeted against over 5,600 gene candidates in the druggable genome was used to transfect the Hepa-1 murine hepatic cancer cell line, which were next treated with TCDD, and then assayed for CYP1A1-dependent ethoxyresorufin-o-deethylase (EROD) enzymatic activity. Following redundant siRNA activity (RSA) statistical analysis we identified 93 hits with a p-value ≤ 0.005 , and confirmed 39 of these as true positive hits in a secondary screening using esiRNAs. We subsequently discovered 12 genes whose expression is necessary for the induction of both CYP1A1-dependent EROD activity and CYP1A1 mRNA. Another 5 candidates tested out of these 12 were also confirmed to be necessary for CYP1A1 protein induction. To further validate our candidates we examined ARNT and AHR expression, which excluded the possibility that diminished ARNT mRNA levels by the indicated siRNA treatments were responsible for our results; however, there were a number of candidates whose expression was necessary for both AHR mRNA and protein expression. By overexpressing AHR in cells treated with the indicated siRNA, we rescued CYP1A1 EROD activity and

determined that the effects on CYP1A1 induction by certain siRNAs were mediated through AHR. These studies revealed that ARMC8, Ube2i, Rab40C, Rbm5, CryGD, Rad50, Tcf20, and DCTN4 are required for both the expression of AHR and CYP1A1. In addition, TMEM5, PDC, CD9, and Sin3A are not required for AHR expression but are required for the induction of CYP1A1, implicating a direct role in *Cyp1a1* transcription. Our methods, although applied to *Cyp1a1*, could be modified for identifying proteins required for the regulation of other inducible genes.

Keywords: *CYP1A1, AHR, RNAi, High Throughput Screening, Hepa-1, TCDD, esiRNA, EROD, expression, Sin3A, CD9, TMEM5, ARMC8, Ube2i, PDC, Rbm5, CryGD, Rad50, Tcf20, DCTN4*

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor which has been shown to have a plethora of physiological roles pertaining to development, the endocrine system, and the immune response (Fujii-Kuriyama and Kawajiri 2010; Kawajiri and Fujii-Kuriyama 2007). Toxicity mediated by AHR lies principally in the dysregulation of its target genes (Okey 2007). *CYP1A1* in particular has been studied as the paradigm target gene of AHR ligand-mediated gene induction, and it is well recognized that xenobiotic metabolism by *CYP1A1* can result in the conversion of procarcinogens into carcinogens (Nebert et al. 2000). Furthermore, in drug development, ligand activation of AHR has been shown to increase the expression and activity of xenobiotic drug efflux pump target genes, including multidrug resistance-associated protein 2, breast cancer resistance polypeptide, and P-glycoprotein. The P-glycoprotein efflux transporter works at the blood-brain barrier and effectively reduces CNS levels of therapeutic drugs (X. Wang et al. 2011). Further characterization of the mechanism(s) of AHR-dependent induction of gene expression is therefore a very important research objective and of particular interest to toxicologists.

Although AHR is promiscuous in ligand binding the most prominent AHR ligands are environmental pollutants and toxicants including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (B[a]P). Humans encounter B[a]P from a wide range of sources in the environment including automobile exhaust, industrial by-products, charbroiled foods, and tobacco smoke (Ma and Lu 2007). Expression of *CYP1A1* has been

found to be increased in the lung (Thum et al. 2006), liver (Chang et al. 2003), placenta (Hakkola et al. 1997), and brain (Miksys and Tyndale 2006) of human cigarette smokers. Upon ligand binding AHR dissociates from its chaperone proteins and enters the nucleus where it can form a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) protein. The AHR/ARNT complex activates transcription by binding to xenobiotic response element (XRE) core consensus sequences (5'-TNGCGTG-3') in genomic DNA, which facilitates nucleosomal disruption, and thereby relieves repression over the promoter region to allow binding of transcription factors and activation of target gene expression (Hankinson 2005; Monostory et al. 2009; Nukaya and Bradfield 2009). Adverse consequences occur when the ensuing CYP1A1 protein acts to bioactivate PAH's to bay region epoxide metabolites which are highly reactive with DNA. Subsequent mutations that alter gene expression and cell cycle progression can lead to carcinogenesis (Androutsopoulos et al. 2009). *CYP1A1* is the AHR target gene which is most active in metabolizing PAH's into reactive oxygen species, making the study of its regulation and overexpression an important anti-cancer target (Androutsopoulos et al. 2009). Of relevance in this regard, overexpression of CYP1A1 has been found in human breast cancer (Brockdorff et al. 2000), esophageal carcinomas (Murray et al. 1994), urinary bladder tumors (Murray et al. 1995), and human lung adenocarcinomas; the later where CYP1A1 was suggested to be used as a prognostic and/or diagnostic tool for patients (Oyama et al. 2007). This makes the complete understanding of the molecular events governing transcriptional regulation of *CYP1A1* of considerable interest, and despite extensive

research it is well accepted that additional studies are necessary (Androutsopoulos et al. 2009; Fujii-Kuriyama and Kawajiri 2010; Hankinson 2005).

In this study we performed a high throughput siRNA library screening to further characterize AHR-ligand mediated induction of *Cyp1a1* gene expression. With the use of an RNAi library we identified 93 proteins with a p-value ≤ 0.005 whose expression appeared to be necessary for induction of CYP1A1-dependent EROD activity by TCDD, and through a secondary screening using in-house produced esiRNAs, we validated the results for 39 of these hits. Additional qPCR analysis revealed 12 previously unidentified proteins which are necessary for the induction of CYP1A1, including Sin3A, CD9, TMEM5, ARMC8, Ube2i, Rab40C, PDC, Rbm5, CryGD, Rad50, Tcf20, and DCTN4. Upon further investigation we determined that our observations are not due to diminished levels of ARNT, and in the case of TMEM5, PDC, CD9, and Sin3A these results are also not a byproduct of effects on AHR activity or expression, indicating a possible direct role for these proteins in the TCDD mediated induction of *Cyp1a1*.

MATERIALS AND METHODS

Cell Culture and Treatment: Hepa1c1c7 (Hepa-1) cells were grown as a monolayer in α -MEM (minimal essential medium) supplemented with 10% FBS, 1% pen/strep and 1% fungizone at 37°C in 5% CO₂. Cells were treated with TCDD dissolved in DMSO for 24 hours at a 10nM concentration, giving a final concentration of 0.01% DMSO.

Construction of esiRNAs: To generate templates for in vitro transcription a two-step PCR procedure was performed with Hepa-1 cDNA used as the primary PCR template. Primers for each esiRNA were chosen from the website RIDDLE (<http://cluster-12.mpi-cbg.de/cgi-bin/riddle/search>), and a T7 anchor tag (GGGCGGGT) was added at their 5' ends to enable annealing of a T7 promoter primer in the second round of PCR. The 400-600bp amplicon was then used for T7 RNA polymerase (NEB, Ipswich, MA) mediated in vitro transcription (Figure 2.1). PCR cycling conditions and reaction contents were performed as described by (Fazio et al. 2008). Each dsRNA was then digested with endoribonuclease RNaseIII to generate a pool of 21bp fragments, and then purified using a two filter purification mirVANA PARIS kit according to manufacturer's protocols (Ambion/ABI, Foster City, CA). GST-RNaseIII was received in a pGEX-2T plasmid as a generous gift from Dun Yang at University of California San Francisco (Yang et al. 2002). Briefly, the plasmid was transformed into E. coli strain BL21 for propagation, isolated with the PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions, and then digested with restriction enzymes to verify the presence of the GST-RNaseIII insert (Figure 2.2a and 2.2b). For protein

expression the plasmid was transformed into TOP10 cells and GST-RNaseIII expression was induced by a 3 hour IPTG treatment (Figure 2.2c). RNaseIII was then purified using reduced glutathione beads (Figure 2.2d).

Transfection of siRNA: Hepa-1 cells were reverse transfected with 25nM siRNA or 25nM esiRNA using lipofectamine RNAiMax (Invitrogen) according to manufacturer's recommendations for 96-well, 6-well, and 10cm dishes. In the 384 multi-well format, black plates (Grenier Bio-One, Monroe, NC) were pre-spotted with 4 (in some cases 8) unique siRNAs for each target gene in the druggable genome (Thermo Scientific Dharmacon siRNA libraries, Lafayette, CO), including a duplicated set of 4 siRNA's targeting CYP1A1 (Qiagen, Valencia, CA) on each plate to serve as positive controls. Each well contained 2uL of 0.5 pmol/uL (or 25nM) siRNA and every plate was run in duplicate. RNAiMax was diluted in OptiMEM at a 1:100 fold dilution, and 10uL was added to each well using a multi-drop dispenser. After the diluted RNAiMax was allowed to incubate with the oligonucleotides for 10-20 minutes, 1500 Hepa-1 cells were seeded per well in a 20uL volume.

Ethoxyresorufin-O-deethylase (EROD) Assay: To assay for CYP1A1 enzymatic activity, cells were treated with 10µM ethoxyresorufin and 500µM dicumarol (Sigma Aldrich, St. Louis, MO) in phenol-red free MEM supplemented with 10% FBS, 1% L-glutamate, and 1% pen/strep. Phenol-red free media reduces light scattering and background fluorescence, whereas dicumarol prevents further metabolic degradation of the fluorescent product, resorufin. Reactions were incubated for the optimized time of 45-60 minutes at 37°C, and resorufin related fluorescence

(530ex/590em) was detected in a multiwell fluorescence reader (FlexStation 3, Molecular Devices).

Cell Viability Assays: Cells were lysed and subjected to an ATP-dependent cell viability assay for each esiRNA/siRNA examined. Cell Titer Glo reagent was used according to the manufacturers protocols (Promega, Madison, WI) in a 1:3 ratio of Cell Titer Glo to medium.

Quantitative PCR: RNA was isolated using RNEasy Mini columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA synthesis of 2 μ g of total RNA was performed with Superscript III reverse transcriptase (Invitrogen) according to manufacturer's protocols with random hexamers for primers (Invitrogen). Synthesized cDNA was used as a template for qPCR at a 1:10 dilution and SYBR Green (Qiagen, Valencia, CA) and used according to standard protocols on an Applied Biosystems 7500 real time PCR machine. Primers for qPCR were designed using Primer Express 3.0 software (Applied Biosystems, CA), and were synthesized by Fisher Scientific, Inc.

Western Blotting: Cellular lysates were resolved on a 4-12% SDS gel in a mini-gel apparatus (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane with a semi-dry apparatus (Thermo Scientific Pierce, Rockford, IL). Blots were then blocked with 3% non-fat milk and incubated with an antibody against CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200, AHR (F. Wang et al. 2006) at 1:200, or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA)

at 1:2000. Donkey anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase were used as secondary antibodies at 1:10,000 or 1:5,000 respectively. Horseradish peroxidase was then detected with a chemiluminescent kit (GE Healthcare, Piscataway, NJ), and densitometry calculations were performed using ImageJ software (NIH).

Transduction for EROD Rescue: Retrovirus was made by cotransfection of 293T cells with the pMSCV-ires/GFP retroviral expression vector (F. Wang et al. 2007) containing the indicated human cDNAs and a pCL-Eco packaging plasmid, using BioT (Bioland Scientific LLC, Paramount, CA) transfection reagent. The following day 293T cells were treated with 10mM Na-Butyrate for 9 hours to enhance viral production, and after an additional 24 hours the viral suspension was harvested. Hepa-1 cells plated in 6-well culture dishes were treated with 2ml of the viral suspension in α MEM supplemented with 8 μ g/mL polybrene, and then centrifuged at 37°C for 1.5 hours at 2,500 rpm. An additional 2ml of fresh α MEM was added, and after 48 hours the cells were trypsinized and plated in growth medium on a 10cm plate. Once confluent, cells successfully transduced and stably expressing human cDNA were isolated by GFP expression using Fluorescence Activated Cell Sorting (FACS). Reverse transfections and EROD experiments were carried out as described.

Transient Transfections and Reporter Gene Assay: Expression plasmids were transfected into Hepa-1 cells cultured in 6-well plates by using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent. The indicated siRNA was

cotransfected with renilla luciferase reporter (*pRL-TK*) (Promega, Madison, WI) and firefly luciferase reporter (*pGL-CYP1A1*) (Beischlag et al. 2002) constructs. *pRL-TK* is driven by the herpes simplex virus thymidine kinase promoter whereas *pGL-CYP1A1* is driven by 4.2 kilobases of the 5' upstream regulatory region of the rat *CYP1A1* gene, including its promoter and multiple XRE's. Cells were harvested and lysed in passive lysis buffer and luciferase activities were measured using the Dual-Luciferase system (Promega, Madison, WI) according to the manufacturer's recommended protocols. Firefly luciferase activity was then normalized to renilla luciferase activity.

Statistical Analysis: Student's two-tailed t-test was performed to determine statistical significance, and data are presented as mean \pm standard deviation. Graphs and blots are representative of at least two separate experiments. Analysis of screening results was performed using the redundant siRNA activity analysis (RSA) algorithm, which was specifically designed for siRNA screens (Konig et al. 2007). Briefly, relative fluorescence units (RFU) and relative luminescence units (RLU) from each well were normalized to their corresponding plate median to reduce variations in plate signals. The normalized RFU pertaining to CYP1A1 EROD activity was then normalized to its respective normalized RLU from Cell Titer Glo viability assays to account for differences in cell densities. A weighting factor WK was calculated to represent the quality of the assay on each plate using positive controls, negative controls, and average standard deviations. From this, a Score' was determined which used WK to calculate a weighted average of each siRNA from the duplicated plates. Utilizing a weighted average permitted that an siRNA would

get a higher Score' when the assay on the plate was of greater quality. Lastly, Score' was scaled based on positive controls from the individual plates such that all positive control signals were 0.1, while keeping the plate median at 1.0. This further reduced plate signal variations and the resulting Score of each siRNA was used for gene ranking in the RSA algorithm with a cutoff threshold of values 3 times the average standard deviation.

RESULTS

Development of an RNAi-based Screen Facilitates the Identification of Proteins Potentially Necessary for Dioxin-Induced CYP1A1 EROD Activity

The basis of our procedure, is that in 384-well plates Hepa-1 cells are reverse transfected with siRNAs for 48 hours, treated with TCDD for an additional 24 hours, and then assayed for CYP1A1-dependent EROD activity (Figure 2.3a). In the EROD assay the chemical ethoxyresorufin is metabolized into the fluorescent product resorufin in the presence of CYP1A1, and thus the amount of resorufin can be measured as a relative indicator of CYP1A1 activity (Figure 2.3b). Diminished levels of relative fluorescent units (RFU) indicate that the targeted gene is necessary for CYP1A1 EROD activity and thereby may reveal factors necessary for induction of *Cyp1a1* gene expression. The RNAi library we used contained 5,600 gene targets including those encoding proteins for transcriptional regulation, GPCR signaling, cell cycle, transporters, nuclear receptors, translational regulators, enzymes, kinases, apoptosis signaling, anti-apoptosis, transmembrane receptors, autophagy and senescence. These gene candidates were chosen based on recent publications and bioinformatics that identified them as potential druggable targets (Thermo Scientific Dharmacon siRNA libraries, Lafayette, CO).

To ensure the most robust results and optimal methodology, we ran several preliminary RNAi experiments on the 384-well plates to calculate the quality of the assay (Z-prime) by comparing results from the 4 individual siCYP1A1 positive controls to Mock negative controls. To this end, the effects of various cell densities on CYP1A1 EROD activity were determined (Figure 2.4a) as well as the cell viability at these densities (Figure 2.4b). Optimal conditions were found to occur at 1500

cells per well where luciferase activity from cell viability assays was at its highest. To establish the reproducibility of the assay between plates on the same day we performed technical replicates of these control plates, and to establish reproducibility of the assay between days we performed biological replicates of our control plates (Figure 2.4c). The incidence of false positives due to low cell density was restricted by normalizing EROD activity to cell viability for each well. Data was analyzed with the redundant siRNA activity (RSA) analysis algorithm which ranked each candidate gene based on the results from duplicated plates containing a group of 4 (or in a few cases 8) individual siRNAs (Konig et al. 2007). The results from this screening revealed 93 hits from over 5,600 candidates in the druggable genome with a p-value ≤ 0.005 (LogP of -2.26) that exhibited a diminished CYP1A1 EROD activity signal (Supplementary Table 2.1).

Validation of Screening Results with esiRNA Expose Top Candidate Protein Hits Required for TCDD-Induced CYP1A1 EROD Activity

To confirm our hits from the high throughput assay a secondary RNAi screen was performed in 96-well plates using endoribonuclease-prepared siRNA (esiRNA). During the production of the esiRNAs, agarose gels were run to determine the quality of the PCR products (Figure 2.5a) and dsRNAs (Figure 2.5b). Digested and purified esiRNAs were run on a 6% non-denaturing polyacrylamide gel to assess their quality compared to a chemically synthesized MapK siRNA positive control (Figure 2.5c). Poor quality or missing PCR/esiRNA products were omitted from the esiRNA screening and followed up with individual siRNAs.

Once production of the esiRNAs was established, we performed validation experiments in 96-well plates to determine optimal cell density (Figure 2.6a) and its corresponding cell viability (Figure 2.6b) using Z' as a measure of the assay quality. Optimized conditions, determined by maximum luminescence signals from cell viability assays, were established as 5000 cells/well. Hepa-1 cells were reverse transfected with 25nM esiRNA for 48 hours, treated with TCDD for an additional 24 hours, and assayed for CYP1A1 EROD activity. Diminished CYP1A1-dependent EROD activity was observed in esiCYP1A1 treatments at concentrations as low as 5nM, but maximum knockdown occurred at 25nM concentrations and was therefore used in our screening (data not shown). Candidate genes that had only a single-hit were excluded from this secondary screening due to the higher risk that these may represent false positive results (Echeverri and Perrimon 2006; Tiedemann et al. 2010). We thus confirmed 39 of the 63 hits tested. Several of the hits identified were general/known transcriptional or translational factors and were omitted from further evaluation. In addition, hits that exhibited a high cell death of $\geq 80\%$ were removed from additional analysis due to the fact that cytotoxicity can cause alterations in the biochemistry of the cells, and produce phenotypes different from the distinct phenotype resulting from the siRNA (Ovcharenko et al. 2005). Supplementary Table 2.2 contains a list of the top 93 hits from the original screening and either provides justifications for excluding each candidate from follow-up experiments, or provides a data summary for each hit we pursued. As another layer of proof, esiRNA confirmed true positive hits were subjected to a 3rd EROD screen using one of the same siRNAs as on the original screen (Table 2.1; Supplementary Table 2.2). In this 3rd screening, 4 interesting candidate genes that

had a $0.005 \leq p\text{-value} \leq 0.01$ were additionally hand selected for follow-up, 3 of which are nuclear proteins and 1 of which is not well-functionally classified.

Altogether this secondary screening led to the discovery of 17 novel proteins which are necessary for TCDD induced CYP1A1 EROD activity including Sin3A, ARMC8, PDC, Tcf20, Cfc1, Sorl1, TMEM5, Cdc20, Agtr2, CD9, Ube2i, Rab40C, Pygl, CryGD, DCTN4, Rbm5, and Rad50 (Table 2.1; Supplementary Table 2.2).

Expression of Sin3A, ARMC8, PDC, Tcf20, TMEM5, CD9, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50 is Necessary for TCDD-induced CYP1A1 Expression

We next sought to discover if the proteins necessary for CYP1A1-dependent EROD activity were also necessary for CYP1A1 mRNA and protein expression. Hepa-1 cells were reverse transfected with siRNA in 6-well plates for 48 hours, and then treated with 10nM TCDD for an additional 24 hours. The mRNA was extracted and quantitative PCR experiments were performed (Figure 2.7; Supplementary Table 2.2). Efficiency of siRNA mediated knockdown from each individual siRNA was initially confirmed (Figure 2.7a; Supplementary Table 2.2), and further qPCR analysis revealed that even though Cfc1, Sorl1, Cdc20, Agtr2, and Pygl are necessary for CYP1A1 enzymatic EROD activity, and therefore included on Table 2.1 as confirmed hits, their effects on CYP1A1 mRNA expression is diminutive in comparison (Table 2.1; Supplementary Table 2.2). Further investigation will reveal if this occurs through inhibition of the translation of CYP1A1, inhibition of the CYP1A1 protein activity, or if the target encodes a factor required for EROD activity (such as P450 reductase). On the other hand, CYP1A1 mRNA expression is

decreased when Sin3A, ARMC8, PDC, Tcf20, TMEM5, CD9, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50 are knocked down, which demonstrates that their expression is vital for *Cyp1a1* transcription (Figure 2.7b; Supplementary Table 2.2). To shed light on their mechanism of action, transcript levels of ALDH3A1 (Figure 2.7c; Supplementary Table 2.2) and NQO1 (Figure 2.7d; Supplementary Table 2.2) were quantified. *ALDH3A1* and *NQO1* are part of the 'AHR battery' of genes that are known for their inducibility by TCDD (Dewa et al. 2008). The results from these studies revealed that ARMC8, TMEM5, CD9, Ube2i, CryGD, and Rbm5 are needed for induced expression of both ALDH3A1 and NQO1, which suggested that they may be required for the expression of AHR-inducible genes in general. Remarkably all of the other hits showed selectivity for either ALDH3A1 or NQO1, which alludes to the possibility that their involvement in CYP1A1 expression may be by a more specific mechanism. Figure 2.7c and 2.7d contains data for the candidates that were confirmed to be required for either ALDH3A1 or NQO1 induction, but a complete list of the results from each siRNA tested can be found on Table 2.1 and Supplementary Table 2.2.

Analogous experiments were performed in 10cm plates and lysates were collected to examine CYP1A1 protein expression. For protein quantification we performed densitometry analysis on western blots (Figures 2.8a and 2.9a; Supplementary Table 2.2) using ImageJ software, and normalized results to the housekeeping control gene GAPDH (Figures 2.8b and 2.9b; Supplementary Table 2.2). When compared to scrambled control we observed a significant decrease in TCDD induced CYP1A1 protein expression in the presence of siRNA targeting Sin3A, ARMC8, TMEM5, Rab40C, and CryGD. For certain candidates, including PDC,

TCF20, CD9, Ube2i, DCTN4, and RAD50, siRNAs were used in their entirety during previous follow-up experiments and therefore their effects on protein expression could not be observed in this study. Collectively these experiments demonstrated that the expression of Sin3A, ARMC8, PDC, Tcf20, TMEM5, CD9, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50 are essential for the TCDD induced expression of CYP1A1 mRNA, and this was established at the protein level for Sin3A, ARMC8, TMEM5, Rab40C, and CryGD.

Influence of the siRNAs on ARNT Expression and AHR Activity/Expression

To rule out the possibility that the results we observed were a byproduct of effects on ARNT or AHR we quantified mRNA levels after siRNA mediated knockdown of our candidate gene expression (Table 2.1; Supplementary Table 2.2). The transcript levels of ARNT were not diminished; however, AHR mRNA was reduced to approximately the same degree as induced CYP1A1 mRNA levels when ARMC8, Tcf20, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50 were knocked down (Table 2.1; Supplementary Table 2.2). As a result, we performed additional studies on AHR, including densitometry analysis of western blots to determine AHR protein levels (Figure 2.8a and 2.9a; Supplementary Table 2.2). In these studies Sin3A, ARMC8, TMEM5, RAB40C, and CRYGD were analyzed while PDC, TCF20, CD9, Ube2i, DCTN4, Rbm5, and Rad50 were excluded for the aforementioned reasons. Analysis of the corresponding proteins corroborated our results for Rab40C and CryGD mRNA (Figure 2.8b; Supplementary Table 2.2). Conversely, AHR transcript levels were diminished less than induced CYP1A1 mRNA levels in

samples treated with siRNA targeting Sin3A, PDC, TMEM5, and CD9 (Table 2.1; Supplementary Table 2.2). These observations were further confirmed for the AHR protein in siSin3A and siTMEM5 treated samples by western blot analysis (Figure 2.9b; Supplementary Table 2.2).

To better define the role of AHR, we next transduced Hepa-1 cells with a pMSCV-ires/GFP retroviral vector containing human AHR cDNA. Isolated transduced cells were then transfected with individual siRNAs to see if the TCDD induced CYP1A1-dependent EROD activity was rescued by overexpression of AHR (Figure 2.10; Supplementary Table 2.2). Consistent with our observed decrease in AHR transcript levels, AHR overexpression rescued TCDD-induced CYP1A1-dependent EROD activity in cells with diminished levels of ARMC8, Rab40C, CryGD, Rbm5, and Rad50 (Figure 2.11; Supplementary Table 2.2). On the contrary, EROD activity was not rescued in the presence of siRNA targeting Sin3A, TMEM5, Tcf20, and CD9 (Figure 2.11; Supplementary Table 2.2). Ube2i, DCTN4, and PDC were not analyzed. These results are consistent with our observations for Sin3A, TMEM5, and CD9 mRNA levels as well as our analysis for effects of siSin3A and siTMEM5 on AHR protein levels, which provides convincing evidence that the role of these proteins in CYP1A1 expression is not mediated through the AHR.

To further understand the mechanism by which a number of our genes function in *Cyp1a1* induction and/or AHR expression, we co-transfected Hepa-1 cells with individual siRNAs to the corresponding cDNAs and the pGL-*CYP1A1* firefly luciferase reporter construct. After 24 hours of treatment with TCDD, we looked at luciferase expression as an indicator of activity of the *CYP1A1* promoter (Figure

2.12a and 2.12b). When compared to pGL-*CYP1A1* cotransfected with either a Mock positive control or an siAHR negative control, we saw a significant influence on luciferase expression in all samples. The most remarkable decrease in luciferase activity was observed with siARMC8 and siCD9 treatments, while a more moderate decrease in luciferase expression was observed in samples treated with siPDC. Luciferase expression was diminished the least, or in some cases increased, in cells treated with siRNA targeting CryGD, Rad50, Ube2i, Rab40C, DCTN4, TMEM5, and Sin3A. Since the pGL-*CYP1A1* construct is not in the native/endogenous chromatin formation a stable luciferase signal in the presence of siRNA may indicate the need of the protein or its downstream factors for chromatin remodeling, whereas a diminished luciferase signal may indicate a direct role for the protein or its downstream factors in binding 5'-flanking *CYP1A1* promoter elements. We would therefore expect proteins that are necessary for AHR expression to display a diminished luciferase signal in these experiments which was not always the case. This lack of effect of a particular siRNA could be because the induction of the luciferase reporter gene is more sensitive and therefore requires less of the corresponding protein than we would observe in the induction of the endogenous *Cyp1a1* gene. Research on AHR-regulation has primarily been focused on downstream target genes, so little information is available regarding the regulation of the *AHR* gene itself (Garrison and Denison 2000). Further investigation is needed to not only elucidate the role that our candidate target genes play in AHR expression but also to elucidate the regulation of AHR in general.

Validation of Candidate Hits ARMC8 and Rbm5

To exclude the possibility that the observed results with ARMC8 and Rbm5 were due to off-target effects from the siRNA, we transduced Hepa-1 cells with the pMSCV-ires/GFP retroviral expression vector containing human ARMC8 or human RBM5 cDNA and isolated successfully transduced cells by FACS sorting of GFP positive cells. These cells were then transfected to see if in each case overexpressing the human cDNA in Hepa-1 cells transfected with its murine siRNA counterpart rescued induced CYP1A1-dependent EROD activity (Figure 2.13). Mouse specific siRNA sequences were BLASTed against the human genome to ensure that human ARMC8 or RBM5 expression would be unaffected. Previous studies revealed that 89% of tested mouse genes were not targeted by the siRNAs against human genes due to the DNA sequence divergence between mouse and human (Neumann et al. 2010). Overexpression of both ARMC8 (Figure 2.12a) and RBM5 (Figure 2.12b) was sufficient to rescue EROD activity. In addition, overexpressing ARMC8 or RBM5 in non-transfected cells significantly increased CYP1A1 EROD activity relative to empty vector control, which further validated the involvement of these proteins in CYP1A1 expression. Similar on-going studies will validate each siRNA used and confirm the results from all of our candidate hits.

DISCUSSION

In this study we performed a series of optimization experiments to develop and implement procedures for an RNAi high throughput screening, which successfully facilitated the discovery of novel gene candidates that are necessary for TCDD induced CYP1A1 expression. High throughput screenings have gained popularity because they enable an expedited means of obtaining an abundance of data, but few of these screenings have focused on transcriptional regulation. The application of these methods could therefore be extended to study the regulation of other inducible genes whose roles and regulations are not clearly understood. The CYP3A family is one such example, and understanding its regulation would have significant clinical importance due to the fact that CYP3A's are not only responsible for metabolizing 50% of currently prescribed drugs, but are also involved in many clinically relevant drug interactions (Qiu et al. 2008).

In this study we used the RSA algorithm which assigns a LogP value to a set of siRNA's targeting one gene and takes into account not only the quantity of hits but also the quality of the assay on each plate. This method of analysis for RNAi screens has proven to be more effective than standard ranking methods, and has higher confirmation rates in secondary screenings (Konig et al. 2007). In addition, we applied a stringent p-value of ≤ 0.005 to minimize false positives to quantities less than 1 in every 100 hits. Utilizing such a rigorous approach of analysis enabled the identification of 93 putative proteins necessary for TCDD induced CYP1A1 EROD activity, although an additional 4 interesting candidates (p-value ≤ 0.01) were later hand-selected for follow-up based on their functional relevance to our screening. These included DCTN4, Rbm5, and Rad50 which serve as nuclear proteins involved

in DNA/RNA binding, and CryGD whose functions and mechanisms have hardly been touched upon, thereby enhancing our prospect of a novel discovery. To confirm these results, we optimized the production of esiRNAs which offer an efficient, cost-effective and specific alternative to siRNAs. Moreover, esiRNAs have been shown to be just as effective if not more effective than synthesized siRNAs in their silencing, yet have a 12-fold reduction in off-target effects (Kittler et al. 2007). Gene targets that had only a single-hit were excluded from this secondary screening in order to further eliminate the presence of false positives (Echeverri and Perrimon 2006; Tiedemann et al. 2010). We thus confirmed our results for 39 of the remaining 63 candidates. Several of the hits identified were general transcriptional or translational factors (Supplementary Table 2.2), as well as known transcriptional regulators of CYP1A1 (Table 2.2). There were 8 known CYP1A1 regulators tested, and of those we observed siRNA hits for each one including AHR, ARNT, eP300, BRG1, TRIP230, ESR1, BRCA1, and MED1 (Table 2.2). Although the plates for 7 of these known regulators exhibited a high Z-prime of 0.7 or higher, TRIP230 had a low Z-prime of 0.29 and 0.27 which may be the reason we did not observe a statistically significant p-value. AHR, ARNT, and eP300 were statistically significant ($p\text{-value} \leq 0.005$), however, and even though these general and known regulators were excluded from additional evaluation, the mere presence of them in our hit list attests to the robustness of the assay and demonstrates the accuracy of our results.

We next aimed to discover which of our candidate hits are necessary for the mRNA and protein expression of CYP1A1. From quantitative PCR and western blot densitometry analysis we found 12 novel proteins that fit this category including

Sin3A, ARMC8, PDC, Tcf20, TMEM5, CD9, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50. Our studies not only provided insight into the regulation of *Cyp1a1* but also AHR-inducible genes *Nqo1* and *Aldh3a1*, as well as *AHR* itself. We thus established that knocking down expression of TMEM5 and CD9 decreased the induction of both NQO1 and ALDH3A1 without having any or much influence on AHR expression, which implicates a role for these proteins in the regulation of AHR-inducible genes in general. CryGD and Rbm5, which were confirmed to be required for CYP1A1 induction due to their effects on AHR expression, were also necessary for NQO1 and ALDH3A1. Although the data for each protein is generally internally consistent, this is not true in some cases where the interpretation of the role of AHR is less straight-forward. For example, ARMC8 is necessary for both the expression of AHR mRNA and induction of luciferase activity from the pGL-*CYP1A1* construct. Furthermore, in the presence of siARMC8 AHR overexpression is sufficient to rescue CYP1A1-dependent EROD activity. These results suggest that ARMC8 is necessary for AHR expression; however, we cannot explain why siARMC8 treatments did not lead to a reduction in AHR protein levels. This is perhaps a drawback of performing a high throughput screening in which an abundance of data is acquired. To address this, Table 2.3 presents a summary of our data to help determine the probability that the activity of any chosen protein is mediated through AHR. Further investigations of these proteins should provide valuable insight.

To define the underlying biology behind our data and outline known pathways relevant in our screening we analyzed our results with GeneGo, Inc©. This software contains a compilation of data from published sources, and therefore serves as a data mining tool for high throughput screenings to identify the most

relevant and known interactions between screening hits, as well as their resulting biological functions. This can be used both for excluding hits and for determining the most relevant hits. As long as the p-values are ≤ 0.05 it is recommended by the developers that approximately 300 hits are necessary for GeneGo, Inc© analysis to deliver the most comprehensive networks, thus, Table 2.4 summarizes the most relevant pathways maps from our top 300 screening hits (p-values ≤ 0.03). From these maps we found 4 significantly relevant transcription based pathways, the top 2 of which both contain one of our most promising hits Sin3A. In addition, pathway analysis revealed that the calcium calmodulin-dependent kinase (Ca²⁺/CaM/CaMKI α) pathway contained a significant number of hits. This is consistent with recent studies in a number of laboratories which have identified that the upregulation of AHR target genes is dependent on the Ca²⁺/CaM/CaMKI α pathway (Han et al. 2009; Lin et al. 2008; Monteiro et al. 2008). Moreover, a recent kinome screening by Gilot et al run concurrently with ours identified that 4 of their 22 kinase hits, which are necessary for CYP1A1 EROD activity, bind calmodulin (Gilot et al. 2011). SPHK1 in particular was first isolated based on its calmodulin-binding ability (Olivera et al. 1998). Overall our results are consistent with their kinome screening for 13 hits of the 17 corresponding kinases we tested.

Specifically SPHK1, CCT2, AK3, and HIPK1 were those of statistical significance but with a $0.005 \geq p\text{-value} \leq 0.05$ we did not investigate them further. Despite the overlap in the kinome portion of these studies, Gilot and coworkers screened only 712 gene candidates meaning that our screening covered nearly 5000 more targets. In addition, we performed extensive secondary screenings which are necessary to validate potential hits, irrespective of the statistical methodology that

is employed (Birmingham et al. 2009; Hirsch 2010). Gilot and coworkers did not perform these secondary screenings on each of their kinome hits, nor did they analyze their most promising candidates nearly as thoroughly as we did. For example, our experiments served to identify the effects of each siRNA on transcript and protein levels of CYP1A1 instead of only EROD activity. EROD data is solely an indicator of protein activity and is not sufficient to determine the need of any particular gene for CYP1A1 expression. In addition, Gilot and coworkers did not explore the impact AHR had on their results. We performed these experiments and further demonstrated that they are essential to discern between a direct involvement in the induction of AHR target genes and an involvement that is merely a byproduct of upstream effects. Overall our studies provide a more complete analysis and give valuable insight into proteins modulating the induction of CYP1A1.

Along with outlining known pathway maps, we used GeneGo, Inc© software to find networks of known interactions between our top 300 hits. From this we discovered that PKC physically and functionally associates with one of our top proteins CD9. Induction of PKC activity synergizes the actions of TCDD-mediated CYP1A1 expression while inhibition of PKC blocks CYP1A1 induction, however the mechanism by which this occurs is still debated (Machemer and Tukey 2005). The presence of CD9 helps in the association of PKC with specific integrins responsible for regulatory signaling, which includes gene induction, and may be a mechanism by which CD9 influences CYP1A1 expression (Akabane et al. 2007; Zhang et al. 2001). Additionally, we found that phosphorylation and subsequent activation of our confirmed hit PDC is dependent on Ca²⁺/CaM (Willardson et al. 1996). Once active, PDC acts to influence transcriptional activity of the CRX transcription factor

through direct binding and translocation of the complex into the nucleus (Zhu and Craft 2000). Co-IP experiments could help determine if PDC influences transcription of *Cyp1a1* in a similar manner by binding and thereby facilitating translocation of AHR. These networks can only begin to help identify how each protein influences CYP1A1 expression; however, they provide a valuable resource for determining essential experiments that will define mechanisms of action.

In summary, we developed and implemented an RNAi high throughput screening and successfully generated esiRNAs to use as a cost-effective tool for subsequent data validation. Our study has established that these are effective methods to investigate the regulation of CYP1A1 induction. This is confirmed by the presence of known *CYP1A1* regulators as some of our top hits, as well as the several follow-up experiments performed which confirmed our screening results. We demonstrated that Rab40C, ARMC8, Ube2i, CD9, Sin3A, PDC, Rbm5, CryGD, Rad50, Tcf20, DCTN4, and TMEM5 are cellular factors necessary for CYP1A1 mRNA expression, and upon additional examination we found that ARMC8, Tcf20, Ube2i, Rab40C, CryGD, DCTN4, Rbm5, and Rad50 are also necessary for AHR mRNA expression. Our studies provide evidence that TMEM5, PDC, CD9, and Sin3A are crucial for the induction of CYP1A1 and that this is not dependent for these effects on AHR expression or function. These proteins have not previously been implicated in CYP1A1 expression and as such this discovery provides novel insights into the induction of AHR-mediated gene expression.

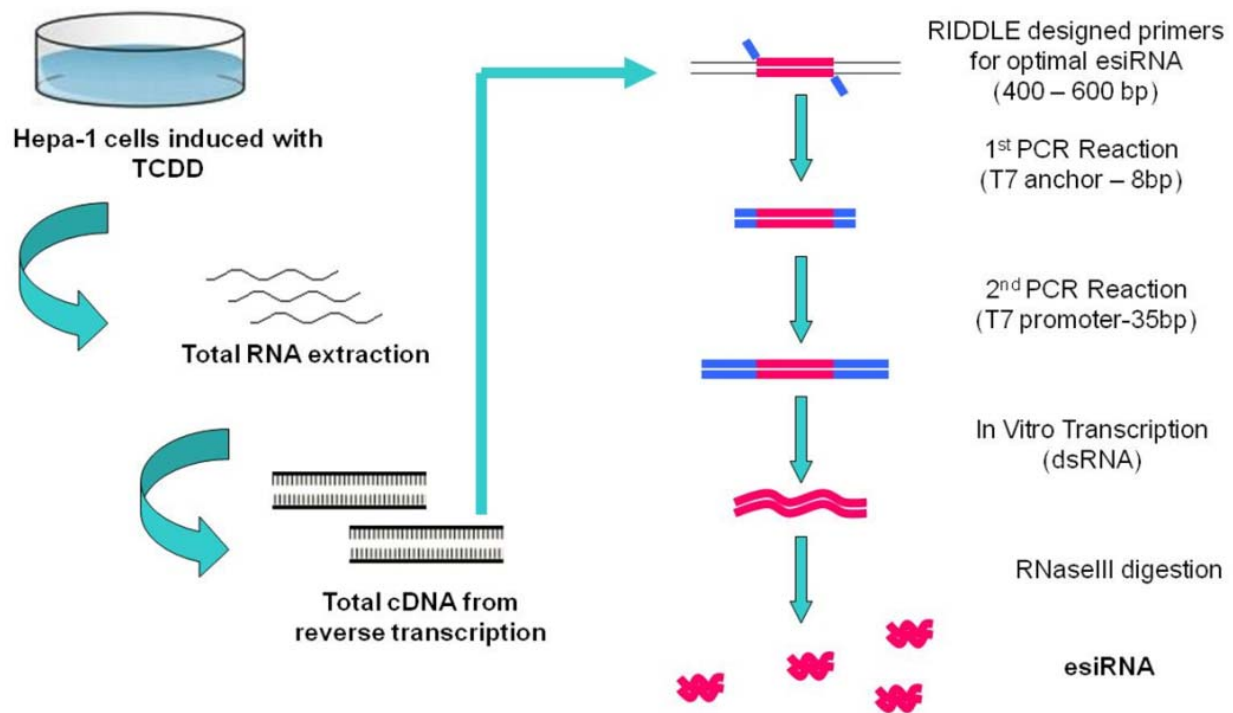
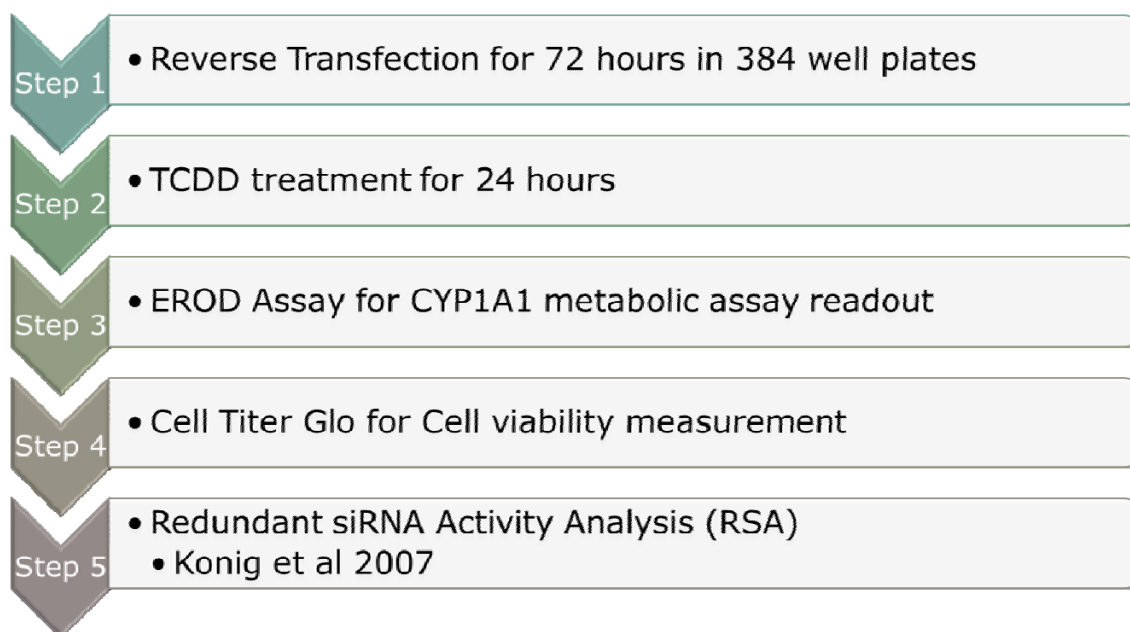
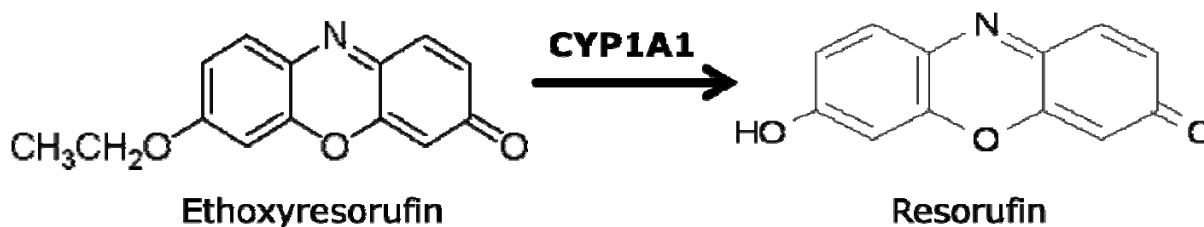


Figure 2.1: esiRNA Production Methods. Total cDNA from Hepa-1 cells was used as a template for a two-step PCR Reaction, which adds on a T7 promoter to a designated cDNA fragment. T7 polymerase mediated in vitro transcription gave a dsRNA product that was subsequently digested to esiRNA with the use of the endoribonuclease RNaseIII.



(a)



(b)

Figure 2.3: RNAi High Throughput Screening Methods. Methods used for the high throughput screening are summarized in this flowchart (a). The EROD readout assay used for this screening takes advantage of the ability of CYP1A1 to O-deethylate the chemical Ethoxyresorufin, which results in the fluorescent product Resorufin (b).

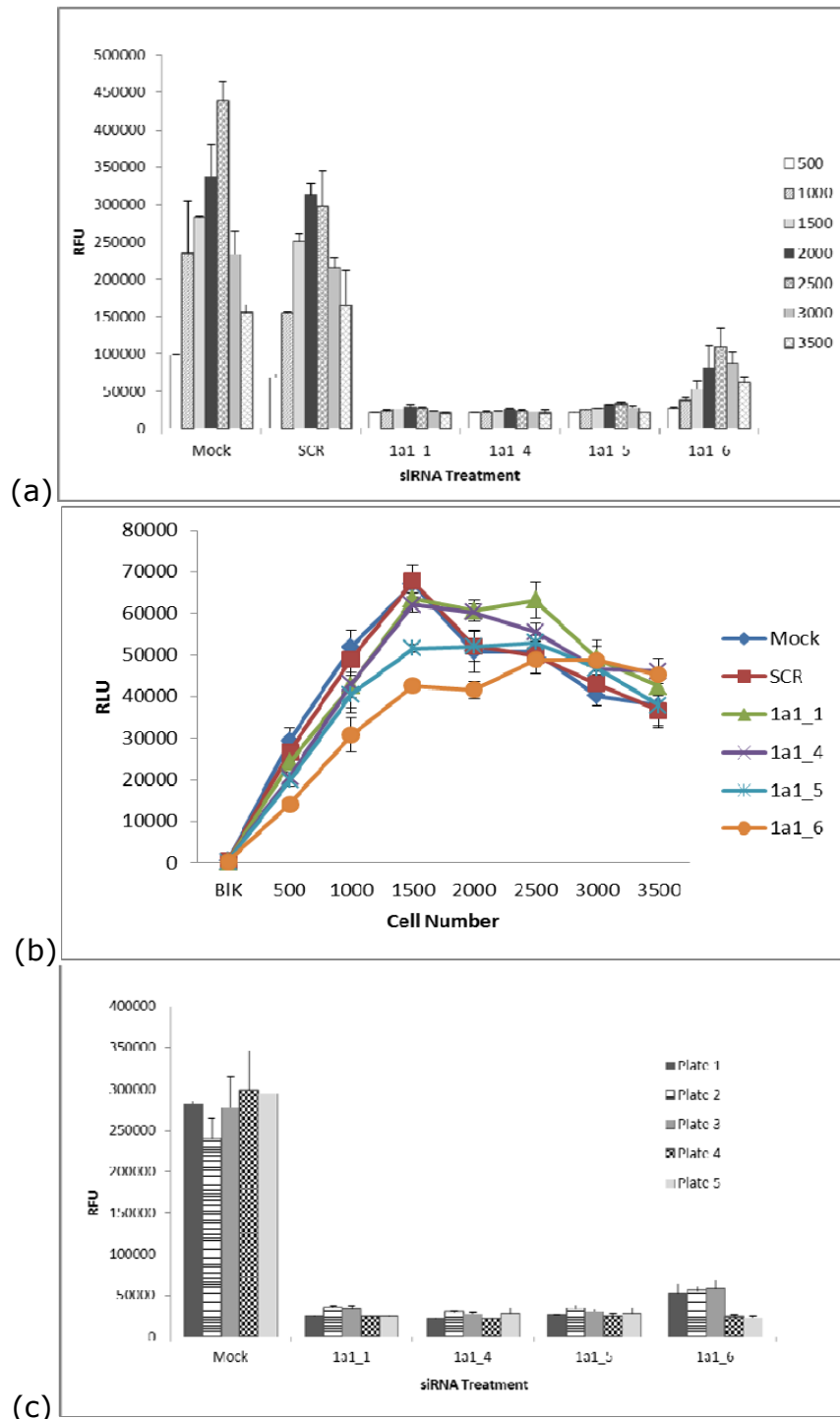


Figure 2.4: RNAi High Throughput Screening Methods Validation.

Preliminary experiments were performed, using 4 individual CYP1A1 siRNAs, to optimize the high throughput methods and examine the effects of varying cell density (a) and the corresponding cell viability (b). Variability of the assay was also examined between plates and between days (c). Plates 2/3 and plates 4/5 were run on the same day. Z-prime average = 0.595.

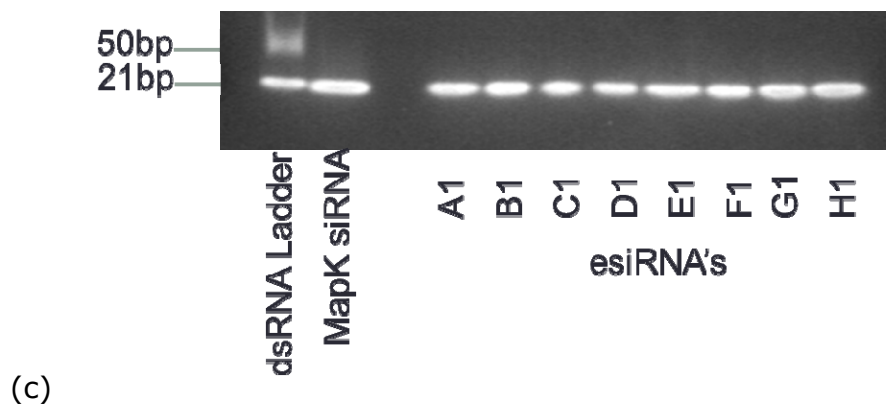
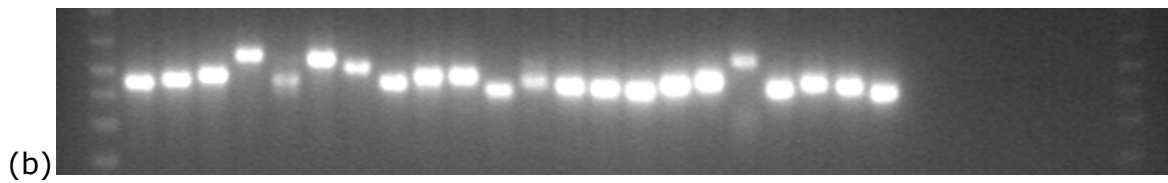
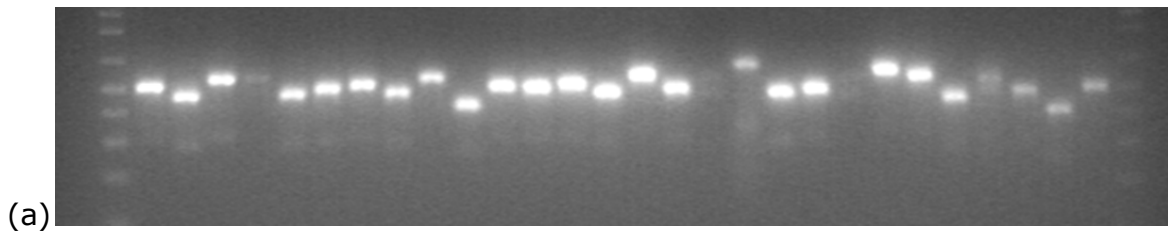


Figure 2.5: esiRNA Production Quality Check. During the production of the esiRNA the quality of the 2nd PCR product (a), and the dsRNA from IVT (b) was verified on a 2% agarose gel. Quality was determined by the intensity of the band and the overall presence of a PCR product. Quality of digested and purified esiRNA's was verified on a 6% non-denaturing gel and compared to the chemically synthesized MapK siRNA (c).

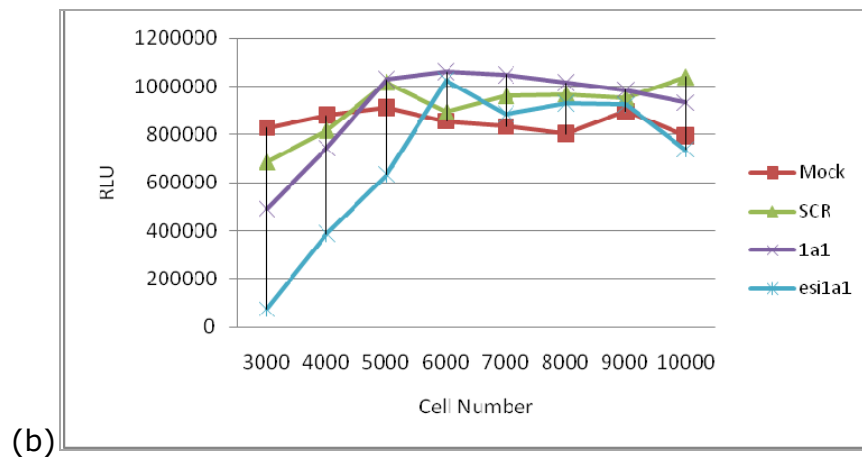
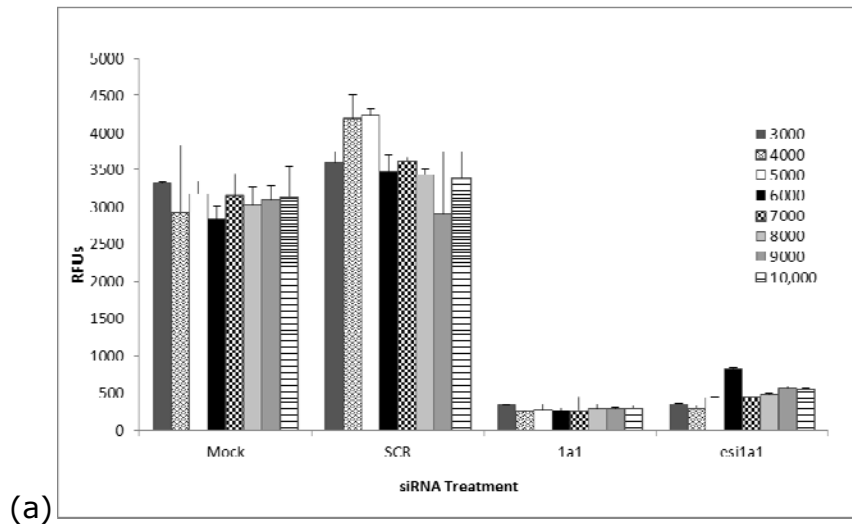
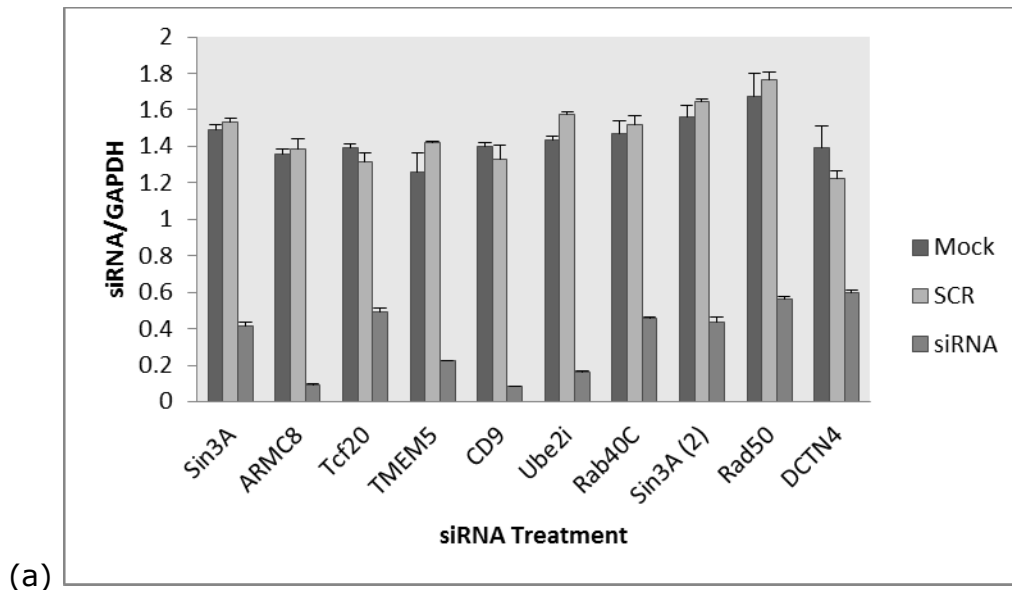
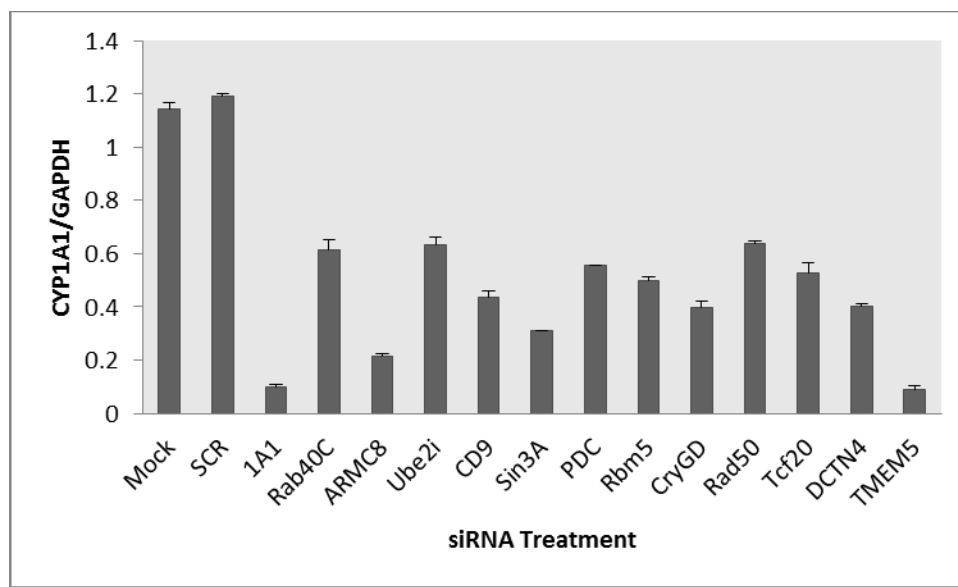


Figure 2.6: esiRNA Screening Methods Validation. Preliminary experiments were performed to optimize the esiRNA screen and the effects of varying cell density (a) and its corresponding cell viability (b) were examined. Z-prime average= 0.773.



(a)



(b)

Figure 2.7: Transcript Levels of siRNA targets, CYP1A1, ALDH3A1, and NQO1 after siRNA Mediated Knockdown and TCDD Induction. Efficiency of transcript knockdown by each individual siRNA was assessed (a), as well as the effects each siRNA had on induced CYP1A1 (b), ALDH3A1 (c), and NQO1(d) mRNA expression. Transcripts were measured using SYBR green real-time PCR and each sample was normalized to the constitutively expressed glycolytic GAPDH gene. All results shown are statistically significant with a p-value ≤ 0.005 relative to Mock and SCR positive controls. See page 59 for Figures 2.7c and 2.7d.

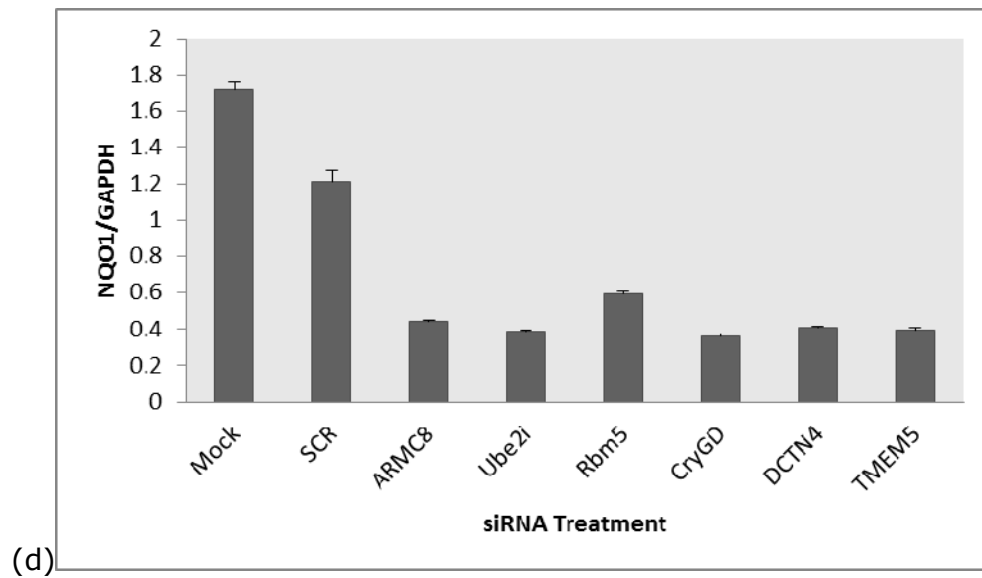
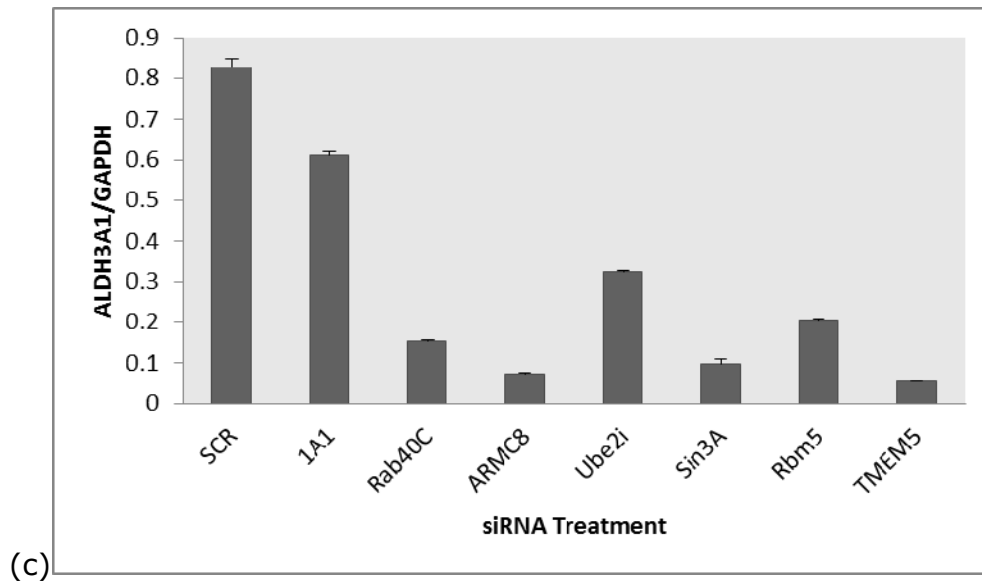


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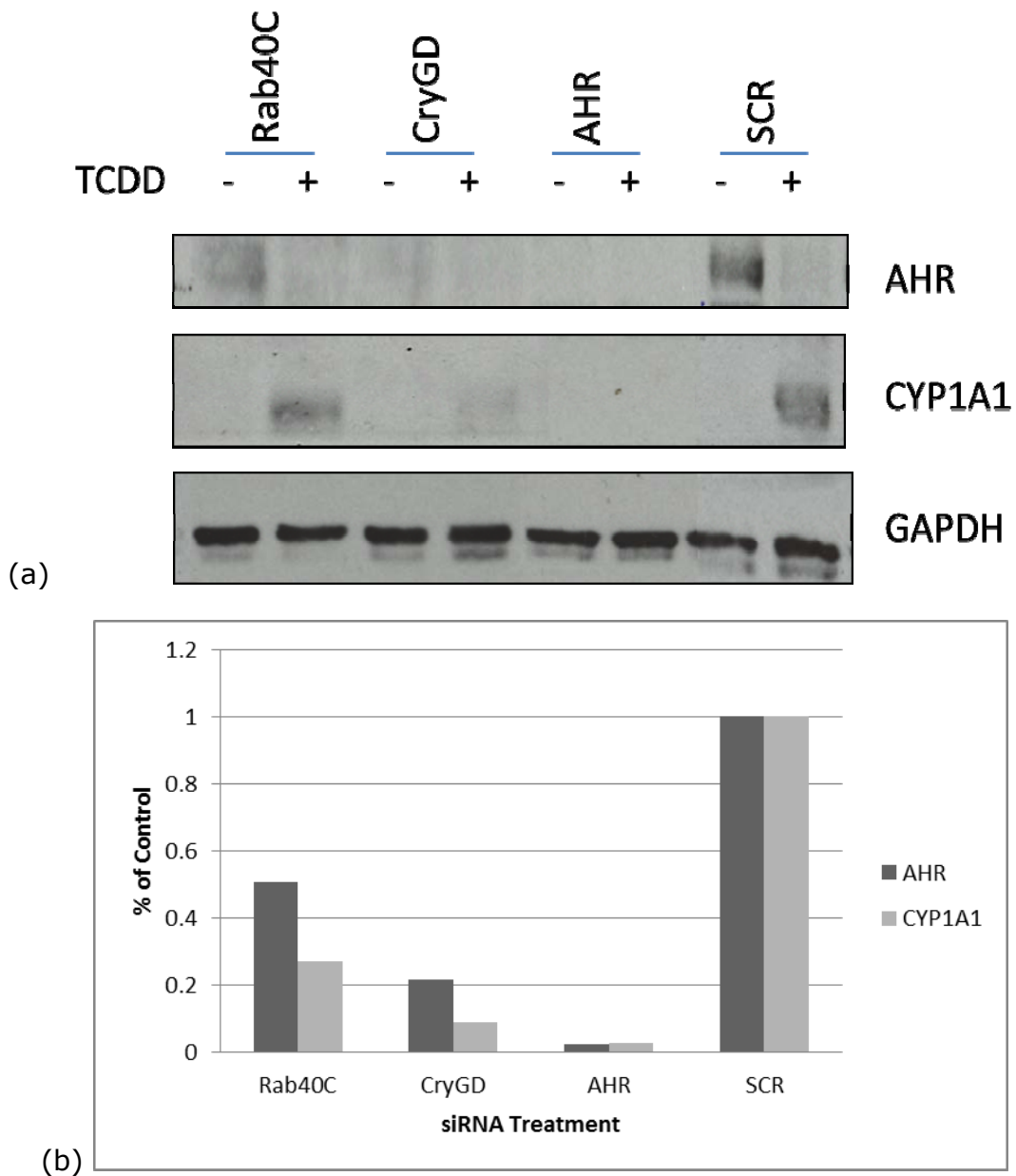


Figure 2.8: Rab40C and CryGD are necessary for TCDD-induced CYP1A1 protein expression and AHR protein expression. (a) Western blots of CYP1A1 and AHR were performed after transfection of Hepa-1 cells with siRNA in the presence of DMSO (-) or TCDD(+). (b) Densitometry quantification of western blots (normalized to GAPDH) revealed the need for Rab40C and CryGD in both CYP1A1 and AHR protein expression.

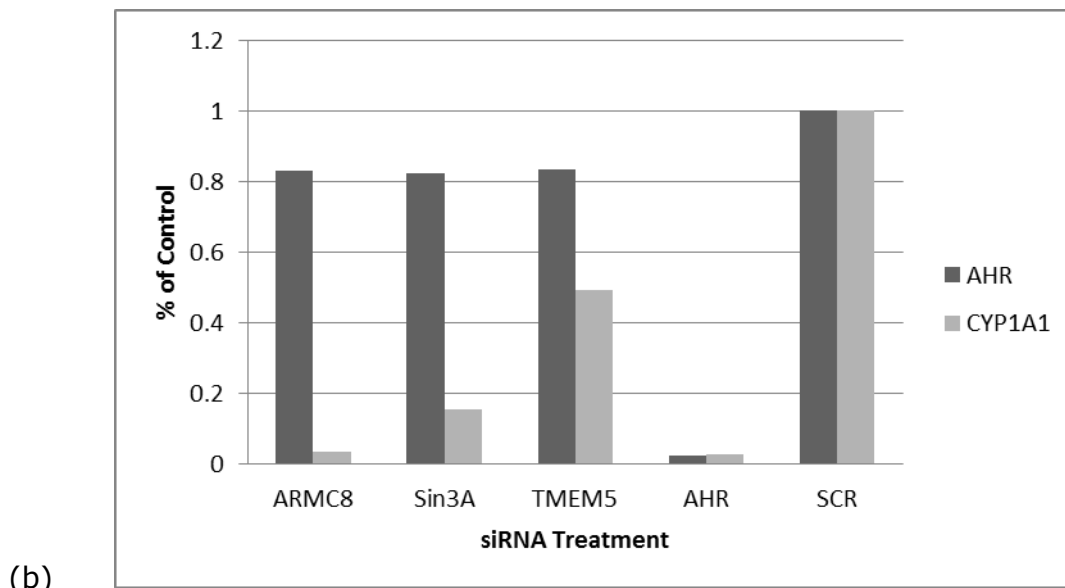
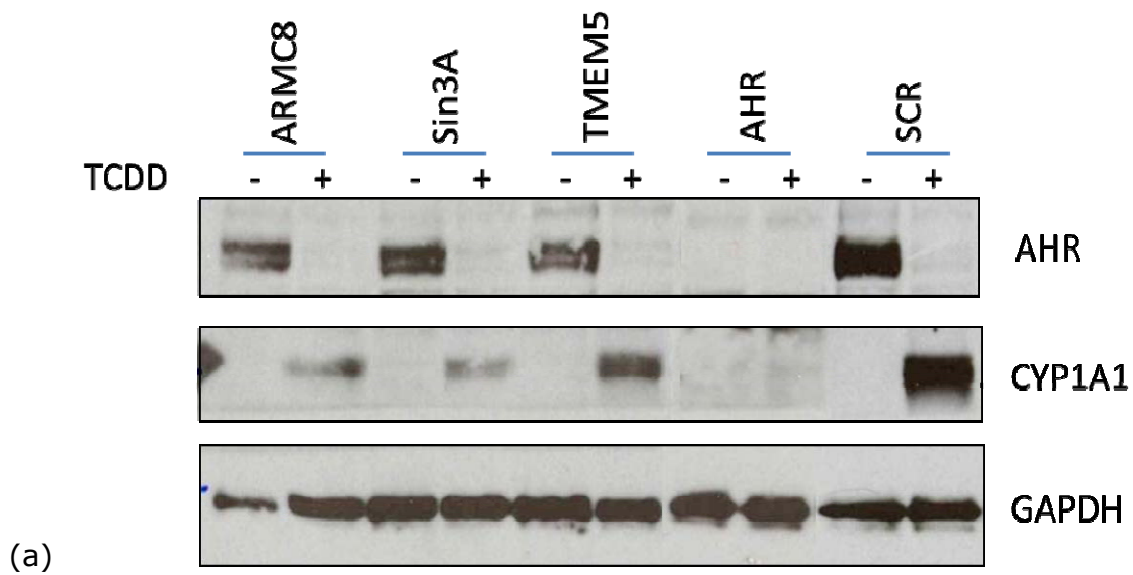


Figure 2.9: ARMC8, Sin3A, and TMEM5 are necessary for TCDD-induced CYP1A1 protein expression but not for AHR protein expression. (a) Western blots of CYP1A1 and AHR were performed after transfection of Hepa-1 cells with siRNA in the presence of DMSO (-) or TCDD(+). (b) Densitometry quantification of western blots (normalized to GAPDH) revealed the need for ARMC8, Sin3A, and TMEM5 in CYP1A1 but not AHR protein expression.

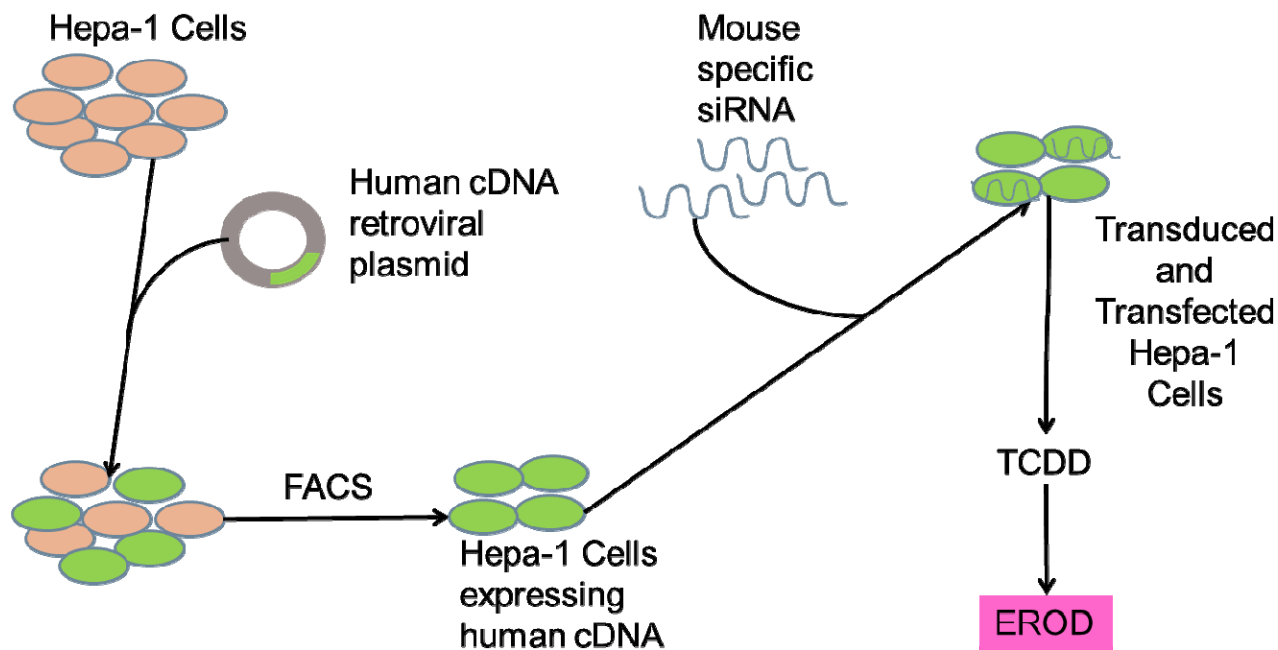


Figure 2.10: EROD Rescue Methods. Hepa-1 cells were transduced with a retroviral pMSCV-ires/GFP vector expressing a human cDNA of interest, and GFP positive cells were isolated using Fluorescent Activated Cell Sorting (FACS). Isolated cells were then transfected with siRNA directed at the mouse cDNA counterpart, treated with TCDD, and assayed for CYP1A1 EROD activity to see if the effects of the siRNA were rescued by overexpressing the cDNA.

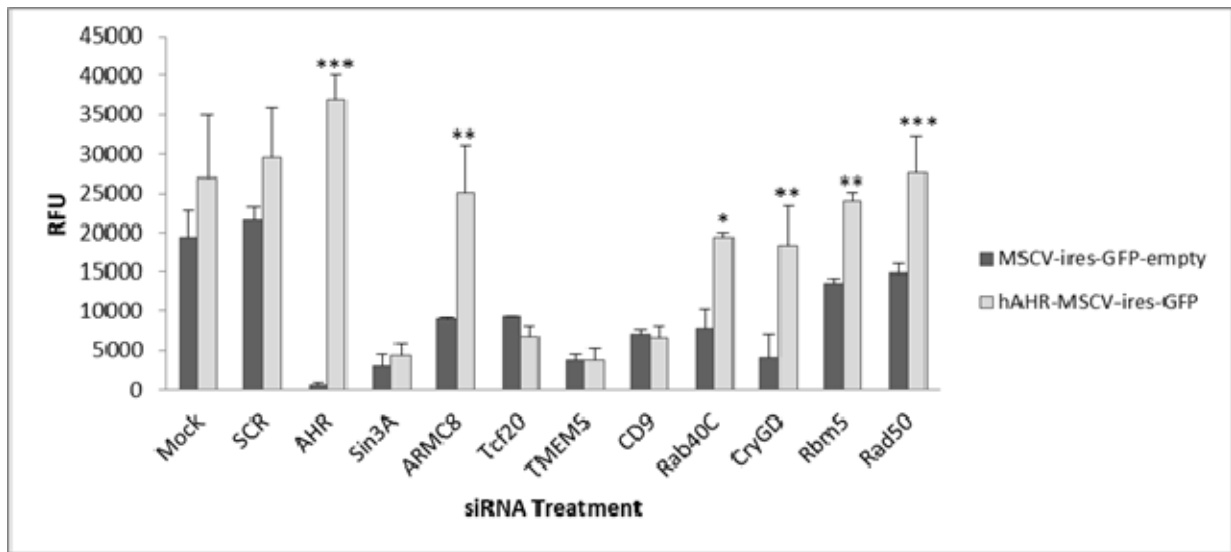


Figure 2.11: Overexpressing AHR Rescues EROD Activity. Hepa-1 cells were transduced with a pMSCV-ires/GFP retroviral expression vector containing human cDNA to overexpress AHR, treated with siRNA, and subsequently treated with 10nM TCDD. CYP1A1 EROD activity was measured to determine if overexpression was enough to rescue CYP1A1 EROD activity in the presence of siRNA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with empty vector control.

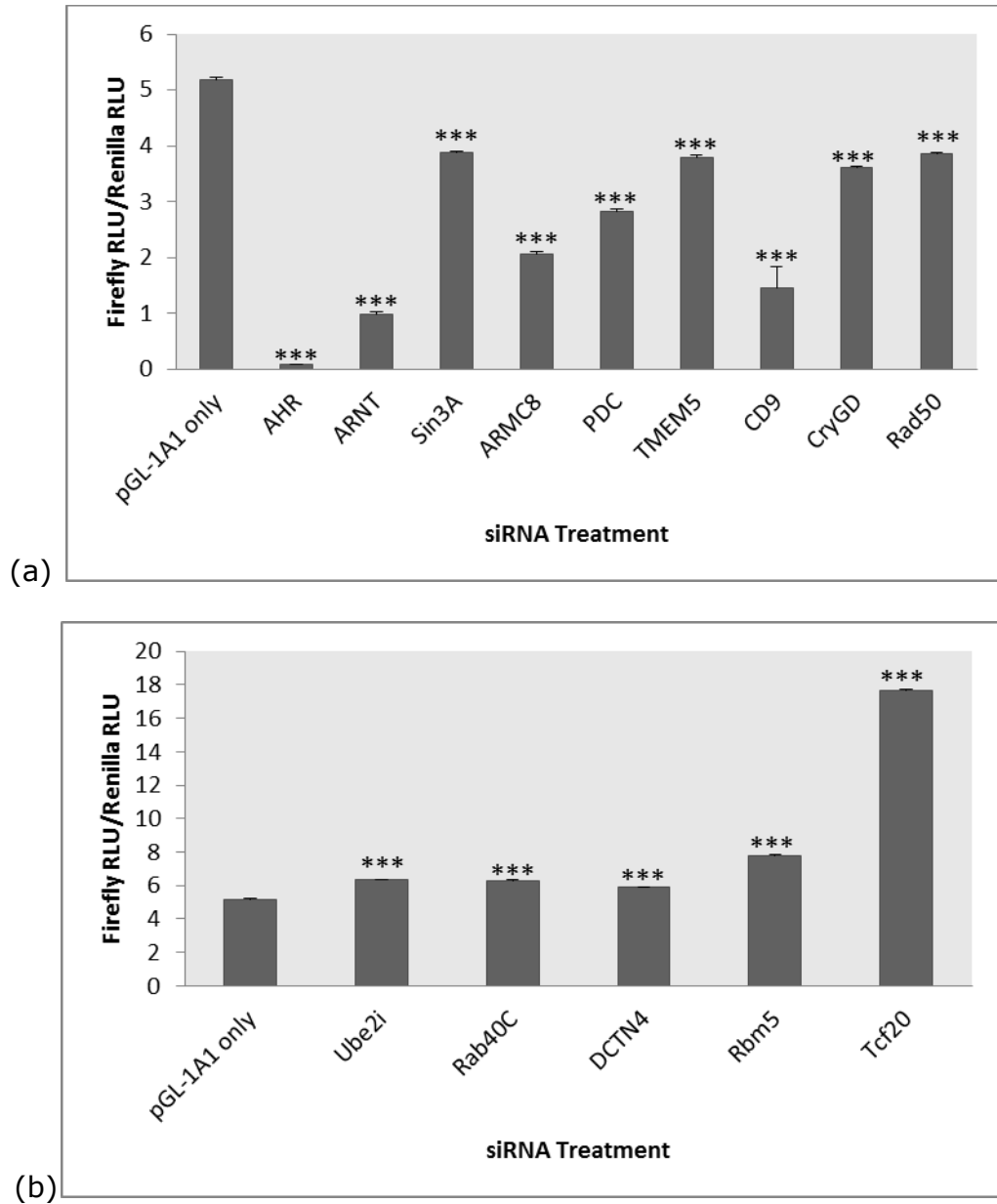


Figure 2.12: Induction of Luciferase Activity by the pGL-CYP1A1 Reporter Construct in the Presence of siRNA. Luciferase activity was measured in Hepa-1 cells that were cotransfected with pGL-*CYP1A1* and siRNA to determine the involvement of the *CYP1A1* promoter. Results revealed a significant influence from each siRNA on luciferase activity, with a most noticeable decrease for ARMC8 and CD9 compared to the untreated control (a), but luciferase activity was not diminished in many samples, and in the case of Tcf20 it was substantially increased (b). Data is representative of 3 experiments.

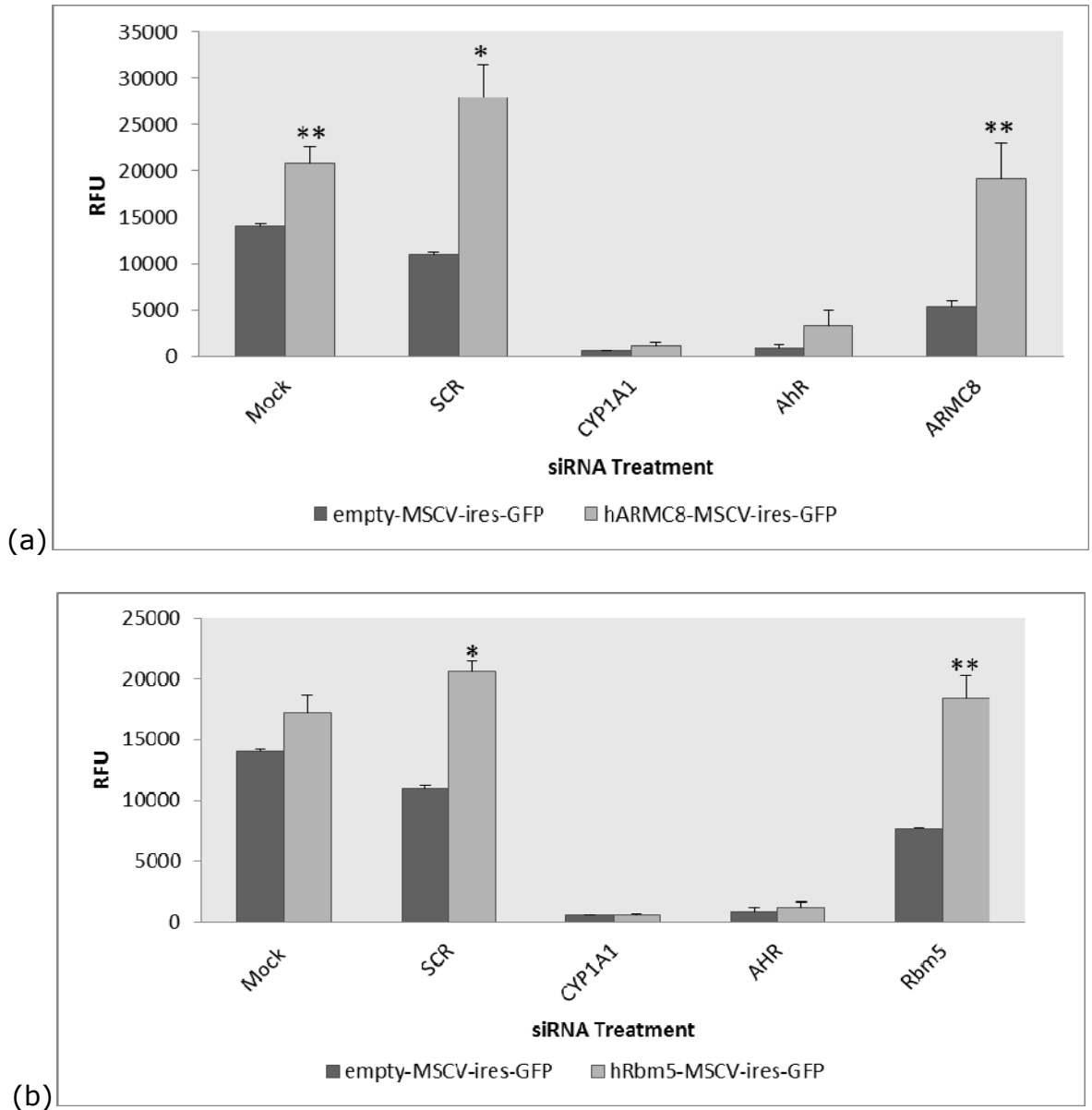


Figure 2.13: EROD Rescue Experiments. Hepa-1 cells were transduced with a pMSCV-ires/GFP retroviral expression vector containing human cDNA to overexpress ARMC8 (a), and RBM5 (b). Cells were subsequently treated with their corresponding siRNA followed by 10nM TCDD, and CYP1A1 EROD activity was measured to determine if overexpression was enough to rescue CYP1A1 EROD activity in the presence of siRNA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with empty vector control.

Rank	Gene Name	Extended Gene Name	Function	EROD (%KD)	qPCR (%KD)				
					CYP1	AHR	ARNT	Nqo1	Aldh3a1
7	Sin3A	SIN3 homolog A, transcription regulator	transcription regulator	62.8%	73.4%	32.2%	1.8%	24.8%	93.3%
23	ARMC8	armadillo repeat containing 8	other	60.7%	81.5%	69.6%	-108.6%	69.9%	94.9%
31	PDC	phosducin	other	54.8%	52.6%	23.2%	-2.0%	-15.5%	42.7%
32	Tcf20	transcription factor 20 (AR1)	transcription regulator	62.0%	55.0%	43.4%	27.0%	43.2%	-8.2%
49	CFC1	cripto, FRL-1, cryptic family 1	other	49.6%	25.6%				
50	SORL1	sortilin-related receptor, L(DLR class)	transporter	49.5%	21.3%				
54	TMEM5	transmembrane protein 5	other	77.1%	92.5%	49.1%	-100.3%	73.1%	96.2%
55	CDC20	cell division cycle 20 homolog (<i>S. cerevisiae</i>)	other	42.3%	38.9%	68.9%	-2.2%	66.3%	55.0%
67	AGTR2	angiotensin II receptor, type 2	GPCR	50.8%	39.2%	18.5%	-5.0%	14.2%	62.0%
76	CD9	CD9 molecule	other	64.8%	62.8%	-10.0%	-12.7%	56.0%	90.2%
87	Ube2i	ubiquitin-conjugating enzyme E2I	enzyme	69.7%	45.8%	55.2%	-34.9%	73.3%	77.8%
88	Rab40C	RAB40C, member RAS oncogene family	enzyme	58.1%	47.3%	51.7%	-5.0%	29.3%	89.6%
92	Pygl	phosphorylase, glycogen, liver	enzyme	53.1%	12.5%				
99	CryGD	crystallin, gamma D	other	79.0%	65.9%	71.7%	13.5%	75.3%	61.3%
103	DCTN4	dynactin 4 (p62)	other	54.9%	65.7%	70.2%	-95.0%	72.4%	21.1%
113	Rbm5	RNA binding motif protein 5	other	49.5%	57.5%	53.8%	-32.4%	59.6%	86.2%
123	Rad50	RAD50 homolog (<i>S. cerevisiae</i>)	enzyme	49.4%	45.5%	33.1%	-7.1%	10.9%	54.7%

Table 2.1: Confirmed Hits Data Summary. A secondary screening confirmed the discovery of 17 novel proteins which are necessary for TCDD induced CYP1A1 EROD activity. Additional quantitative PCR experiments were performed to determine effects of siRNA on expression of *Cyp1a1*, *Aldh3a1*, *Nqo1*, *AHR*, and *ARNT*. Data from these experiments are expressed as percentage knockdown (%KD) compared to scrambled and mock siRNA controls.

	Gene	siRNA Hits	p-value	Rank
1	AHR	3	0.001	29
2	eP300	4	0.002	33
3	ARNT	4	0.003	63
4	BRG-1	2	0.06	482
5	TRIP230	3	0.09	791
6	ESR1	2	0.09	805
7	BRCA1	2	0.42	2618
8	MED1	1	0.69	3448
9	CoCoA	Not Available in Library		
10	GAC63			
11	RIP 140	Not Tested		
12	NCOA1			
13	NCOA2			
14	NCO3			

Table 2.2: RNAi High Throughput Screening Results from Known CYP1A1 Transcriptional Regulators. Several known regulators of CYP1A1 induction were part of the druggable genome RNAi library, and were therefore examined for their requirement in induction of CYP1A1 EROD activity. A total of 8 known regulators were screened, each of which demonstrated to have at least 1 siRNA hit out of the possible 4. The most statistically significant hits included AHR, eP300, and ARNT.

siRNA Target	Reduced Levels of AHR mRNA	hAHR Overexpression Rescues EROD Induction	Reduced Levels of AHR Protein	Induction of pGL-CYP1A1 Luciferase Reporter Gene by TCDD
Sin3A	-	-	-	25%
ARMC8	+	+	-	60%
PDC	-			42%
Tcf20	+/-	-		-240%
TMEM5	-/+	-	-	27%
CD9	-	-		72%
Ube2i	+			-23%
Rab40C	+	+	+	-21%
CryGD	+	+	+	30%
DCTN4	+			-14%
Rbm5	+	+		-50%
Rad50	+/-	+		25%

Table 2.3: AHR Data Summary. A summary table of experimental results addresses the potential that the activity of any particular protein is mediated through AHR. Data specifies if the siRNA knockdown of each target gene affects AHR (+), does not or only slightly reduces AHR mRNA (-), effects AHR relative to CYP1A1 expression (+/-), or does not effect AHR relative to CYP1A1 expression (-/+). Observations for AHR mRNA expression levels were relative to that of CYP1A1 mRNA expression levels, and are defined as: (- corresponding to <50%); (+ corresponding to >80%); (-/+ corresponding to 50%<-/+< 65%); and (+/- corresponding to 65%<+/- <80%).

1	Cell cycle	Spindle assembly and chromosome separation
2	Transcription	Ligand-Dependent Transcription of Retinoid-Target genes
3	Regulation of lipid metabolism	RXR-dependent regulation of lipid metabolism via PPAR, RAR
4	Transcription	Sin3 and NuRD in transcription regulation
5	Cell cycle	Role of APC in cell cycle regulation
6	Cytoskeleton remodeling	TGF, WNT and cytoskeletal remodeling
7	Transcription	PPAR pathway
8	Cell Cycle	Regulation of G1 to S transition part 1
9	Development	Role of HDAC and calcium calmodulin-dependent kinase in control of skeletal myogenesis
10	Cytoskeleton remodeling	Neurofilaments
11	Transcription	Role of Akt in hypoxia induced HIF1 activation
12	Cell Cycle	The metaphase checkpoint
13	Regulation of lipid metabolism	Regulation of fatty acid synthase activity in hepatocytes

Table 2.4: GeneGo® Pathway Maps. Candidate hits were uploaded into GeneGo® software to determine relevant known pathway maps that contain a significant number of these hits. These pathway maps are listed.

ACKNOWLEDGEMENTS

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Chapter 3:

A Novel Role for Sin3A in the Induction of *Cyp1a1* Expression by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

ABSTRACT

CYP1A1 is a target gene of the aryl hydrocarbon receptor (AHR) transcription factor which plays a role in the bioactivation of several procarcinogens and in detoxification of several xenobiotic compounds. In order to elucidate the mechanisms of AHR-dependent induction of *Cyp1a1* gene expression we performed an RNAi high throughput screening in mouse Hepa-1 cells, which were treated with the AHR ligand 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) and then subjected to the ethoxyresorufin-o-deethylase (EROD) assay to quantify activity of the CYP1A1 protein. From this screening we discovered that Sin3A is necessary for induction of CYP1A1-dependent enzymatic activity. Secondary screenings with siRNA and endonuclease-prepared siRNA (esiRNA) validated the results, and upon further investigation we determined that Sin3A is also necessary for TCDD induction of CYP1A1 mRNA and protein. Sin3A mediates histone deacetylase enzymatic activity through its association with HDAC1/HDAC2, and can thereby influence gene expression. Through chromatin immunoprecipitation (ChIP) assays we established that Sin3A physically associates with the 5' flanking regulatory region of *CYP1A1* in TCDD-induced mouse Hepa-1 cells and human HepG2 and MCF-7 cells. In addition, induction of a pGL-*CYP1A1* luciferase reporter gene construct, which is driven by the 5' upstream regulatory sequence of *CYP1A1*, was significantly reduced in cells treated with siSin3A, although not to the same degree as the reduced expression of the endogenous *Cyp1a1* gene, further implicating that Sin3A is acting on the regulatory regions of *CYP1A1* to influence its expression. These studies indicate that Sin3A is essential for *Cyp1a1* induction and exposes a novel role for Sin3A in transcriptional activation of AHR-mediated gene expression.

Keywords: *Cyp1a1, AHR, Sin3A, HDAC, Hepa1, histone deacetylase, expression, RNAi, high throughput screen, EROD, CHIP, MCF-7, HepG2*

INTRODUCTION

CYP1A1 is a phase I metabolizing enzyme involved in either the detoxification or bioactivation of xenobiotics and environmental pollutants, including polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) (Hankinson 1995). The expression of *CYP1A1* is induced upon exposure to these PAHs/HAHs, such as benzo[a]pyrene (B[a]P), which can come from a number of environmental sources including tobacco smoke, automobile exhaust, industrial by-products, and charbroiled foods (Ma and Lu 2007). The most robust induction of *CYP1A1*, however, comes from 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is a persistent environmental pollutant and one of the most potent carcinogens known (Mandal 2005). The mechanism of induction, and thereby proposed mechanism of toxicity, is through the aryl hydrocarbon receptor (AHR) pathway, where TCDD serves as a ligand for AHR. Ligand binding enables the translocation of AHR into the nucleus where it can then form a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) protein, and subsequently bind xenobiotic response element (XRE) core consensus sequences within genomic DNA to activate transcription (Monostory et al. 2009; Nukaya and Bradfield 2009). There have been considerable advances in the field through the years, but further investigation is needed to completely understand the events governing *CYP1A1* transcription (see reviews by Androutsopoulos et al. 2009; Fujii-Kuriyama and Kawajiri 2010; Hankinson 2005).

In this study, we aimed to further elucidate the mechanism leading to induction of *Cyp1a1*. To this end, we performed an RNAi high throughput screening, which revealed Sin3A as a possible protein involved in TCDD induced

CYP1A1 EROD activity. Sin3A is a transcriptional regulator, and as the core component of a multiprotein complex, it serves as a platform for several protein interactions (Silverstein and Ekwall 2005). Sin3A has no known intrinsic enzymatic activity, and is devoid of any known DNA-binding motifs, which is indicative of the fact that the function of Sin3A is dependent on its ability to bind multiple proteins and form specialized complexes (Grzenda et al. 2009). Recruitment of Sin3A to targeted gene promoters is mediated through its interaction with other DNA-binding factors, whereas the histone deacetylase enzymatic activity of Sin3A is exhibited via its association with HDAC1/HDAC2 (Silverstein and Ekwall 2005). The formation of several diverse complexes permits Sin3A to be involved in an assortment of signaling pathways and associated biological processes, including embryonic development, DNA repair, mitochondrial metabolism, gene transcription, and chromatin modifications (Dannenberg et al. 2005). The role of chromatin modifications, which can affect either the interaction of the histones with the DNA or the interaction between adjacent histones, have not been fully explored in *CYP1A1* transcriptional regulation. Many types of modifications exist, such as phosphorylation, ADP-ribosylation, deimination, proline isomerization, and acetylation. Of these modifications, deacetylation activity like that from the Sin3A/HDAC complex serves to restore the basic charge of lysine necessary for histone compaction, and is therefore thought to restrict access to the DNA and to ultimately inhibit transcription (Kouzarides 2007). Despite this canonical role of transcriptional repression, however, recent studies provide accumulating evidence that Sin3A also plays a role in transcriptional activation, including that for MAPK Hog1 target genes, as well as TMEM71 (De Nadal et al. 2004; Silverstein and Ekwall

2005; Ythier et al. 2010). Early evidence that Sin3A can also act as a positive regulator dates back to when Sin3A was found to be necessary for the upregulation of GAM3/ADR6, thereby leading to the activation of STA1, an extracellular glucosamylase (Yoshimoto et al. 1992). Work in our lab has previously demonstrated the importance of such chromatin modifications in CYP expression by revealing that methylation at the *CYP1B1* promoter in HepG2 cells prevented its induction (Beedanagari et al. 2010b). The requirement of various chromatin modifications in *CYP1A1* induction therefore needs further exploration.

In this study we investigated the role of the Sin3A transcription factor in AHR-mediated induction of *Cyp1a1*, which has not been previously explored. Our RNAi high throughput screening presented us with Sin3A as a factor necessary for TCDD induced CYP1A1 enzymatic EROD activity, and through our follow-up studies we further discovered that Sin3A is necessary for CYP1A1 induction in Hepa-1 cells, and binds upstream regulatory regions of *CYP1A1* in multiple cell lines. Transcriptional activation by Sin3A has not been as widely explored as transcriptional repression, and the results obtained from these studies shed light on these novel mechanisms of Sin3A in the induction of AHR-mediated gene expression.

METHODS

Cell Culture and Reagents: Cells were grown as a monolayer in α -MEM (Minimal Essential Media) (Hepa1c1c7 and MCF-7) or in DMEM (HepG2), supplemented with 10% FBS, 1% penicillin-streptomycin and 1% fungizone (Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂. Tissue culture dishes used to grow HepG2 cells were pre-treated with 50µg/ml poly-L-lysine. Treatments with TCDD (Wellington Laboratories, Guelph, Ontario, Canada) dissolved in DMSO were at either 10nM (Hepa-1) or 100nM (MCF-7 and HepG2) concentrations, and the final concentration of DMSO in the medium was 0.1%.

Transfection of siRNA: Hepa-1 cells were transfected for 72 hours with 25nM siRNA (Thermo Scientific Dharmacon, Lafayette, CO) using Lipofectamine RNAiMax transfection reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Construction of esiRNAs: To generate templates for in vitro transcription a two-step PCR procedure was performed with Hepa-1 cDNA used as the primary PCR template. Primers for esiSin3A were chosen from the website RIDDLE (<http://cluster-12.mpi-cbg.de/cgi-bin/riddle/search>), and a T7 anchor tag (GGGCGGGT) was added at their 5' ends to enable annealing of a T7 promoter primer in the second round of PCR. The 400-600bp amplicon was then used for T7 RNA polymerase (NEB, Ipswich, MA) mediated in vitro transcription. PCR cycling conditions and reaction contents were performed as described by (Fazio et al. 2008)). The dsRNA was then digested with endoribonuclease RNaseIII to generate

a pool of 21bp fragments, and then purified using a two filter purification mirVANA PARIS kit according to manufacturer's protocols (Ambion/ABI, Foster City, CA). GST-RNaseIII was received in a pGEX-2T plasmid as a generous gift from Dun Yang at University of California San Francisco (Yang et al. 2002). Briefly, the plasmid was transformed into E. coli strain BL21 for propagation, isolated with the PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions, and then digested with restriction enzymes to verify the presence of the GST-RNaseIII insert. For protein expression the plasmid was transformed into TOP10 cells and GST-RNaseIII expression was induced by a 3 hour IPTG treatment. RNaseIII was then purified using reduced glutathione beads.

Ethoxyresorufin-O-deethylase (EROD) Assay: To assay for CYP1A1 enzymatic activity, cells were treated with 10 μ M ethoxyresorufin and 500 μ M dicumarol (Sigma Aldrich, St. Louis, MO) in phenol-red free MEM supplemented with 10% FBS, 1% L-glutamate, and 1% pen/strep. Phenol-red free media reduces light scattering and background fluorescence, whereas dicumarol prevents further metabolic degradation of the fluorescent product, resorufin (530ex/590em). Reactions were incubated for the optimized time of 45-60 minutes at 37°C, and resorufin related fluorescence was detected in the Modulus Microplate fluorescence reader (Turner Biosystems, Madison, WI).

Cell Viability Assays: Cells were lysed and subjected to the Cell Titer Glo ATP-dependent cell viability assay, according to the manufacturer's protocols (Promega, Madison, WI) in a 1:3 ratio of Cell Titer Glo to medium.

Quantitative PCR: RNA was isolated using RNEasy Mini columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA synthesis of 2 μ g of total RNA was performed with Superscript III reverse transcriptase (Invitrogen) according to manufacturer's protocols with random hexamers for primers (Invitrogen). Synthesized cDNA was used as a template for qPCR at a 1:10 dilution and SYBR Green (Qiagen, Valencia, CA) used according to standard protocols on an Applied Biosystems 7500 real time PCR machine. Primers for qPCR were designed using Primer Express 3.0 software (Applied Biosystems, CA), and were synthesized by Fisher Scientific, Inc. Quantities were normalized to those for the *Gapdh* glycolytic housekeeping gene.

Western Blotting: Cellular lysates were resolved on a 4-12 % SDS gel in a mini-gel apparatus (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane with a semi-dry apparatus (Thermo Scientific Pierce, Rockford, IL). Blots were then blocked with 3% non-fat milk and incubated with an antibody against CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200, AHR (F. Wang et al. 2006) at 1:200, or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2,000. Donkey anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase were used as secondary antibodies at 1:10,000 or 1:5,000 respectively. Horseradish peroxidase was then detected with a chemiluminescent kit (GE Healthcare, Piscataway, NJ), and densitometry calculations were performed using ImageJ software (NIH).

Transduction for EROD Rescue: Retrovirus was made by cotransfection of 293T cells with the pMSCV-ires/GFP retroviral expression vector (F. Wang et al. 2007) containing the human AHR cDNA, and a pCL-Eco packaging plasmid, using BioT (Bioland Scientific LLC, Paramount, CA) transfection reagent. The following day, transfected 293T cells were treated with 10mM Na-Butyrate for 9 hours to enhance viral production, and after an additional 24 hours the viral suspension was harvested. In 6-well culture dishes, Hepa-1 cells were treated with 2ml of this viral suspension in α MEM supplemented with 8 μ g/mL polybrene, and then centrifuged at 37°C for 1.5 hours at 2,500 rpm. An additional 2ml of fresh α MEM was subsequently added and after 48 hours the cells were trypsinized and plated in a 10cm plate. Once confluent, cells successfully transduced were isolated using Fluorescence Activated Cell Sorting (FACS) for GFP expression. Reverse transfections and EROD experiments were carried out as described.

Transient Transfections and Reporter Gene Assay: Expression plasmids were transfected into Hepa-1 cells cultured in 6-well plates by using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent. siRNA targeting Sin3A was cotransfected with renilla luciferase reporter (pRL-TK) (Promega, Madison, WI) and firefly luciferase reporter (pGL-*CYP1A1*) (Beischlag et al. 2002) constructs. pRL-TK is driven by the herpes simplex virus thymidine kinase promoter whereas pGL-*CYP1A1* is driven by 4.2 kilobases of the 5' upstream regulatory region of the rat *CYP1A1* gene including its promoter and multiple xenobiotic response elements (XRE). XREs serve as a recognition motif for targeting the AHR/ARNT complex to the DNA. Cells were harvested and lysed in passive lysis buffer and luciferase

activities were measured using the Dual-Luciferase system (Promega, Madison, WI) according to the manufacturer's recommended protocols. Firefly luciferase activity was then normalized to renilla luciferase activity.

Chromatin Immunoprecipitation Assay: Confluent cells were treated with TCDD for 60 minutes then treated with 1% formaldehyde solution for 10 minutes at 37°C to crosslink DNA to proteins and proteins to proteins. All subsequent steps were performed at 4°C. Crosslinking was stopped by the addition of 0.125M Glycine for 5 minutes. The cells were then rinsed twice and collected in ice-cold PBS supplemented with 1x protease inhibitor solution (Affymetrix / USB, Cleveland, OH). Centrifugation was performed at 1500 x g for 10 minutes in a Beckman tabletop centrifuge and the pelleted cells were resuspended in 420 µl of lysis buffer (Affymetrix / USB, Cleveland, OH). After a 10 minute incubation on ice, cell lysates were sonicated using an ultra sonicator (Diagenode, NJ, USA) on a high amplitude with 30 second pulses for a total of 13 minutes to shear DNA fragments to sizes between 200 to 900 base pairs. Cellular debris was precipitated by centrifugation at 13,000 rpm for 10 minutes, and supernatants were diluted 1:5 in lysis buffer supplemented with 1 x protease inhibitor solution. For immunoclearing, a 50% slurry of protein-A sepharose beads in Tris-EDTA/2.5 µg of sonicated salmon sperm DNA/bovine serum albumin solution (TE/SSDNA/BSA) was added to the supernatant and incubated on a rotator for 1 hour. Supernatants were isolated, treated with 2µg of the antibodies of interest overnight on a rotator, and then treated with a 50% slurry of protein-A sepharose beads in TE/SSDNA/BSA solution for 2 hours on a rotator. The beads were then pelleted, sequentially washed in

buffers, and chromatin complexes were then eluted from the beads according to manufacturer's protocols (Affymetrix / USB, Cleveland, OH). The crosslinking was reversed by incubating samples in high salt conditions at 65°C overnight. DNA was extracted using DNeasy mini columns (Qiagen) according to manufacturer's protocols. Real-time PCR analysis of the DNA was performed using the methods and primers previously described for our lab (Beedanagari et al. 2010a). Background signals represented by the IgG negative controls were subtracted from all samples, and analysis of the ChIP real-time PCR results were reported relative to that of total inputs. Primers used for the murine CYP1A1 promoter region were 5'-AATGGAGGCCCCAGTACTTAC-3' and 5'-AGAACTACCACCTTCAGGGTTAGG-3' (S. Wang et al. 2004) and primers for the human CYP1A1 promoter were 5'-CCCGCCTATAAAGGTGGCA-3' and 5'-AGCAACTCACCTGAGGTACTG-3' (Beedanagari et al. 2009).

Statistical Analysis: The student's two-tailed t-test was performed to obtain statistical significance and data are presented as mean \pm standard deviation. Graphs and blots are representative of at least two separate experiments.

RESULTS

Sin3A is Necessary for Induction of CYP1A1 EROD Activity and Expression

We previously performed an RNAi high throughput screening for identifying proteins necessary for induction of CYP1A1-dependent EROD activity. In this screening 4 individual siRNAs per target gene were tested in duplicate, and each candidate gene was then ranked by the RSA algorithm according to both the quality of the assay and the quantity of hits (see Chapter 2). One candidate of particular interest was Sin3A, which ranked 7th out of the 5,600 different gene candidates tested, with 4 out of the possible 4 siRNA hits, and a p-value of 0.0002; a ranking higher than both AHR and ARNT. Furthermore, gene expression pathway analysis of our screening results, which was performed using GeneGo© software with the manufacturer's recommended quantity of the top 300 hits (p-value≤0.03), revealed 2 of the most relevant known pathways were transcription based pathways that contained Sin3A (see Chapter 2 for complete list). To confirm our screening results we constructed endoribonuclease-prepared siRNA (esiRNA), which was created in-house through an RNaseIII digestion of in-vitro transcribed dsRNA corresponding to the Sin3A mRNA. Independent EROD experiments to validate Sin3A as a hit were performed, which utilized esiRNA in a secondary screening, and then one of the same chemically synthesized siRNAs as on the original screen for a 3rd screening (Table 3.1). Data from all 3 EROD screenings were consistent, which validated the quality and reproducibility of our screening methods, and provided further evidence that Sin3A expression is necessary for TCDD induced CYP1A1 enzymatic EROD activity.

We next sought to discover if Sin3A was necessary for induction of AHR-mediated gene expression. Hepa-1 cells were reverse transfected with siRNA in 6 well plates for 48 hours, and treated with 10nM TCDD for an additional 24 hours. The mRNA was extracted and quantitative PCR experiments performed. We first confirmed that both our siRNAs knocked down expression of Sin3A (Figure 3.1a) by 76.9%. We then demonstrated that both siRNAs reduced the induction of CYP1A1 (Figure 3.1b) and ALDH3A1 (Figure 3.1c), but not NQO1 (Figure 3.1d) mRNAs. *ALDH3A1* and *NQO1* are part of the 'AHR battery' of genes that are known for their inducibility by TCDD (Dewa et al. 2008). These results indicate that the need for Sin3A in TCDD inducible expression of AHR target genes is selective. Similar experiments were performed to measure protein levels of CYP1A1. Western Blot experiments were performed (Figure 3.2a), and ImageJ software was used to quantify the relative amounts of protein (Figure 3.2b). When compared to scrambled control we observed a significant decrease of 85% in CYP1A1 protein levels in the presence of siRNA directed at Sin3A. Collectively these experiments provide evidence that Sin3A expression is essential for the TCDD mediated induction of CYP1A1.

Effects of Sin3A on ARNT and AHR Expression

To rule out the possibility that the results we observed were secondary effects, we quantified the mRNA levels of both AHR (Figure 3.1e) and ARNT (Figure 3.1f). We found no significant changes in expression of ARNT, but did observe a significant decrease in AHR mRNA. As such, we next examined AHR protein levels

(Figure 3.2) after transfection with siSin3A and demonstrated that there was no significant effect on AHR protein levels, thus confirming that diminished AHR expression is not the source of loss of *Cyp1a1* induction. To further exclude the likelihood of AHR playing a role in our results, we overexpressed AHR to see if this would rescue CYP1A1 enzymatic EROD activity. Hepa-1 cells were transduced with a pMSCV-ires/GFP retroviral expression vector containing human AHR cDNA and GFP positive cells were isolated using FACS. These cells were then transfected with siSin3A, and after a 24 hour treatment with TCDD EROD assays were performed. Overexpression of AHR was not sufficient to rescue CYP1A1 EROD activity in the presence of siRNA to Sin3A, indicating that the need for Sin3A in CYP1A1 induction is not mediated through a decrease in activity or expression of AHR (Figure 3.3).

Sin3A Binds *Cyp1a1* Regulatory Regions

To define the role of Sin3A in the TCDD-induced expression of CYP1A1 we performed chromatin immunoprecipitation (ChIP) assays on the promoter region of *CYP1A1* in both human and mouse cell lines. Our results demonstrated that the recruitment of Sin3A to the promoter region of the *Cyp1a1* gene in Hepa-1 cells was significantly increased in the samples treated with 10nM TCDD, compared to the samples treated with DMSO alone (Figure 3.4a). We also studied the recruitment of Sin3A to the promoter regions of *CYP1A1* in the human cell lines MCF-7 (Figure 3.4b) and HepG2 (Figure 3.4c) after treatment with 100nM TCDD, and discovered that Sin3A physically associates with the 5' flanking regulatory regions of *CYP1A1* in the presence of TCDD in both cell lines.

To elucidate the mechanism by which Sin3A regulates *Cyp1a1* induction after binding upstream regulatory regions, we co-transfected Hepa-1 cells with siRNA directed at Sin3A and the pGL-*CYP1A1* firefly luciferase reporter. This construct is driven by 4.2 kilobases of the 5' upstream regulatory region of the rat *CYP1A1* gene, which includes its promoter and multiple xenobiotic response elements. After 24 hours of treatment with TCDD we measured luciferase expression as an indicator of activity of the *CYP1A1* promoter and observed a significant decrease in luciferase expression in samples treated with siSin3A (Figure 3.5). In comparison to the diminished induction of the pGL-*CYP1A1* reporter gene in the presence of siAHR and siARNT (98% and 81%, respectively), however, there was a much less impact on induction of the *CYP1A1* construct (25% reduction) by siSin3A treatments. In addition, decreases in induction of the endogenous *Cyp1a1* gene in the presence of siSin3A (73% reduction) are considerably higher than decreases in induction of the pGL-*CYP1A1* construct, which suggests that Sin3A plays an important role in induction of the gene in its native chromatin configuration. These data demonstrate that Sin3A binds upstream regulatory regions of the endogenous *CYP1A1* gene, and is required for the induction of a transiently transfected *CYP1A1* driven reporter gene, which implicates that Sin3A exerts its effects on gene expression via the upstream regulatory regions of *Cyp1a1*.

DISCUSSION

In this study, we report on a novel role for Sin3A in induction of the *Cyp1a1* gene. CYP1A1 has been studied as the hallmark of AHR-mediated gene induction and overexpression has been widely accepted to lead to carcinogenesis (Androutsopoulos et al. 2009). Understanding the regulation of *Cyp1a1* will therefore contribute not only to an understanding of the dysregulation of AHR-target genes but also the resulting toxicity of this dysregulation. Through our experiments we confirmed results from an initial RNAi high throughput screening and revealed that Sin3A is necessary for TCDD-mediated CYP1A1 enzymatic EROD activity and expression. We not only established the capacity of Sin3A to influence *CYP1A1* induction, we also showed a physical interaction between Sin3A and upstream regulatory regions of the *CYP1A1* gene. Thus binding of Sin3A occurs after treatment with TCDD in Hepa-1 cells as well as in human HepG2 (hepatocellular) and MCF7 (breast) carcinoma cell lines.

Sin3A is known to associate with several proteins to exert activities on transcription, primarily through nucleosomal remodeling (Grzenda et al. 2009). Although generally associated with repression, HDAC activity characteristic of the Sin3A/HDAC complex has been known to alter chromatin domains that can activate gene transcription (Fischle et al. 2003). Structural analysis of Sin3A has revealed the presence of 4 paired α -helices (PAH), a histone interaction domain (HID), and a highly conserved region (HCR). As a scaffold protein, different interaction regions on Sin3A have been established for various proteins. HDACs and Sin3A associated proteins (SAPs) bind to the HID motif of Sin3A whereas the HCR motif is considered important for binding to proteins necessary for repression, such as the nuclear

receptor hormone corepressor Alien (Fleischer et al. 2003; Moehren et al. 2004). The PAH domains however are thought to be the main interacting domains of Sin3A, and PAH2 in particular has been predominantly identified as binding transcription factors (Silverstein and Ekwall 2005). Although there is little sequence homology, the structural similarity between the PAH motifs of Sin3A and the helix-loop-helix protein dimerization domains of certain transcription factors allows for Sin3A to be involved in multiple protein-protein interactions (Silverstein and Ekwall 2005). One such example is the basic helix-loop-helix (bHLH) transcription factor Mad, which forms a heterodimer with Max in order to associate with Sin3A and ultimately bind DNA. This interaction requires the PAH2 domain of Sin3A, and the first 25 residues of Mad comprising one of its basic DNA-binding α -helices (Ayer et al. 1995). In addition, HERP1 and HERP2, which are also members of the same family of bHLH transcription factors as AHR and ARNT, interact with Sin3A and HDAC1 through their bHLH domain (Iso et al. 2001). The structural homology and the interaction of Sin3A with other bHLH transcription factors suggests Sin3A may similarly bind AHR and/or ARNT to influence CYP1A1 expression, and this likely occurs through the PAH motif. Co-IP experiments will confirm this interaction, and subsequent yeast two-hybrid assays with the bHLH region of AHR/ARNT and/or the PAH motif of Sin3A will help determine interacting domains. Additional investigation to determine amino acids essential for binding can be performed through the creation of point mutations that will eliminate the interaction with Sin3A.

Despite the robust results obtained in our siRNA screening for Sin3A, our library contained siRNAs to several subunits of Sin3A including Rbbp4, Rbbp7,

Sap18, and Sap30 which did not significantly reduce CYP1A1 induction (see Chapter 2). These subunits may not be essential for CYP1A1 induction, but it is noteworthy that data from these siRNAs may have not been statistically significant because of the nature of the analysis which incorporated the quality of each plate (Z-prime). A marginal Z-prime of 0.32 and 0.37 was observed for Rbbp4 and Rppb7 and may be the reason we did not see a low p-value for these targets, but further investigation will help determine this. Our library also contained siRNAs targeting HDAC1 and HDAC2, and despite their high Z-prime values of 0.71 and 0.75 we did not see a single siRNA hit in our screening. Through association with HDAC1/HDAC2, histone deacetylase activity is thought to be the main driving engine for Sin3A's effect on transcription activity, which contrasts with our observations. Our results, however, do coincide with the studies performed by Schnekenburger et al, where they demonstrated that knocking down expression of HDAC1 had no effect on induced or uninduced CYP1A1 expression (Schnekenburger et al. 2007). Despite these observations they did, however, show HDAC1 binding to the promoter of *Cyp1a1* in uninduced Hepa-1 cells (Schnekenburger et al. 2007). These findings would appear to conflict with our observation that Sin3A binding to the CYP1A1 promoter increases in induced cells, and contrasts with a study by Suzuki and Nohara, which demonstrated that upon exposure to TCDD the presence of HDAC1 increases at the promoter of *Cyp1a1* in Hepa-1 cells (Suzuki and Nohara 2007). In lieu of the conflicting experiments we aimed to elucidate the requirement of histone deacetylation activity by Sin3A in the induction of CYP1A1. To this end we performed siSin3A experiments and treated Hepa-1 cells with varying concentrations of the global histone deacetylation inhibitors Trichostatin-A (TSA) or

sodium butyrate (data not shown). Our lab has previously treated Hepa-1 cells with TSA in the absence of siRNA to determine the effects of histone acetylation on CYP1A1 expression (Beedanagari et al. 2010a). Deacetylation generally favors gene expression, and although the results from the study by Beedanagari et al revealed that global acetylation causes an enhancement in CYP1A1 induction, no significant results could be concluded. TSA is a global inhibitor influencing the expression of many genes including AHR, and it was noted in these studies that the increase in AHR expression after TSA treatment may contribute to the increased levels of expression of CYP1A1. In congruence with these results, however, we would expect in our experiment that since HDAC activity is already inhibited by the treatment with deacetylation inhibitors, additional treatments with siSin3A would have little influence on the increased levels of induced CYP1A1 detected. If, however, the activity of Sin3A is acting through some other mechanism than HDAC activity we may expect to see a reduction in the increased levels of CYP1A1 observed. Nevertheless, even at the lowest concentrations the treatments were significantly toxic to the cells in fewer than 3 hours (data not shown). No conclusions could be drawn from these experiments and further inhibition studies cannot be explored unless a more specific inhibitor is discovered. Additional experiments are therefore necessary to elucidate the role of the Sin3A/HDAC complex in *CYP1A1* induction. Irrespective of the inconclusive results obtained from these HDAC experiments we now know that Sin3A can form several complexes with other enzymatic proteins that are capable of histone methylation, DNA methylation, chromatin remodeling, and N-acetyl-glucosamine transferase activity (Ellison-Zelski and Alarid 2010). Even though Sin3A is predominantly known to

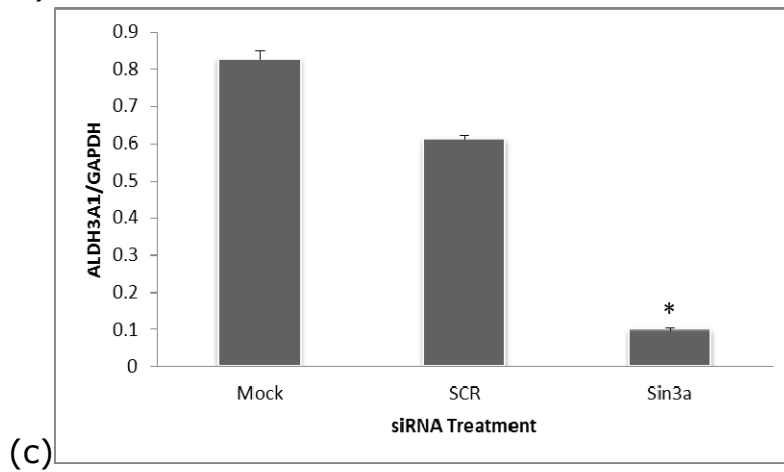
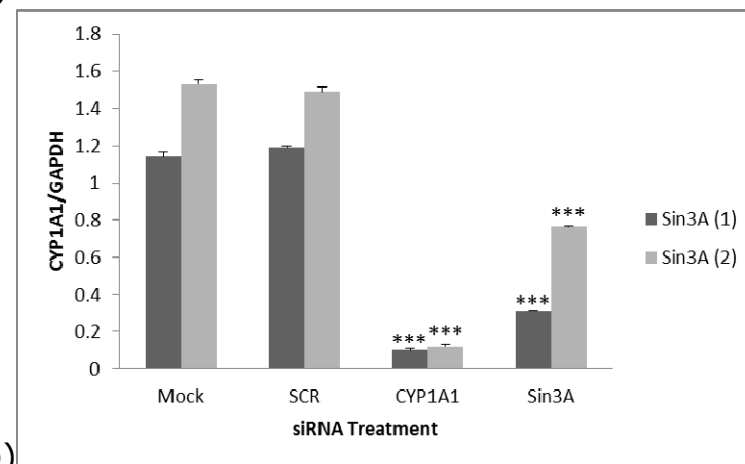
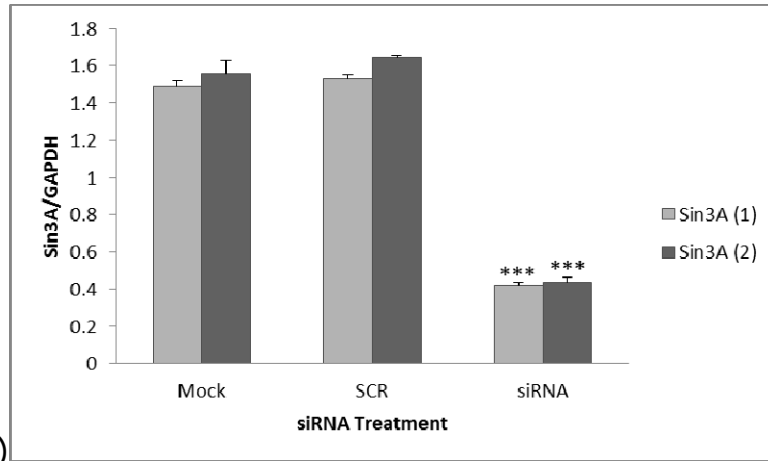
exert its effects through HDAC activity in the Sin3A/HDAC complex, we cannot rule out the possibility that the role of Sin3A in TCDD induction of CYP1A1 is a result of enzymatic activity other than HDAC activity. For example, two independent laboratories have found that Sin3 associates with subunits of the BRG-based SWI/SNF chromatin remodeling complex (Kuzmichev et al. 2002; Sif et al. 2001). BRG-1 has ATPase enzymatic activity which is necessary for its ATP-dependent chromatin remodeling activity. Furthermore, our lab has previously reported the functional involvement of BRG-1 in TCDD induction of CYP1A1 transcription (S. Wang and Hankinson 2002), and the association of BRG-1 with Sin3A could be the means by which Sin3A is required for CYP1A1 induction. Taken together, we cannot presume it is HDAC that is the enzymatic subunit responsible for our observations and further investigation is needed to determine interacting proteins of Sin3A that are required for its function in chromosomal rearrangements and subsequent induction of CYP1A1 expression.

In summary, this study established an essential role for Sin3A in the regulation of CYP1A1 induction, and provides additional support of the notion that Sin3A can act as a transcriptional activator of gene expression. Further studies focusing on identifying the binding domains of Sin3A with AHR/ARNT, as well as the role of HDAC or other enzymatic proteins in the Sin3A complex will provide insight into the mechanism by which Sin3A positively influences AHR-mediated induction of CYP1A1.

Original Screening				esiRNA	siRNA
Gene	p-value	Hits	EROD (%KD)	EROD (% KD)	EROD (%KD)
Sin3a	0.00020	4	79.2%	67.8%	62.8%

Table 3.1: Sin3A is Necessary for Induction of CYP1A1 EROD Activity.

RNAi high throughput screening results (original screening) revealed Sin3A as a promising candidate necessary for TCDD induced CYP1A1 EROD activity. Follow-up EROD experiments using both endoribonuclease prepared siRNA (esiRNA) as well as a separate chemically synthesized siRNA confirmed the results from the screening.



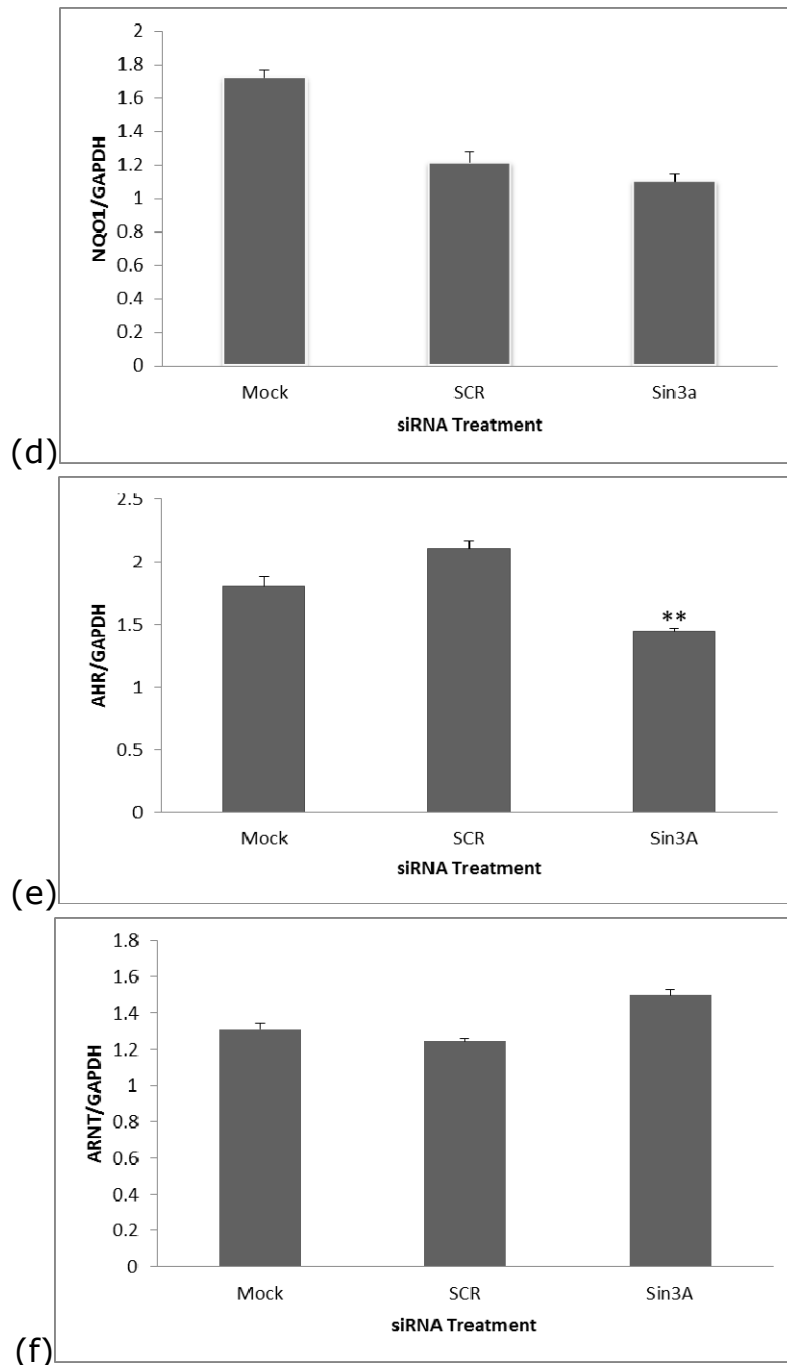


Figure 3.1: Quantitative PCR in Hepa-1 cells Treated with siSin3A. siRNA transfection of Hepa-1 cells to knockdown Sin3A expression (a) reveals that Sin3A is necessary for AHR-mediated induction of CYP1A1 (b) and ALDH3A1(c), but not NQO1(d). There was a modest reduction in AHR (e) expression by siSin3A, but not ARNT (f) expression remained unaffected. Data was normalized to GAPDH. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to Mock + SCR controls.

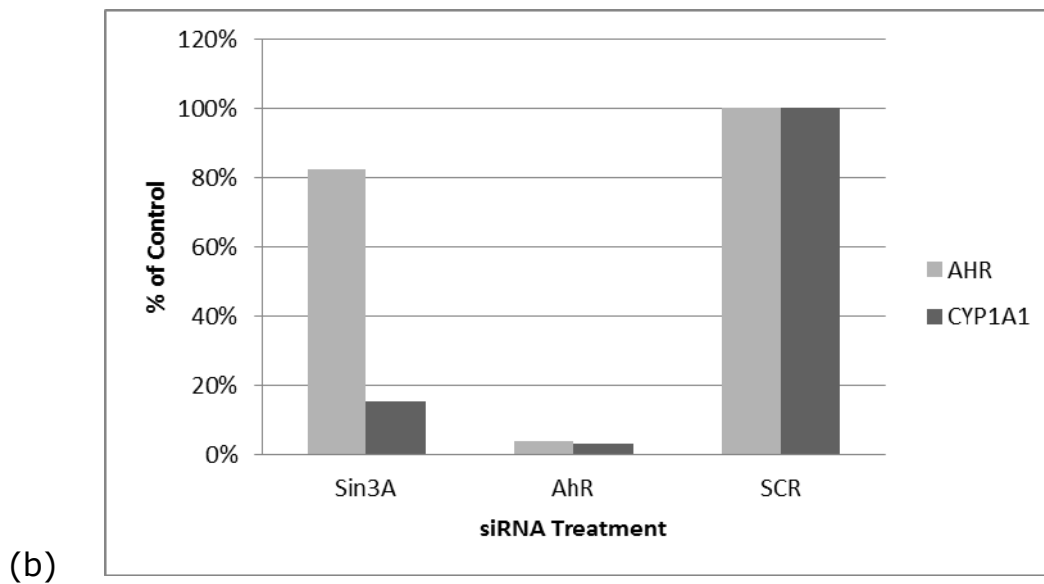
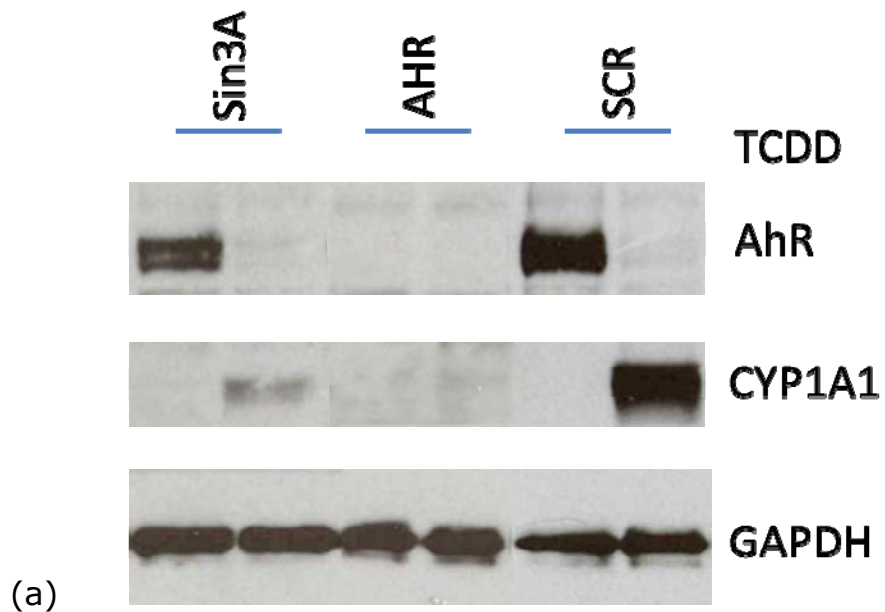


Figure 3.2: Sin3A is Necessary for TCDD Induced CYP1A1 Protein Expression but not for AHR Protein Expression. (a) Western blot of CYP1A1 and AHR after transfection of Hepa-1 cells with siSin3A in the presence of DMSO (-)for AHR or TCDD(+) for CYP1A1. Densitometry quantification of Western blot was normalized to GAPDH (b).

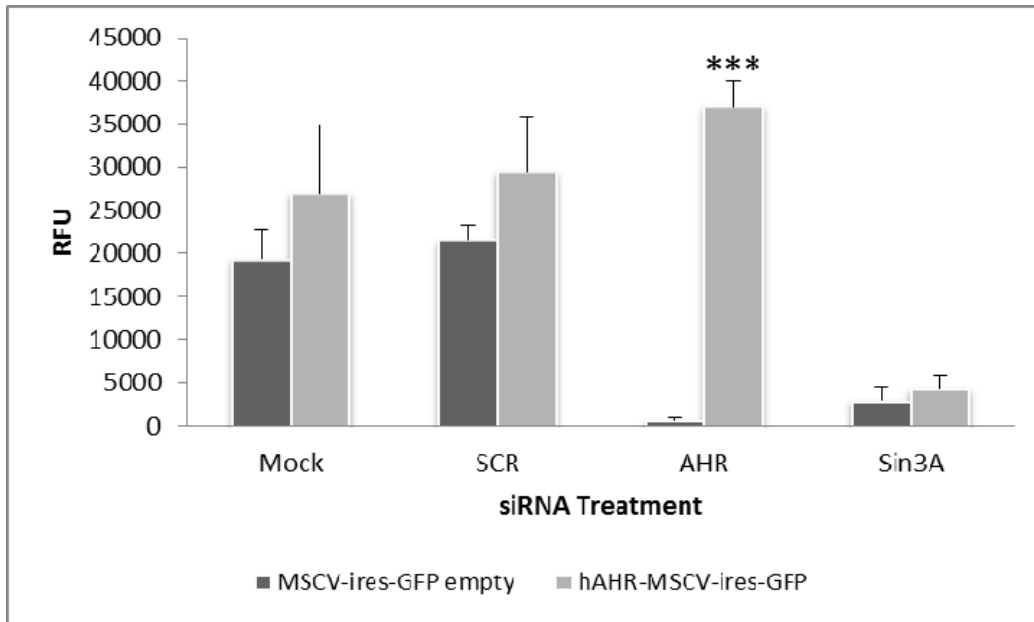


Figure 3.3: Overexpression of AHR does not Rescue the Effects of siSin3A on Induction of CYP1A1 EROD Activity. Hepa-1 cells were retrovirally transduced with a pMSCV-ires/GFP expression vector containing a human AHR cDNA insert. Cells successfully transduced and thereby overexpressing AHR were isolated using FACS and were subsequently transfected with siRNA. CYP1A1 EROD activity was measured to determine if overexpression of AHR was sufficient to overcome the effects of siSin3A. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with empty vector control.

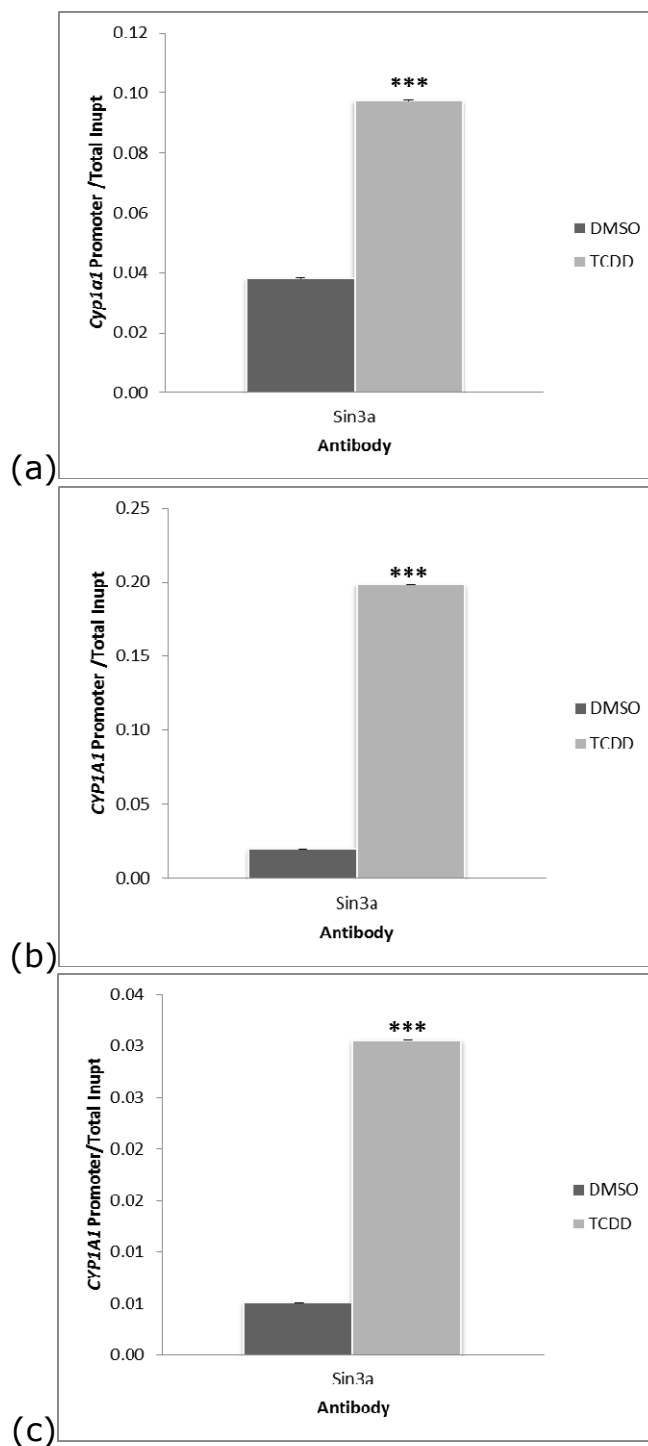


Figure 3.4: Sin3A recruitment to the *CYP1A1* promoter in Hepa-1, HepG2, and MCF-7 cells. Cells were subjected to ChIP analysis after 1 hour of TCDD treatment. The recruitment of Sin3A to the promoter of the *CYP1A1* genes was measured by real-time PCR of ChIP samples from Hepa-1 (a), HepG2 (b), and MCF-7 (c) cells. Data was normalized to the total input. ** $p < 0.01$, and *** $p < 0.001$ compared with DMSO control.

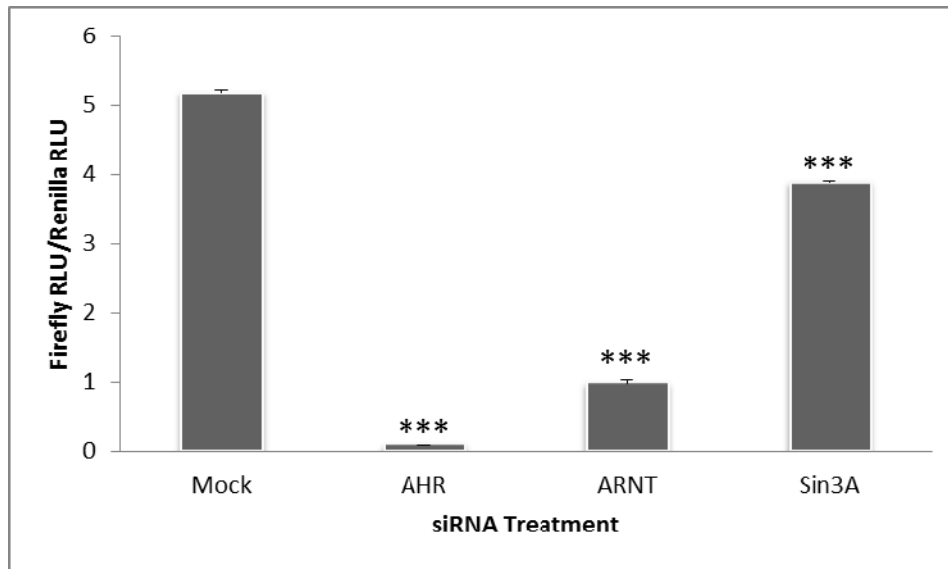


Figure 3.5: Induction of Luciferase Activity by the pGL-*CYP1A1* Reporter Construct in the Presence of siRNA Targeting Sin3A. Hepa-1 cells were transiently transfected with siSin3A and the pGL-*CYP1A1* luciferase reporter driven by 4.2kb fragment containing the promoter and enhancer regions (including functional XREs) of the rat *CYP1A1* gene. siSin3A significantly reduces induction of the pGL-*CYP1A1* construct. *** $p < 0.001$ in comparison to pGL-*CYP1A1* Mock controls.

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Chapter 4

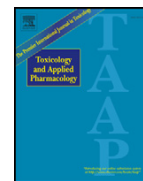
**2,3,7,8-Tetrachlorodibenzo-p-dioxin
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2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment alters eicosanoid levels in several organs of the mouse in an aryl hydrocarbon receptor-dependent fashion

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) adversely affects many mammalian organs and tissues. These effects are mediated by the aryl hydrocarbon receptor (AHR). CYP1A1, CYP1A2 and CYP1B1 are upregulated by the liganded AHR. These (and other) cytochromes P450 can metabolize arachidonic acid into a variety of bioactive eicosanoids. Towards investigating a potential role of eicosanoids in TCDD toxicity, arachidonic acid, two other unsaturated long-chain fatty acids, and up to twenty-five eicosanoids were measured in five organs/tissues of male and female wild-type and *Ahr* null mice treated or untreated with TCDD. TCDD generally increased the levels of the four dihydroxyeicosatrienoic acids (DHETs) and (where measured) 5,6-epoxyeicosatrienoic acid and 18-, 19- and 20-hydroxyeicosatrienoic acids (HETEs) in the serum, liver, spleen and lungs, but not the heart, of both sexes, and increased the levels in the serum, liver and spleen of several metabolites that are usually considered products of lipoxygenase activity, but which may also be generated by cytochromes P450. TCDD also increased the levels of the esterified forms of these eicosanoids in the liver in parallel with the corresponding free forms. The levels of prostanoids were generally not affected by TCDD. The above changes did not occur in *Ahr* null mice, and are therefore mediated by the AHR. TCDD increased the mRNA levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1* and the *Pla2g12a* form of phospholipase A₂ to varying degrees in the different organs, and these increases correlated with some but not all the changes in eicosanoids levels in the organs, suggesting that other enzymes may also be involved.

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Introduction

Eicosanoids are derivatives of twenty carbon polyunsaturated fatty acids, particularly arachidonic acid (AA). Equivalent derivatives of eighteen and twenty-two carbon unsaturated fatty acids, such as linoleic (LA) acid and docosahexaenoic acid (DHA), may have similar biological activities, although less is known about the biological effects of these latter compounds. Eicosanoids are generally short-lived *in vivo*. They have effects on many organs and tissues (Nebert and Karp, 2008). Drugs targeting the arachidonate cascade represent over 25% of the world's pharmaceutical sales (Li et al., 2011).

Arachidonic acid (and the other aforementioned polyunsaturated fatty acids) are metabolized via three pathways: the cyclooxygenase, lipoxygenase and cytochrome P450 epoxidation/hydroxylation

pathways (although cytochromes P450 are or can be involved in all three pathways). The least is known about the last pathway. Mammalian cytochromes P450 from many different subfamilies, including the CYP1A, CYP1B, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, CYP2J, CYP2K, CYP2L, CYP2M, CYP2R, CYP2S, CYP2U, CYP3A, CYP3A4, CYP3A5, CYP3A7, CYP3A9, CYP3A10, CYP3A11, CYP3A12, CYP3A13, CYP3A14, CYP3A15, CYP3A16, CYP3A17, CYP3A18, CYP3A19, CYP3A20, CYP3A21, CYP3A22, CYP3A23, CYP3A24, CYP3A25, CYP3A26, CYP3A27, CYP3A28, CYP3A29, CYP3A30, CYP3A31, CYP3A32, CYP3A33, CYP3A34, CYP3A35, CYP3A36, CYP3A37, CYP3A38, CYP3A39, CYP3A40, CYP3A41, CYP3A42, CYP3A43, CYP3A44, CYP3A45, CYP3A46, CYP3A47, CYP3A48, CYP3A49, CYP3A50, CYP3A51, CYP3A52, CYP3A53, CYP3A54, CYP3A55, CYP3A56, CYP3A57, CYP3A58, CYP3A59, CYP3A60, CYP3A61, CYP3A62, CYP3A63, CYP3A64, CYP3A65, CYP3A66, CYP3A67, CYP3A68, CYP3A69, CYP3A70, CYP3A71, CYP3A72, CYP3A73, CYP3A74, CYP3A75, CYP3A76, CYP3A77, CYP3A78, CYP3A79, CYP3A80, CYP3A81, CYP3A82, CYP3A83, CYP3A84, CYP3A85, CYP3A86, CYP3A87, CYP3A88, CYP3A89, CYP3A90, CYP3A91, CYP3A92, CYP3A93, CYP3A94, CYP3A95, CYP3A96, 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CYP771, CYP772, CYP

hydrolase, which converts them to the dihydroxyeicosatrienoic acids (DHETs). DHETs are generally less biologically active, although some activities have been ascribed to them (Buczynski et al., 2009; Nebert and Karp, 2008).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) elicits a wide variety of toxic, teratogenic and carcinogenic responses in animals and in humans. Most if not all the effects of dioxin are mediated by the aryl hydrocarbon receptor (AHR), which after binding TCDD, translocates to the nucleus, dimerizes with the aryl hydrocarbon nuclear translocator protein (ARNT) and activates transcription of a large number of genes, including those for CYP1A1, CYP1A2 and CYP1B1, and represses others (White and Birnbaum, 2009; Ma et al., 2009; Denison et al., 2011). A wide variety of other compounds, including halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH) can bind to, and activate the AHR. Many if not most of the biological effects of the AHR are probably mediated by its effect on these transcriptional responses (Bunger et al., 2008; Fujii-Kuriyama and Kawajiri, 2010). Nebert and Karp pointed out that “the myriad AHR-mediated processes mirror the vast universe of action of the eicosanoids” and they proposed that many of the biological effects of AHR are mediated by synthesis of eicosanoids by the CYP1 subfamily (Nebert and Karp, 2008). Compatible with this notion, several investigators have shown that microsomes from organs of TCDD- or PAH-treated mammals catalyze the conversion of arachidonic acid to certain eicosanoids at different rates compared with microsomes from non-treated mammals (Capdevilla et al., 1990; Lee et al., 1998; Aboutabl et al., 2009). Furthermore, Dalton and coworkers showed that TCDD exposure increased the levels of three cyclooxygenase-derived arachidonic metabolism in the urine of mice (Dalton et al., 2001). However, to directly address the potential role of eicosanoids

in TCDD toxicity, it would be highly advantageous to measure these compounds in the relevant organs and tissues. Recent advances in liquid chromatography-tandem mass spectrometry (LC-MS/MS) have allowed for the identification and quantitation of a large number of eicosanoids in tissue extracts (Blaho et al., 2009; Yang et al., 2009). We report here such a “lipidomics” approach to quantitate up to twenty-five eicosanoids and three polyunsaturated fatty acids (Fig. 1) in five different organs/tissues from TCDD-treated and untreated mice. Our results demonstrate that the levels of eicosanoids derived from the cytochrome P450-dependent epoxidation/hydroxylation pathway were the most widely and markedly elevated, and that the levels of some of mid-chain hydroxides and other metabolites generally considered to be products of the lipoxygenase pathways were also increased, although there were differences in these regards between organs/tissues. Products of the cyclooxygenase pathway were generally not affected by TCDD treatment. By utilizing an *Ahr*^{-/-} null mouse, we also demonstrate that the changes in eicosanoids levels elicited by TCDD are dependent upon the AHR. Altogether, these studies lay the foundation for future experiments addressing the potential role of eicosanoids in mediating the toxic effects of TCDD and other ligands of the AHR.

Materials and methods

Chemical and reagents

HPLC solvents (HPLC grade) were obtained from Sigma Aldrich (St. Louis, MO). The C18 reversed-phase column (Discovery® C18, Supelco, 2.2 mm × 150 mm, 5 μm) was purchased from Supelco Sigma Alrich (St. Louis, MO). 9α,11α-epidioxy-15S-hydroxy-prosta-5Z,13E-dien-1-

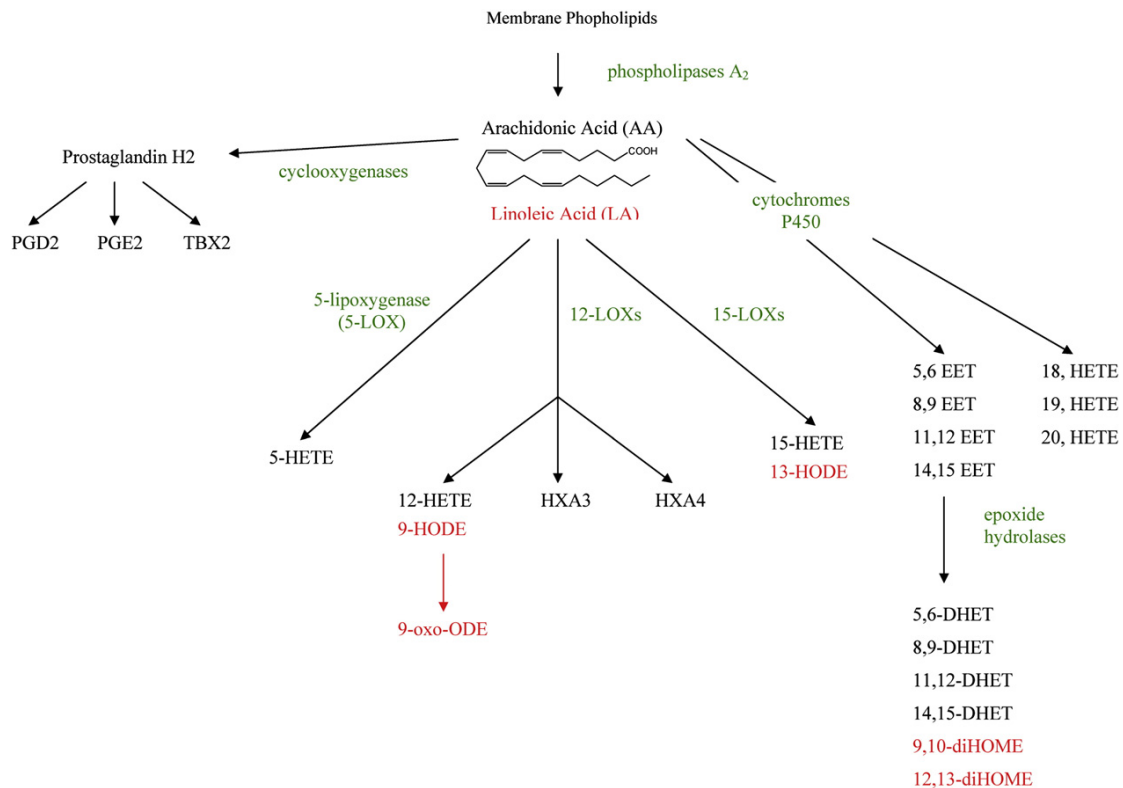


Fig. 1. Metabolism of arachidonic acid (and linoleic acid) by the cyclooxygenase, lipoxygenase and cytochrome P450 pathways, showing the metabolites that were measured. Linoleic acid metabolites are shown in red. Enzymes are shown in green. Abbreviations here and in Table 1 are as follows: TBX, thromboxane; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; 9-oxo-ODE, 9-oxo-octadecadienoic acid; HX, hepxilin; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; diHOME, dihydroxyoctadecenoic acid.

oic acid (PGH2), 9-oxo-11 α ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (pGE2), 9 α ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (PGD2), 9 α ,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid (TXB2), 6-oxo-9 α ,11 α ,15S-trihydroxy-prosta-13E-en-1-oic acid (6-k-PGF1 α), 9 α ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic-3,3,4,4-d4 acid (PGD2-d4), 9,15-dioxo-11 α -hydroxy-prosta-5Z,13E-dien-1-oic acid (15-keto-PGE2), 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid (LTB4), 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (Resolvin D1), 10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (Protectin D1), 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME), (\pm)5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-DHET), (\pm)8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-DHET), (\pm)11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-DHET), (\pm)14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-DHET), (\pm)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5(6)-EET), (\pm)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8(9)-EET), (\pm)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11(12)-EET), (\pm)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14(15)EET), 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxo-ETE), 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-oxoETE), 15-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-oxoETE), 13-oxo-9Z,11E-octadecadienoic acid (13-oxoODE), 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), 15S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-HETE), 13S-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), (\pm)18-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (18-HETE), (\pm)19-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (19-HETE), (\pm)20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (20-HETE), (\pm)17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17-HDOHE), Linoleic acid (LA), Docosahexaenoic Acid (DHA), Arachidonic acid (AA), 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid (5-HETE-d8), 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid (12-HETE-d8), 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid (15(S)-HETE-d8), and 13-HODE-d4 were purchased from Cayman Chemical (Ann Arbor, MI). Oasis HLB (1 cc/10 mg, 30 μ m) was purchased from Waters Corporation (Milford, MA, USA). The protease inhibitor cocktail was purchased from Roche. TCDD was purchased from Wellington Laboratories, Guelph, ON, Canada and handled with extreme caution.

Animals

Ahr $^{-/-}$ null mice were a kind gift of Christopher Bradfield (Schmidt et al., 1996). They were backcrossed at least seventeen times to C57BL/6 mice and therefore were of the C57BL/6 genetic background. Male and female Ahr $^{-/-}$ null mice and their sibling Ahr $^{+/+}$ wild type mice were obtained from crossing heterozygous Ahr $^{+/-}$ mice. Genotyping of mice was performed by PCR as described by the Jackson Laboratories (http://jaxmice.jax.org/protocolsdb/f?p=116:2:4212526675950722::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:195,002831). All mice were housed and bred at UCLA in a specific-pathogen-free facility. Mice were allowed free access to food (chow diet) and water before being used for the experiments. Mice were kept under a 12-h light/dark cycle and house at 25 °C. Two to three-month-old mice were used for all experiments.

Administration of TCDD to mice and harvesting of tissues/organs

TCDD was received in a pre-weighed vial. 50 μ g/kg of TCDD in corn oil was administered by intraperitoneal injection. Corn oil was used as vehicle control. Five to 8 animals were used per treatment group. Mice were euthanized with carbon dioxide on day 3 after injection. Immediately, blood was collected via cardiac puncture using heparin-coated needles; 0.2% of BHT (butylated hydroxytoluene) and TPP (triphenylphosphine) were added directly to the blood to

prevent auto-oxidation of fatty acids. Serum was prepared using serum separator tubes (BD). The heart, lung, liver and spleen were also collected and stored in -80° C for later analysis. Hearts from only the wild type mice were collected and analyzed. All TCDD procedures with mice, including exposure, euthanasia and dissections were performed in two dedicated laboratories, constituting a “Carcinogen Suite” in the containment area of the UCLA vivarium. The suite is under negative pressure. Isolation of mouse tissues from the TCDD-treated mice was done in a vertical laminar flow hood (Class II B1) certified for use with chemical carcinogens. Cages which had been used with TCDD-treated mice were wiped down with methanol and then water. Bedding was treated as hazardous waste. Mouse carcasses were placed in a designated refrigerator for disposal by UCLA’s Environmental Health and Safety Office (E.H. & S.). After use, solutions containing TCDD were extracted with chloroform for disposal. The chloroform fraction was sent to the UCLA Division of Environmental (E.H. & S.) for disposal by incineration. These procedures have been approved by the UCLA Division of Laboratory Animal Medicine, the UCLA Division of E.H. & S., and the UCLA Office for the Protection of Research Subjects.

Extraction of fatty acids from different tissues

Serum. A 150 μ l volume of serum was transferred to a 2 ml polypropylene tube. The sample was spiked with 100 μ l of internal standards mixture (PGD2-d4, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8, 13-HODE-d4, 10 ng/ml each) in methanol. Subsequently, 1.75 ml of water (0.1% acetic acid, pH 3.0) was added. The samples were left for 15 minutes on ice for complete acidification and equilibration. The resulting sample was then extracted using the solid-phase extraction method described below.

Liver, lung, spleen and heart. Each tissue was cut into pieces and transferred into 2 ml polypropylene tubes. 450 μ l of 50 mM Tris-HCl, pH 7.5 plus protease inhibitors (complete mini protease inhibitor cocktail, Roche, Nutley, New Jersey) was added into each tube. Samples were homogenized for 60 seconds with the medium setting, followed by sonication with 7–8 pulses at 50% output and a 5 pulser setting using a VibraCell sonicator (Sonics and Materials, Inc., Newtown, CT). 250 μ l of lysate was then mixed with 100 μ l of MeOH (0.2% BHT and TPP) followed by centrifugation at 10,000 \times g for 15 minutes. The supernatant was then transferred to 2 ml tube containing 1700 μ l 0.1% acetic acid, pH3.0 and 100 μ l of internal standards mixture (see above) were added. The supernatant was then treated as for the serum samples above. For female mice, the liver tissue was treated the same as for the male mice except that the liver was suspended in 250 μ l H₂O plus 250 μ l of MeOH (0.2% BHT and TPP).

Total fatty acid extraction

Only wild type male mice liver samples were used for total lipid extraction. 250 μ l of liver lysate samples were transferred to a 2 ml tube containing 250 μ l water. 500 μ l of 1 M KOH was added to hydrolyze fatty acids from their conjugates, and the tubes incubated at 37 °C for 30 minutes. The samples were then acidified with 500 μ l of 1 M HCl followed by centrifugation at 10,000 \times g for 15 min. The supernatant was transferred to a 15 ml tube containing 1500 μ l H₂O and 100 μ l of internal standard mixture (10 ng/ml in methanol). The samples were left for 15 min on ice for complete acidification and equilibration. The sample was then extracted using the solid-phase extraction method.

Solid phase extraction

Processed samples from different tissues were loaded onto a pre-conditioned 1 cc Oasis HLB solid-phase extraction (SPE) cartridge on a vacuum manifold (Waters). The SPE cartridge was equilibrated with 2 ml methanol followed by 2 ml water (0.1% acetic acid, pH 3.0) before sample loading. After slowly loading the cartridge, it was washed with 2 ml 5% methanol in water (0.1% acetic acid). Fatty acid analytes were subsequently eluted with 2 ml methanol. The eluate was then evaporated to dryness under a stream of argon. 100 μ l of methanol was added to the dried extract, vortexed for 30s, and the reconstituted extract was centrifuged at 13,200 rpm for 20 min at 4 °C.

LC/MS/MS analysis

LC/MS/MS was performed using a quadrupole mass spectrometer (4000 QTRAP; Applied Biosystems, Foster City, CA) equipped with electrospray ionization (ESI). The HPLC system utilized an Agilent 1200 series LC pump equipped with a thermostated autosampler (Agilent Technologies, Santa Clara, CA). Chromatography was performed using C18 reversed-phase column, (Discovery® C18, Supelco, 2.2 mm \times 150 mm, 5 μ m), plus a C18 guard column held at 45 °C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The autosampler was set at 4 °C. The injection volume was 20 μ l and the flow rate was controlled at 0.4 mL/min. Chromatography was optimized to separate all analytes in 30 minutes. The gradient is given in Table S-1 in the supplemental material. Data acquisition and instrument control were accomplished using Analyst 1.4.2 software (Applied Biosystems). Detection was accomplished by using the multiple reaction monitoring (MRM) mode with negative ion detection. The parameter settings are described in table S-2. Mass spectrometry measurement was divided into 4 periods to increase dwell time and lower the limits of detection. Dwell time was found to play a very important role in increasing the signal-to-noise ratio with a shorter dwell time resulting in a much higher noise (Yang et al., 2009). In our method, the dwell times were set above 75 milli-seconds which is longer than the dwell time used by Yang and coworkers (Yang et al., 2009) (Table S-2). In addition, MRM transitions, collision energy, declustering potential, and collision cell exit potential were optimized for each compound by direct infusion into the mass spectrometer to obtain optimum sensitivity (see Table S-3). For several analytes for which we did not possess a standard, MRM transition parameters were adopted from previous publications (Blaho et al., 2009; Yang et al., 2009). For most analytes, four concentrations (5, 10, 20, and 40 ng/ml) were used to create standard curves. In the case of docosahexaenoic acid (DHA), 10, 20, 40, and 80 ng/ml were used, whereas 50, 100, 200, and 400 ng/ml were used for arachidonic acid (AA) and linoleic acid (LA). All the standard curves had R² values (for linear regression analysis) of 0.990 or greater for each analyte. The limit of detection for each standard was below 0.5 ng/ml. The inter- and intra-day accuracy and precision were determined based on the % CV (coefficient of variation) using data for 13-HODE and 12-HETE (see supplemental material Table S-4-5). In addition, to ensure the consistency of the instrument during every experimental run, the standards were always submitted first and resubmitted again at the end of each run. The % CV between the two submissions of the same sample were routinely less than 15%.

RNA extraction, cDNA synthesis, and real-time PCR

RNA was isolated using RNEasy Mini columns (Qiagen, Valencia, CA), according to the manufacturer's instructions for frozen animal tissues, and quantified on a NanoPhotometer (Implen, Westlake Village, CA). One microgram of total RNA was used for cDNA synthesis

in a total reaction volume of 20 μ l. Reverse transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen) and primed with random hexamers (Invitrogen) according to the manufacturer's instructions. Following cDNA synthesis, all reactions were diluted into a total of 200 μ l of RNase-free water, and were then used as templates for real-time PCR.

SYBR Green real-time PCR was performed according to standard protocols using the 7500 Fast (Applied Biosystems, Foster City, CA) and quantities were normalized to those for the GAPDH glycolytic enzyme housekeeping gene. All primers were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA), and purchased from Fisher Scientific (Pittsburgh, PA). The forward and reverse primers used for quantification are listed in Supplementary Table S-6.

Data were analyzed using the ABI software and Microsoft Excel, and significance was evaluated using Student's *t*-test.

Results

The effects of TCDD on eicosanoid levels

Adult C57BL/6 wild-type or knockout mice were injected intraperitoneally with 50 μ g/kg TCDD or with vehicle (corn oil). Three days later their organs/tissues were harvested and subsequently analyzed for the levels of certain free (i.e. non-esterified) eicosanoids by HPLC followed by MS/MS (see Materials and Methods). This dose and duration of exposure were used as they are known to maximally induce most TCDD-responsive genes (Hayes et al., 2007; Forgacs et al., 2012). Three separate experiments were performed. In each independent experiment, 5 to 8 mice were analyzed per gender per genotype. In experiment 1, male and female wild-type and *Ahr*^{-/-} knockout mice were analyzed for the levels of certain free eicosanoids in serum, liver, lung, spleen, and heart (wild-type males only). The data for experiment 1 are presented in Supplementary Table S-7. In experiment 2 (Supplementary Table S-8), the levels of total eicosanoids as well as free eicosanoids were measured in the livers of wild-type male mice. To obtain total eicosanoids, liver extracts were treated with KOH in order to hydrolyze membrane phospholipids. In the third experiment, free eicosanoids were measured in the serum from wild-type mice (data not shown). The results from all three experiments are summarized as a "heat map" in Fig. 2. The major points that can be made from these data are as follows:

- (i) TCDD treatment increased the levels of arachidonic acid in the liver
- (ii) In wild-type mice, TCDD had little if any effect on the levels of the cyclooxygenase pathway metabolites in any of the five organs/tissues examined.
- (iii) TCDD increased the levels of many metabolites of the cytochrome P450 epoxidation/hydroxylation pathway in the serum, liver, lung and spleen of wild-type mice, but not in the heart.
- (iv) In the serum, liver and spleen but not the lungs or heart of wild-type mice, TCDD treatment increased the levels of many metabolites of arachidonic acid that are generally categorized as lipoxygenase products (but which can also be generated by particular cytochromes P450).
- (v) The levels of the total metabolites in the liver were in all cases greater than the levels of the corresponding free metabolites, generally exceeding them by 2- to 10-fold (Supplementary Table S-8). The levels of total prostanoids could not be measured as they are degraded by KOH.
- (vi) For those metabolites in the liver affected by TCDD treatment, total levels generally increased in parallel with the free levels of the same metabolite. This was particularly striking for the cytochrome P450-derived DHETs and terminal hydroxides.
- (vii) TCDD significantly increased the levels of only four metabolites and only in one tissue/organ examined (male serum) of male

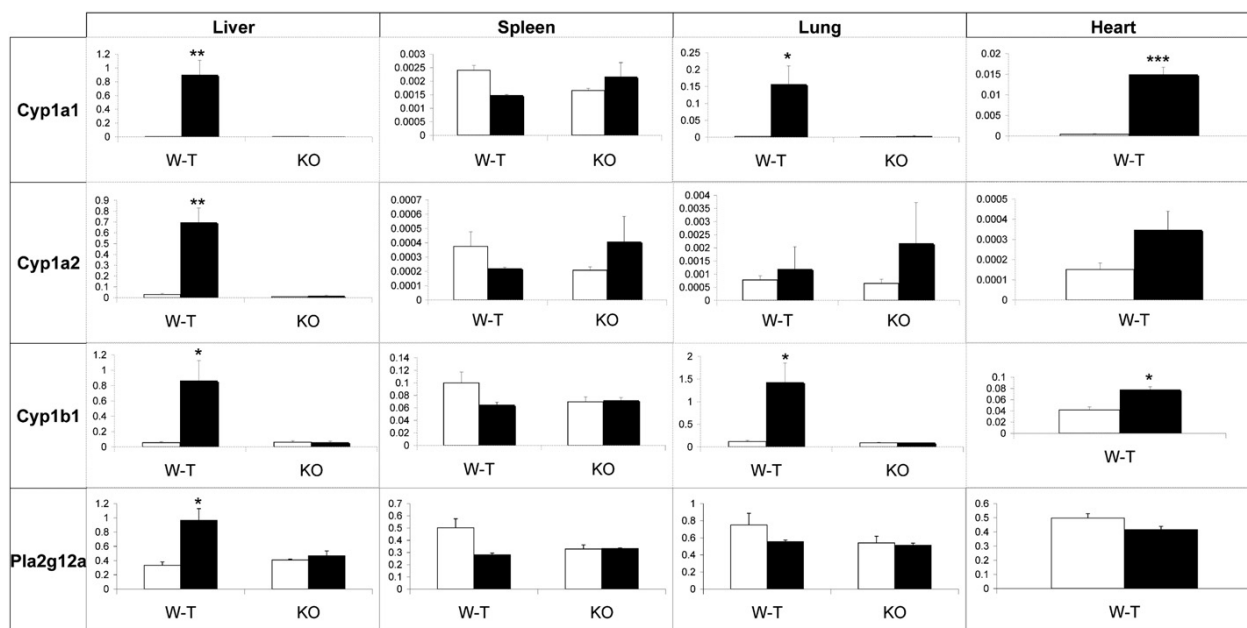


Fig. 3. Effect of TCDD on Cyp1a1, Cyp1a2, Cyp1b1, and Pla2g12a mRNA levels in liver, spleen, lung and heart of male wild-type mice. The levels of each mRNA are represented relative to the levels of the constitutively expressed GAPDH glycolytic enzyme in each organ. The means and standard errors were derived from four mice treated with TCDD (filled bars) or vehicle (open bars). *, ** and *** represent significantly different from the DMSO control at $p < 0.05$, $p < 0.01$ and 0.001 , respectively.

The levels of the Cyp1a1 and Cyp1b1 mRNAs were increased by TCDD treatment in the wild-type male mouse in the liver, lung and heart (although the mRNA levels were 10-fold or more lower in the heart). Cyp1a2 increased only in the liver. None of the mRNA levels were increased by TCDD in the spleen. Interestingly, the levels of Cyp1a1 mRNAs in the different organs correlate with the known distribution of TCDD to these organs (De Jongh et al., 1995). No induction of any of these enzymes occurred in the *Ahr*^{-/-} mouse (Fig. 3). It should be noted that TCDD characteristically affects the levels of these enzymes exclusively at the transcriptional level, and increases in their mRNAs are reflected in increases in the corresponding proteins (Hankinson, 1995). The mRNA for the phospholipase A2 form Plag2g12a has been reported to be inducible by TCDD in the liver of both male and female C57BL/6 mice in whole genome microarray studies (Tijet et al., 2005; Boutros et al., 2008; Kopec et al., 2010). We found that Plag2g12a mRNA was inducible in the male liver about 2.5-fold in our studies (Fig. 3). Plag2g4a was reported to be increased by TCDD treatment in the Hepa-1 mouse hepatoma cell line (Kinehara et al., 2009). This enzyme has been reported to represent a major form of phospholipase A2 with regard to the metabolism of arachidonic acid to eicosanoids (Kita et al., 2006). However, neither this enzyme, nor Ephx2 were induced by TCDD in our studies in mice (Supplementary Fig. S1). The levels of the prostaglandin endoperoxide synthase 2 mRNA was not increased in any of the organs after a three day treatment with (50 $\mu\text{g}/\text{kg}$) TCDD (Supplementary Fig. S1). This is in agreement with the results of Vogel and coworkers (Vogel et al., 1998). However, those workers did find that 10 $\mu\text{g}/\text{kg}$ TCDD transiently increased cyclooxygenase 2 mRNA levels: inducing the mRNA maximal 4.5-fold and 3.5-fold in the lung and spleen, respectively, of female C57BL/6 mice after 24 hours of treatment. (No increase occurred in the liver.) Nevertheless, any increase in cyclooxygenase that might have occurred at earlier time points had minimal effects on the levels of prostanoids in our experiments. In conclusion, TCDD induction of Cyp1a1, Cyp1a2, Cyp1b1 and Plag2g12a correlated with TCDD-induced increases in eicosanoid levels in some organs (lung and liver), but not others (spleen and heart).

Discussion

In this study, we show that TCDD increases the levels of a number of eicosanoids in several organs/tissues of the mouse. DHETs were elevated in many of the organs/tissues by TCDD. The levels of the DHETs we measured in serum fell in the 4 to 40 nM range in the absence of TCDD. These values are similar to those previously reported for mouse serum (Kubala et al., 2010). Although these compounds are generally considered to be inactive metabolites of EETs, they do exhibit some of the same properties as EETs, including vasodilatory activity (Oltman et al., 1998), and they are ligands for peroxisome proliferator activated receptors (PPAR) α and γ (Buczynski et al., 2009; Konkkel and Schunck, 2011). Importantly, they are rapidly generated from the corresponding EETs by epoxide hydrolase, and so their levels probably reflect the levels of the more biologically active EETs. We did not report the levels of the EETs themselves in most organs/tissues either because they were below the level of detection, or because we had not developed the means for their analysis. Nevertheless, we found that 5,6-EET was increased by TCDD in the liver and spleen. Schlezinger and coworkers previously reported that TCDD treatment increased the levels of three EETs in the liver of fish (Schlezinger et al., 1998). The equivalent derivatives of linoleic acid, 9,10-diHOME and 12,13-diHOME, which are proinflammatory (Slim et al., 2001), were increased by TCDD in some organs.

We did not possess the means for measuring the terminal hydroxides in most experiments. However, where measured, these metabolites were consistently elevated by TCDD in all organs/tissues studied (except the heart). 20-HETE is known to be biologically active, for example exhibiting potent vasoconstrictive activity (Ishizuka et al., 2007). Little is known about the potential functions of 18-HETE and 19-HETE, however, they can induce vasodilation by inhibiting the effects of 20-HETE (Carroll et al., 1996). Interestingly, many metabolites that are traditionally considered products of lipoxygenase metabolism, including 5-HETE, 12-HETE, 9-HODE, HXA3, HXB3, 15-HETE and 13-HODE were increased by TCDD treatment in serum, liver

and spleen. These compounds all exhibit biological activity, of varying potency (Buczynski et al., 2009).

TCDD increased the level of free arachidonic acid in the liver of wild-type mice, as has been described previously (Lin et al., 2011), and this may explain to some degree the increases in eicosanoids that occur after TCDD treatment in this organ. However the increases in these metabolites are not likely to be completely due to the increase in arachidonic acid, because (i) increases did not occur in the levels of the prostanoid products of arachidonic acid metabolism, (ii) increases in the many arachidonic acid metabolites occurred in female liver (although to a lesser extent), despite the fact that no increase free in arachidonic acid occurred in this organ, and (iii) an increase did not occur in the amount of total arachidonic acid in male liver. The total levels of several eicosanoids were increased by TCDD in male liver. Since the metabolites are likely generated from free arachidonic or linoleic acid rather than the esterified forms, the free metabolites are probably incorporated rapidly into phospholipids, and the phospholipids so formed therefore represent a reservoir for these metabolites.

The TCDD-induced increases in the levels of the eicosanoids in the spleen and other solid organs cannot be ascribed to the (relatively small amount of) blood co-harvested with them, as the levels in the organs were generally of the same magnitude or greater than in serum. Nevertheless, it is very possible that, since several of the eicosanoids were elevated by TCDD in serum, increases in one organ may be transmitted to another via transport of the compound in the blood.

Since there was little effect of TCDD in eicosanoids levels in *Ahr*^{-/-} mice, the Ahr mediates most if not all the effects of TCDD on the eicosanoids. Cyp1a1, Cyp1a2 and Cyp1b1 are known to be among the most highly TCDD-induced genes in the liver in vertebrates. Purified (human) CYP1A1, CYP1A2, and CYP1B1 can metabolize arachidonic acid to midchain HETEs, terminal HETEs and EETs (Choudhary et al., 2004; Schwarz et al., 2004; Fer et al., 2008; Rifkind, 2006), and so these cytochromes P450 may be responsible for much of the increases in these metabolites in the liver after TCDD treatment. However, in some (but not all) microarray experiments, TCDD has been shown to elevate the levels of the arachidonic acid-metabolizing Cyp2c29, Cyp2c47 and Cyp2c50 enzymes (Hayes et al., 2007; Forgacs et al., 2011), and benzo(a)pyrene, a ligand for the AHR has been shown to increase the levels of the mRNAs for the arachidonic acid-metabolizing Cyp4f4 and Cyp4f5 enzymes in the rat (Aboutabl et al., 2009). Therefore other cytochromes P450 may contribute to the increase in eicosanoids levels occurring upon TCDD treatment. The heart did not exhibit an increase in any eicosanoids after TCDD treatment, despite the fact that Cyp1a1 and Cyp1b1 mRNAs were inducible in this organ. However, this may be explained by the fact that the induced levels of these cytochromes P450 were considerably lower than they were in the liver and lung.

It should be noted that although analysis of arachidonic acid metabolism by purified cytochromes P450 is useful, the metabolic profile of arachidonic acid in a particular organ is difficult to predict from its content of cytochromes P450, for several reasons, including the effects of regulatory interactions between metabolic products of some cytochromes P450 (Rifkind, 2006). Interestingly, Schlezinger and coworkers found that TCDD had a stronger effect on the *in vivo* metabolism of arachidonic acid than its *in vitro* metabolism by liver microsomes (Schlezinger et al., 1998). Lipidomic analysis as we report here is therefore essential for advancing understanding of the roles of the eicosanoids in biological processes in the whole organism.

In the absence of TCDD, *Ahr*^{-/-} mice exhibited different levels of only a few eicosanoids compared with wild-type mice. (Supplementary Table S-7). Furthermore, the number of compounds involved was much less than the number increased by TCDD treatment in wild-type mice; more compounds showed elevated levels

in knockout mice than showed reduced levels; and the five metabolites that were significantly elevated in wild-type mice did not exhibit an obvious expression pattern (i.e. they were not focused in any particular metabolic group or organ). Thus, it seems unlikely that the eicosanoids that were measured contribute to the known differences in the physiological phenotypes of *Ahr*^{-/-} and wild-type mice in the absence of exogenous ligand (Fujii-Kuriyama and Kawajiri, 2010), although this needs further study.

TCDD causes toxicity in all the organs we analyzed. TCDD induces many adverse effects in the liver, including hepatocellular hypertrophy and hyperplasia, fatty change, necrosis, inflammation, portal fibrosis, and liver tumor promotion and progression (Yoshizawa et al., 2007; Bock and Kohle, 2009). TCDD causes several forms of pulmonary disease, such as keratinizing epithelioma, bronchiolar metaplasia, and squamous metaplasia of the alveolar epithelium (Yoshizawa et al., 2007). TCDD causes cardiomyopathy, defects in several heart functions, and elevated arterial blood pressure (Korashy and El-Kadi, 2006; Yoshizawa et al., 2007). AHR agonists cause modest splenic lymphoid atrophy in rodents in some but not all studies (Sulentic and Kaminski, 2010; Yoshizawa et al., 2007). (We observed a decrease in spleen weight of 44% and 27% in male and female mice, respectively, in the current study that was dependent on the AHR {data not shown}). It is important to consider whether increases in eicosanoids levels cause or contribute to any of the TCDD-induced toxicities of these and other organs. Insights into this question can be provided by studies addressing the potential roles of Cyp1a1, Cyp1a2 and Cyp1b1 in TCDD toxicity, since these cytochromes P450 are probably responsible for a large portion of the increased levels of the eicosanoids after TCDD treatment. Many toxic responses to a high TCDD dose, including lethality and wasting, were found to be abrogated in *Cyp1a1*^{-/-} or *Cyp1a2*^{-/-} null mice demonstrating that these cytochromes P450 are essential for these toxic responses to TCDD (Smith et al., 2001; Uno et al., 2004). Certain rat strains that were resistant to about half of the multi-organ toxicities of TCDD that were analyzed exhibit normal induction of Cyp1a1, Cyp1a2 and Cyp1b1, indicating that although they may be necessary, these cytochromes P450 are not sufficient for the development of these toxic manifestations (Pohjanvirta et al., 2011). With regard to the hepatic toxicity, induction of Cyp1a1 and Cyp1a2 appear to protect against some toxic responses but to enhance others (Nukaya et al., 2009, 2010a,b). TCDD induction of splenic lymphoid atrophy appears not to depend upon induction of Cyp1a1 (Uno et al., 2004). Interestingly, studies with *Cyp1a1* knockout mice demonstrated that this enzyme is required for vascular dysfunction and hypertension induced by TCDD (Kopf et al., 2010).

These studies lay the foundation for future experiments addressing the potential role of eicosanoids in mediating the toxic effects of TCDD and other ligands of the AHR. Comparing the kinetic and dose–response parameters for the TCDD-mediated increases in eicosanoids levels with those for the induction of potentially relevant enzymes could provide insight into the identities of the enzymes involved. Such studies would be complemented by studies using knockout mice for the relevant genes. It will be also of interest to ascertain whether other ligands for the AHR, particularly nutrient-derived ligands such as indole-3-carbinol have the same effect as TCDD. Our studies on whole organs may mask much greater changes in eicosanoids levels in individual cell types in these organs, and this warrants examination. In addition, analysis of additional eicosanoids may identify metabolites that are even more elevated by TCDD than those studied here. It will also be of interest to investigate the effect of TCDD on the levels of eicosanoids in other mouse organs that are targets of TCDD toxicity. Finally, it will be most important to determine whether elevated eicosanoids levels contribute to the deleterious effects of TCDD and other toxic agonists of the AHR.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2011.12.009.

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Conflict of interest statement

The authors of “2,3,7,8-tetrachlorodibenzo-p-dioxin treatment alters eicosanoids levels in several organs of the mouse in an aryl hydrocarbon receptor-dependent fashion” declare that there are no conflicts of interest. None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence (bias) their work.

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Supplementary Table S-1:

Step	Total Time (min)	Flow Rate(μ l/min)	A (%)	B (%)
0	0	400	60	40
1	3	400	60	40
2	20	400	5	95
3	21	400	5	95
4	22	400	60	40
5	30	400	60	40

S-1. *LC gradient. HPLC run was set for 30 minutes at 400 μ L/min with different mixture of solution A (H₂O, 0.1% formic acid) and solution B (acetonitrile, 0.1% formic acid)*

Supplementary Table S-2:

Parameters	Period 1	Period 2	Period 3	Period 4
Scans in Period:	222	161	166	300
Relative Start Time:	0.00 min	6.01 min	9.11 min	14.3
CUR:	20	20	25	20
IS:	-4500	-4500	-4500	-4500
TEM:	550	550	525	550
GS1:	50	50	45	50
GS2:	30	30	55	30
ihe:	ON	ON	ON	ON
CAD:	High	High	Medium	High
EP	-10	-10	-10	-10

S-2. Optimized mass spectrometric parameters

Supplementary Table S-3:

Compounds	Parent Ions	Product Ion	Period	CE	Internal Standard
TBX2	369.1	169.1	1	-24	PGD2-d
PGE2	351.1	270.9	1	-24	PGD2-d
PGD2-d	355	237	1	-18	PGD2-d
PGD2	351.1	271.2	1	-24	PGD2-d
15-keto-PGE2	349	234.8	1	-22	PGD2-d
12,13-DiHOME (Isoleukotoxin diol)	313.2	128	2	-18	PGD2-d
HXA3 (hepoxilin A3)	335	127	2	-24	PGD2-d
9,10 diHOME (Leukotoxin diol)	313	201	2	-28	PGD2-d
14,15-DHET	337.2	206	3	-26	15-HETE-d
11,12-DHET	337.1	166.5	3	-26	12-HETE-d
8,9-DHET	337.1	126.9	3	-30	12-HETE-d
19-HETE	319.1	275	3	-24	15-HETE-d
HXB3 (Hepoxilin B3)	335	183	3	-24	15-HETE-d
20-HETE	319	289	3	-26	15-HETE-d
18-R-HETE	319.1	260	3	-22	15-HETE-d
5,6-DHET	337.2	144.8	3	-24	5-HETE-d
13-HODE-d	299	197.9	3	-26	
13-HODE	295	194.8	3	-28	13-HODE-d
9-HODE	295	171	3	-24	13-HODE-d
15-HETE	319	219	3	-18	15-HETE-d
15-HETE-d	327	226	3	-20	
9-oxoODE	293	185	3	-16	13-HODE-d
12-HETE-d	327.1	184	3	-22	
12-HETE	319	179	3	-20	12-HETE-d
5-HETE-d	327.1	115.9	3	-22	
5-HETE	319	115	3	-20	5-HETE-d
14,15-EET	319	219	4	-16	5-HETE-d
11,12-EET	319	166.7	4	-16	5-HETE-d
8,9-EET	319	154.8	4	-20	5-HETE-d
5,6-EET	319	191	4	-16	5-HETE-d
LA (Linoleic acid)	279	96.9	4	-40	5-HETE-d
DHA (Docosaehaenoic Acid)	327	282.9	4	-18	5-HETE-d
AA (Arachidonic Acid)	303.5	259	4	-18	5-HETE-d

S-3. *Optimized Mass Transitions, Collision Energy for Each Compound, and Internal standards used to normalize each compound measurement*

Supplementary Table S-4:

	13-HODE			12-HETE		
Animal ID	Replicate1 (area)	Replicate2 (area)	% CV	replicate1 (area)	Replicate 2 (area)	% CV
21-1	9.74E+04	1.07E+05	6.4	16900	18600	6.77
22-1	5.63E+04	6.26E+04	7.49	134000	136000	1.05
23-1	3.62E+04	3.47E+04	2.99	38800	39400	1.09
21-2	1.11E+05	1.25E+05	8.39	17800	16400	5.79
22-2	6.85E+04	7.53E+04	6.69	144000	139000	2.5
23-2	4.04E+04	3.90E+04	2.49	40100	41000	1.57

S-4. *Inter-assay validation. Comparison between technical replicates using % CV.*

Supplementary Table S-5:

Sample ID	13-HODE			12-HETE		
	Day 1 (area)	Day 10 (area)	%CV	Day1 (area)	Day 10 (area)	% CV
1	7.61E+04	6.34E+04	12.87	5.64E+03	6.05E+03	4.96
2	8.89E+04	8.31E+04	4.77	3.87E+03	3.13E+03	14.95
3	2.51E+05	2.44E+05	2	2.49E+04	2.44E+04	1.43
4	1.63E+05	1.47E+05	7.3	3.31E+04	3.12E+04	4.18
5	1.47E+05	1.32E+05	7.6	1.98E+04	2.18E+04	6.8
6	1.42E+05	1.24E+05	9.57	8.78E+03	8.84E+03	0.48
7	2.82E+05	2.56E+05	6.83	1.86E+04	2.01E+04	5.48
8	1.41E+05	1.24E+05	9.07	1.52E+04	1.48E+04	1.89
9	2.40E+05	2.32E+05	2.4	2.08E+04	1.87E+04	7.52
10	4.09E+05	3.93E+05	2.82	2.37E+04	2.46E+04	2.64
11	4.11E+05	4.09E+05	0.34	1.11E+04	1.11E+04	0
12	1.89E+05	1.76E+05	5.04	4.27E+03	5.98E+03	23.59
13	7.06E+05	6.92E+05	1.42	6.27E+04	7.87E+04	16

S-5. Intra-assay validation. Comparison of the same samples that was measured on two different days based on % CV values

Supplementary Table S-6:

	Forward Sequence	Reverse Sequence
GAPDH	5'-CATTGTGGAAGGGCTCATGA-3'	5'-GGAAGGCCATGCCAGTGAGC-3'
Cyp1a1	5'-CCCCGGCTTTCTGACAGA-3'	5'-CCGGAAGGTCTCCAGAATGA-3'
Cyp1a2	5'-TGGAGCTGGCTTTGACACAGT-3'	5'-GCCATGTCACAAGTAGCAAAATG -3'
Cyp1b1	5'-ACCAGAAGTCCTCCTACCAAGAGA-3'	5'-GCCTCATCCAGGGCTATAAAGG -3'
PLA2g4a	5'-TCACCCCGATTTTCCAGAGA-3'	5'-TGTGGCTGACATTTTTCATTAGCT-3'
Ephx2	5'-TTGAGTATGCCTGTGCCATCA-3'	5'-GGACTGCCCCATACCTTCCT-3'
Ptgs2	5'-TGCCTCCCACTCCAGACTAGA-3'	5'-CAGCTCAGTTGAACGCCTTTT-3'
PLA2g12a	5'-GCTCCTCTTTGACAGCGTCAT-3'	5'-CCGCTGGCTGTCCAGGTA-3'

S-6. Real time primer sequences

	SERUM ng/ml							
	Male				Female			
	KO		WT		KO		WT	
	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD
AA	161 ± 17	134 ± 7	153 ± 5	105 ± 12	90 ± 9	98 ± 10	89 ± 12	123 ± 9*
LA	34 ± 4	36 ± 8	46 ± 11	39 ± 7	39 ± 12	32 ± 13	20 ± 3	32 ± 12
DHA	2792 ± 419	2172 ± 215	2108 ± 211	1549 ± 132	786 ± 77	977 ± 132	643 ± 67	1148 ± 111**
TXB2								
PGE2								
PGD2								
5-HETE	3.5 ± 0.4	3.4 ± 0.1	2.0 ± 0.31	3.4 ± 0.3*	2.8 ± 0.3	2.8 ± 0.2	2.9 ± 0.2	4.6 ± 0.3*
12-HETE	274 ± 9	502 ± 41***	372 ± 48	956 ± 282	227 ± 55	303 ± 52	318 ± 25	603 ± 91*
9-HODE	32 ± 7	39 ± 2	21 ± 2.2	39 ± 3**	19 ± 2	17 ± 2	14 ± 1	21 ± 2*
9-oxo-ODE								
HXA3								
HXB3	78 ± 19	110 ± 9	100 ± 23	130 ± 31	41 ± 8	89 ± 22	56 ± 4	120 ± 20*
15-HETE	1.5 ± 0.2	2.1 ± 0.2	1.8 ± 0.2	2.5 ± 0.3	1.9 ± 0.2	2 ± 0.2	2.3 ± 0.2	3.0 ± 0.2*
13-HODE	123 ± 22	132 ± 9	82 ± 10	167 ± 27*	109 ± 21	103 ± 21	100 ± 10	127 ± 11
5,6-EET								
8,9-EET								
11,12-EET								
14,15-EET								
5,6-DHET	3.4 ± 0.1	4 ± 0.2	3.1 ± 0.1	5.7 ± 0.4*	2.9 ± 0.4	2.5 ± 0.2	3.3 ± 0.4	8.2 ± 0.3***
8,9-DHET	5.1 ± 0.4	6.9 ± 0.5*	4.0 ± 0.4	11 ± 1***	6 ± 0.7	5.5 ± 0.3	5.4 ± 0.6	12 ± 0.7***
11,12-DHET	4.5 ± 0.5	7.1 ± 0.7*	2.5 ± 0.2	22 ± 2***	4.9 ± 1.2	3.4 ± 0.2	3.6 ± 0.7	16 ± 1***
14,15-DHET	11 ± 1	19 ± 2*	6.8 ± 1.3	24 ± 2***	12 ± 4	8 ± 1	8.0 ± 0.18	28 ± 3***
9,10 diHOME	9.2 ± 0.8**	11 ± 1	4.4 ± 0.2	6.4 ± 0.6*	11 ± 2*	7.5 ± 1	5.6 ± 0.8	7 ± 2
12,13-diHOME	148 ± 33**	233 ± 22	28 ± 2	70 ± 9*	113 ± 24**	90 ± 11	53 ± 6	63 ± 11
18-R-HETE								
19-HETE								

Supplementary Table S-7A. Data for Experiment 1. The means and standard errors were obtained from 5 to 8 mice per gender per treatment. Values are presented as ng compounds per g tissue, except for those presented in red, which are presented as arbitrary units/gm tissue. *, ** and *** represent significantly different from the DMSO control at $p < 0.05$, $p < 0.01$ and 0.001 , respectively. and : the values for the knockout mice treated with TCDD are significantly different from those of the knockout mice treated with DMSO at $p < 0.05$ and $p < 0.001$ respectively. and : The values for the DMSO treated knockout mice are significantly different from those for the DMSO treated wild-type mice at $p < 0.05$ and $p < 0.01$, respectively.

	LIVER							
	Male				Female			
	KO		WT		KO		WT	
	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD
AA	5.9 ± 2	8.3 ± 1.6	4.9 ± 1.0	12 ± 2*	4.5 ± 1	1.7 ± 0.3	3.5 ± 0.4	1.6 ± 0.34**
LA	69 ± 11	62 ± 10	57 ± 5	68 ± 18	31 ± 6	19 ± 2	25 ± 4	38 ± 12
DHA	353 ± 88	397 ± 72	272 ± 28	750 ± 121**	216 ± 30	154 ± 18	129 ± 14	222 ± 13*
TXB2	12 ± 2	7.5 ± 1.1	12 ± 1	12 ± 1	12 ± 5	10 ± 2	6.4 ± 1.2	10 ± 2.1
PGE2	19 ± 2	15 ± 2	18 ± 3	21 ± 3	7.6 ± 1.5	6.8 ± 0.7	4.6 ± 0.4	5.3 ± 0.6
PGD2	41 ± 12	29 ± 2	33 ± 7	34 ± 6	35 ± 8*	30 ± 4	15 ± 1.5	18 ± 2
5-HETE	1.9 ± 3	1.8 ± 0.3	1.2 ± 0.2	3.9 ± 0.4**	2.1 ± 0.1	1.9 ± 0.06	1.9 ± 0.07	2.1 ± 0.09*
12-HETE	5.6 ± 1.3	5.7 ± 0.8	6.5 ± 1.6	10 ± 3	11 ± 2	8.3 ± 1.3	7.1 ± 1.5	5.2 ± 0.5
9-HODE	36 ± 2*	47 ± 5	77 ± 18	75 ± 14	39 ± 11	46 ± 11	29 ± 12	43 ± 11
9-oxo-ODE								
HXA3	1.3 ± 0.4	1.2 ± 0.5	2.1 ± 0.8	6.7 ± 1.0*	6.4 ± 0.2	3.8 ± 1.0	3.8 ± 0.2	8.8 ± 1.3**
HXB3								
15-HETE	8.2 ± 1.3	7.6 ± 1.1	5.8 ± 0.5	12 ± 1*	3.7 ± 0.6	2.4 ± 0.4	2 ± 0.45	1.5 ± 0.51
13-HODE	49 ± 5*	84 ± 19	115 ± 24	122 ± 22	79 ± 40	119 ± 30	57 ± 24	71 ± 22
5,6-EET	2.8 ± 1	5.7 ± 2	2.7 ± 0.2	12 ± 1.1***				
8,9-EET								
11,12-EET					9.1 ± 1	8.4 ± 1.3	9.2 ± 0.9	10 ± 1
14,15-EET								
5,6-DHET	1.6 ± 0.2	1.4 ± 0.07	1.0 ± 0.23	3.9 ± 0.5**	1.8 ± 0.1*	1.4 ± 0.04	1.3 ± 0.07	1.9 ± 0.05***
8,9-DHET	5.8 ± 0.6	3.9 ± 0.3	4.2 ± 0.4	9.6 ± 1**	8 ± 1*	5.8 ± 0.2	5.3 ± 0.2	6.4 ± 0.3**
11,12-DHET	14 ± 2	10 ± 1	12 ± 2	29 ± 3***	14 ± 3*	4.8 ± 0.5	4.6 ± 0.9	12 ± 1***
14,15-DHET	31 ± 4	23 ± 2	25 ± 2	51 ± 4***	41 ± 10*	17 ± 1	16 ± 3	28 ± 2**
9,10 diHOME	5.3 ± 1.0	6.6 ± 1	6.2 ± 1.0	16 ± 2**	9.5 ± 2.9	7.4 ± 1.6	4.2 ± 1.0	9.2 ± 2.4*
12,13-diHOME	34 ± 3	43 ± 6	37 ± 5	106 ± 11***	60 ± 1*	38 ± 0.4	37 ± 0.4	49 ± 10
18-R-HETE	0.74 ± 0.31	0.19 ± 0.02	0.51 ± 0.19	9.7 ± 2.3**				
19-HETE	0.49 ± 0.25	0.76 ± 0.21	1.0 ± 0.47	7.3 ± 1.1***	0.81 ± 0.27	0.70 ± 0.01	0.73 ± 0.1	1.3 ± 0.1**

Supplementary Table S-7B.

	ng/g tissue							
	SPLEEN Male KO				Female KO			
	DMSO	TCDD	WT DMSO	TCDD	DMSO	TCDD	WT DMSO	TCDD
AA	1.6 ± 0.2	0.96 ± 0.18	2.0 ± 0.7	0.76 ± 0.28	1.1 ± 0.2	0.56 ± 0.10	2.0 ± 0.7	0.97 ± 0.20
LA	184 ± 49	192 ± 44	312 ± 70	103 ± 114	617 ± 224	333 ± 69	418 ± 157	479 ± 70
DHA	50 ± 5	49 ± 9	52 ± 12	19 ± 3.7	67 ± 15	40 ± 3	40 ± 7	24 ± 3
TXB2	422 ± 30	454 ± 57	359 ± 28	550 ± 53*	864 ± 102*	594 ± 44	512 ± 41	754 ± 78*
PGE2	350 ± 34*	401 ± 64	223 ± 45	324 ± 29	1362 ± 197*	1097 ± 54	759 ± 119	854 ± 65
PGD2	3012 ± 195*	3064 ± 282	2009 ± 259	2641 ± 129	5711 ± 372*	4222 ± 328	3269 ± 486	3906 ± 345
5-HETE	11 ± 2	14 ± 5	6.7 ± 1.6	8.8 ± 1.4	16 ± 2*	14 ± 1	8.6 ± 1.5	13 ± 1*
12-HETE	1741 ± 139	1007 ± 283	1477 ± 324	1779 ± 321	2570 ± 548	2403 ± 390	2060 ± 511	2005 ± 138
9-HODE	2084 ± 463	1353 ± 158	1221 ± 164	4466 ± 488***	1552 ± 257	1824 ± 189	1244 ± 385	3305 ± 389**
9- α -ODE								
HXA3								
HXB3	91 ± 10**	79 ± 13	300 ± 120	710 ± 280	520 ± 140	250 ± 18	540 ± 48	1100 ± 100*
15-HETE	76 ± 5*	89 ± 10	40 ± 6	46 ± 5.3	143 ± 22**	108 ± 8	46 ± 9	81 ± 7
13-HODE	2720 ± 769	1438 ± 226	1171 ± 86	6043 ± 733***	1826 ± 325	2587 ± 187	1616 ± 484	3306 ± 548*
5,6-EET								
8,9-EET								
11,12-EET								
14,15-EET								
5,6-DHET								
8,9-DHET	3.2 ± 0.2	3.3 ± 0.2	3.9 ± 0.4	4.6 ± 0.71	9.0 ± 0.3	8.9 ± 0.3	9.1 ± 0.5	9.2 ± 0.3
11,12-DHET	0.77 ± 0.14	0.91 ± 0.12	2.5 ± 0.69	5.2 ± 0.83*	3.9 ± 0.9	2.4 ± 0.3	3.2 ± 0.4	6.1 ± 0.4***
14,15-DHET	3.9 ± 0.3*	4.6 ± 0.3	5.2 ± 0.5	7.5 ± 0.58*	6.4 ± 1.4	5.1 ± 0.6	5.2 ± 0.4	6.8 ± 0.4*
9,10 diHOME	0.93 ± 0.46	1.0 ± 0.39	1.4 ± 0.6	0.55 ± 0.12	1.2 ± 0.3	0.94 ± 0.22	1.4 ± 0.4	3.1 ± 0.5
12,13-diHOME	120 ± 28	125 ± 19	138 ± 52	416 ± 29**	155 ± 33	139 ± 33	175 ± 44	371 ± 55*
18-R-HETE								
19-HETE								

Supplementary Table S-7C.

	LUNG ng/g tissue							
	Male				Female			
	KO		WT		KO		WT	
	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD	DMSO	TCDD
AA	2.0 ± 0.3	2.4 ± 0.3	1.8 ± 0.2	1.9 ± 0.2	2.4 ± 0.4	1.8 ± 0.1	2.9 ± 0.3	2.1 ± 0.3
LA	68 ± 10	73 ± 16	60 ± 19	48 ± 11	56 ± 12	54 ± 5	50 ± 8	49 ± 7
DHA	27 ± 3	34 ± 3	21 ± 2	25 ± 4	51 ± 5	32 ± 3*	50 ± 5	34 ± 7
TXB2	<u>83 ± 4*</u>	73 ± 7	118 ± 12	109 ± 9	168 ± 22	120 ± 7	159 ± 12	199 ± 11*
PGE2	772 ± 22	780 ± 46	943 ± 101	805 ± 47	1390 ± 149	1134 ± 53	1157 ± 68	1360 ± 62
PGD2	297 ± 27	250 ± 18	320 ± 16	326 ± 24	363 ± 13	322 ± 11	366 ± 15	439 ± 14*
5-HETE	5.7 ± 0.7	4.3 ± 0.7	6.1 ± 1.6	8.5 ± 1.3	3.8 ± 0.6	2.6 ± 0.3	4.1 ± 0.9	5.2 ± 1
12-HETE	552 ± 35	504 ± 76	901 ± 127	595 ± 70*	1001 ± 183	457 ± 50	1516 ± 206	883 ± 120*
9-HODE	273 ± 25	239 ± 20	328 ± 71	310 ± 49	509 ± 67	367 ± 79	451 ± 79	677 ± 94
9-oxo-ODE								
HXA3								
HXB3								
15-HETE	28 ± 1	23 ± 2	34 ± 2	25 ± 3	81 ± 15*	43 ± 5	62 ± 8	59 ± 3
13-HODE	232 ± 39	176 ± 23	316 ± 75	249 ± 49	499 ± 103	357 ± 94	317 ± 66	540 ± 82
5,6-EET								
8,9-EET	10 ± 1	9.9 ± 1.2	11 ± 0.6	9.3 ± 1.1	13 ± 1	11 ± 1	8.1 ± 1.9	12 ± 1
11,12-EET	12 ± 1	12 ± 2	12 ± 1	12 ± 1	7.8 ± 1.0	10 ± 1	9.0 ± 0.8	12 ± 2
14,15-EET								
5,6-DHET	3.8 ± 0.03	3.8 ± 0.03	3.8 ± 0.03	4.1 ± 0.03*	3.9 ± 0.08	3.9 ± 0.03	3.8 ± 0.03	4.0 ± 0.06*
8,9-DHET	3.0 ± 0.2	3.8 ± 0.4	4.3 ± 0.7	6.3 ± 0.4*	9.3 ± 1	4.7 ± 0.4	10 ± 0.8	7.3 ± 0.4
11,12-DHET	4.5 ± 0.1	5.0 ± 0.3	5.4 ± 0.3	9.2 ± 0.4**	7.1 ± 0.4	5.3 ± 0.3	8.2 ± 0.8	14 ± 1***
14,15-DHET	9.2 ± 0.3	9.0 ± 0.1	9.6 ± 0.3	12 ± 0.3***	12 ± 0.5	10 ± 0.3	11 ± 0.2	15 ± 0.9***
9,10 diHOME	0.23 ± 0.01	0.40 ± 0.05	0.28 ± 0.06	0.55 ± 0.07	0.16 ± 0.02	0.23 ± 0.04	0.11 ± 0.01	0.55 ± 0.14
12,13-diHOME	16 ± 3	19 ± 2	17 ± 3	21 ± 2	18 ± 4	12 ± 3	9.4 ± 1	18 ± 2*
18-R-HETE								
19-HETE	3.8 ± 0.3	3.7 ± 0.4	4.1 ± 0.3	9.2 ± 0.4***	3.9 ± 0.3	3.9 ± 0.3	3.7 ± 0.2	12 ± 1***

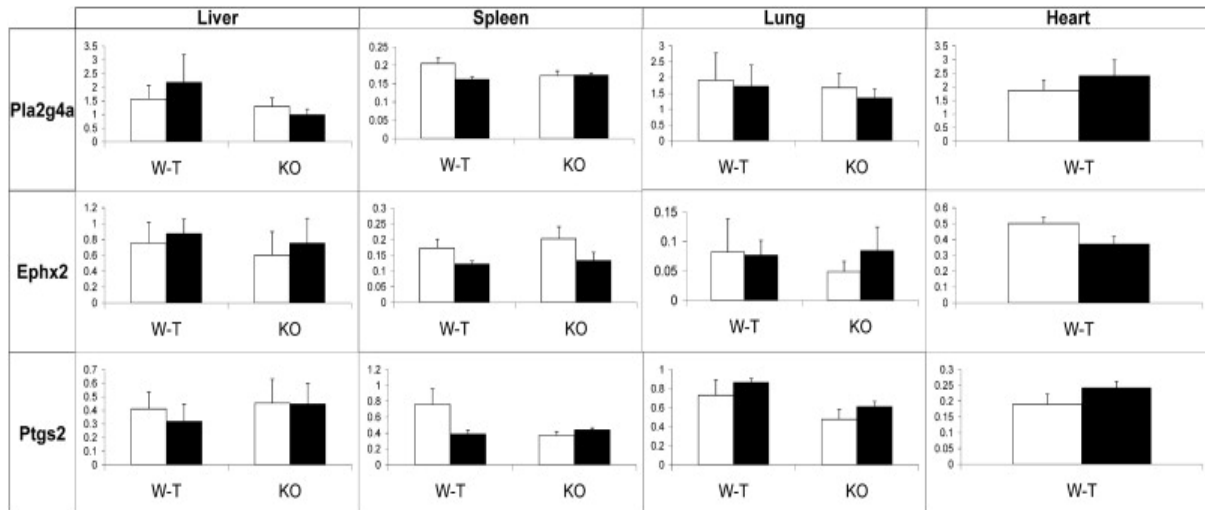
Supplementary Table S-7D.

	HEART Male WT DMSO	ng/g tissue TCDD
AA	26 ± 3	25 ± 2
LA	13 ± 5	12 ± 3
DHA	227 ± 45	210 ± 20
TXB2		
PGE2	10 ± 0.7	7.7 ± 0.6*
PGD2	29 ± 2	21 ± 1**
5-HETE	10 ± 0.7	11 ± 0.7
12-HETE	1256 ± 126	1113 ± 314
9-HODE	344 ± 34	298 ± 35
9-oxo-ODE		
HXA3		
HXB3	66 ± 6	24 ± 6***
15-HETE	16 ± 0.6	13 ± 1*
13-HODE	774 ± 82	639 ± 133
5,6-EET	33 ± 6	39 ± 4
8,9-EET	10 ± 1	9.3 ± 3.1
11,12-EET	6.0 ± 1.5	7.6 ± 1.1
14,15-EET		
5,6-DHET	1.4 ± 1.0	1.0 ± 0.3
8,9-DHET	3.6 ± 0.4	3.3 ± 0.4
11,12-DHET	2.3 ± 0.4	1.7 ± 0.2
14,15-DHET	2.3 ± 0.3	1.3 ± 0.05*
9,10 diHOME	9.1 ± 1.6	6.2 ± 0.96
12,13-diHOME	68 ± 11	43 ± 6
18-R-HETE		
19-HETE	77 ± 9	62 ± 18

Supplementary Table S-7E.

	MALE LIVER ng/g tissue			
	Free DMSO	TCDD	Total DMSO	TCDD
AA	20 ± 5	210 ± 51**	224 ± 16	232 ± 16
TXB2	128 ± 30	101 ± 12	223 ± 35	166 ± 21
5-HETE	1.2 ± 0.5	15 ± 1***	38 ± 3	63 ± 3***
12-HETE	13 ± 4	33 ± 18	55 ± 5	107 ± 20*
9-HODE	40 ± 8	26 ± 7	83 ± 23	175 ± 10**
HXA3	1.6 ± 0.5	5.0 ± 0.5***	10.1 ± 1.6	13.7 ± 1.9**
15-HETE	5.2 ± 0.5	2.5 ± 0.7	47 ± 18	109 ± 12**
13-HODE	55 ± 11	56 ± 22	186 ± 48	336 ± 24**
5,6-DHET	0.97 ± 0.30	2.3 ± 1.1	40 ± 11	138 ± 13***
8,9-DHET	8.1 ± 1.9	14 ± 4*	103 ± 27	433 ± 36***
11,12-DHET	9.0 ± 2.2	42 ± 11**	103 ± 26	514 ± 71***
14,15-DHET	40 ± 13	84 ± 10*	200 ± 40	429 ± 55**
9,10 diHOME	5.7 ± 0.6	38 ± 5***	62 ± 7	164 ± 15***
18-R-HETE	0.0 ± 0.0	5.7 ± 2.9***	2.5 ± 2.5	45 ± 5***
19-HETE	0.10 ± 0.10	9.9 ± 3.8*	1.9 ± 1.9	40 ± 9**
20-HETE			8.2 ± 3.0	27 ± 5**

Supplementary Table S-8. Data for Experiment 2. Data were obtained from seven TCDD-treated and six vehicle (DMSO)-treated mice. Values are presented as ng compounds per g tissue, except for those presented in red, which are presented as arbitrary units/gm tissue. *, ** and ***: the values for the wild-type mice treated with TCDD are significantly different from those of the wild-type treated with DMSO, at $p < 0.05$, < 0.01 and < 0.001 , respectively.



Supplementary Fig. S1. Effect of TCDD on *PLA2g4a*, *Ephx2* and *Ptgs2* mRNA levels in male wild-type mice treated with TCDD (filled bars) or vehicle (open bars). The levels of each mRNA are represented relative to the levels of the constitutively expressed GAPDH glycolytic enzyme in each organ. The means and standard errors were derived from four mice

Chapter 5

**CYP2S1 is negatively regulated by
corticosteroids in human cell lines**



CYP2S1 is negatively regulated by corticosteroids in human cell lines

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ABSTRACT

Cytochrome P450s are monooxygenase proteins involved in the metabolism of both exogenous and endogenous compounds. CYP2S1 can metabolize eicosanoids in the absence of both NADPH and NADPH cytochrome P450 reductase, and can also activate the anticancer agent 1 AQ4N [1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxy anthracene-9,10-dione]. CYP2S1 is mainly expressed in extrahepatic tissues such as the trachea, lung, stomach, small intestine, spleen, skin, breast, kidney and placenta. Furthermore, increased expression of CYP2S1 occurs in several tumors of epithelial origin, making the characterization of CYP2S1 regulation relevant to the treatment of disease. We report that the synthetic glucocorticoid receptor ligand dexamethasone (DEX) represses CYP2S1 expression. The ED₅₀ is between 1 nM and 3 nM and maximal repression is reached by 48 h. Other corticosteroids are also effective at repressing CYP2S1. We show that repression by DEX is mediated by the glucocorticoid receptor and requires histone deacetylase activity.

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1. Introduction

Cytochromes P450 are evolutionarily conserved phase I monooxygenase proteins involved in metabolism of xenobiotics (drugs, carcinogens or environmental toxicants) and endogenous compounds such as steroid hormones, bile acids, fatty acids and eicosanoids (Lewis, 2004). There are 57 cytochromes P450 in the human, while the mouse has 88. CYP2S1 was identified in our laboratory as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible gene in mouse Hepa-1 hepatoma cells (Rivera et al., 2002), and also by Rylander et al. (2001) by performing a homology search in a sequence database. Human CYP2S1 has been localized to chromosome 19q13-2 in a cluster with other cytochrome P450s of the CYP2 family. Unlike many other cytochrome P450s, CYP2S1 is expressed at low levels in the liver but at high levels in epithelial tissues, such as those found in the lung, skin, and colon (Saarikoski et al., 2005). Human CYP2S1 is inducible in skin by coal tar and UV light, and is expressed in psoriatic skin (Smith et al., 2003). TCDD upregulates CYP2S1 in Hepa-1 cells, human A549 cells and mouse liver. Hypoxia

upregulates CYP2S1 in Hepa-1 cells (Rivera et al., 2002, 2007). CYP2S1 mRNA is also upregulated by TCDD and 3-methylcholanthrene (3-MC) in certain rat tissues (Deb and Bandiera, 2010).

Recently, our group demonstrated that human CYP2S1 can metabolize certain endogenous fatty acids and eicosanoid derivatives in the absence of both NADPH and NADPH cytochrome P450 reductase (Bui et al., 2011). Nishida et al. (2010) also recently showed that CYP2S1 can reduce and thereby activate the anticancer agent AQ4N [1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxy anthracene-9,10-dione], but only under anoxic or hypoxic conditions.

There is evidence that differential expression of certain cytochrome P450s occurs in tumors and that some forms of cytochrome P450s show tumor-type selectivity. The CYP2S1 protein was found to be expressed at significantly higher levels in colorectal cancer, and this was associated with a poor prognosis (Kumarakulasingham et al., 2005). The protein was found at significantly elevated levels in 37% of breast cancers and this was correlated with shorter patient survival time (Murray et al., 2010). Therefore, the characterization of the regulation of CYP2S1 may lead to potential strategies for the treatment of certain cancers.

Based on previous findings that CYP2S1 can be regulated by the aforementioned agents, we decided to test several additional agents, including certain nuclear hormone receptors agonists, for their ability to regulate CYP2S1. This analysis resulted in the finding that the synthetic glucocorticoid dexamethasone (DEX) is a novel repressor of CYP2S1 expression in the A549 human lung

Abbreviations: CYP2S1, cytochrome P450 2S1; GR, glucocorticoid receptor; DEX, dexamethasone.

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epithelial cell line. DEX, a glucocorticoid receptor (GR) ligand, is widely used to treat inflammatory diseases and mediates the expression of anti-inflammatory genes when used in A549 cells and other lung epithelial cell lines (Huang et al., 2009; Pujolsa et al., 2009).

A549 cells are considered a good model for the study of xenobiotic metabolism in the human lung epithelium as they have retained many metabolic characteristics of normal type II alveolar cells, including expression of most of the major constitutive and inducible cytochrome P450s found in the lung epithelium (Hukkanen et al., 2000), such as CYP1A1, CYP1B1 and CYP2B6 (Dohr et al., 1997; Foster et al., 1998). They also form DNA adducts when treated with B(a)P (Feldman et al., 1978). In this study we observed repression of CYP2S1 in a time and dose-dependent manner. This repression was mediated by the GR and required histone deacetylase. We also found that DEX similarly repressed CYP2S1 in a human pancreatic cell line.

2. Methods

2.1. Materials

Actinomycin D, dexamethasone, prednisolone, triamcinolone acetonide, flunisolide, beclomethasone, and hydrocortisone were purchased from Sigma (St. Louis, MO). The anti-CYP2S1 (goat; cat# sc-49426), anti-glucocorticoid receptor (cat# sc-8992) and anti-GAPDH (mouse; cat# sc-32233) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cells

A549 cells were grown in DMEM at 37 °C and 5% CO₂. All media contained 10% fetal bovine serum (FBS), 1% fungizone, and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA). For treatments with DEX and other corticosteroids, cells were cultured in phenol-red free media and charcoal-stripped FBS (Gemini Bioproducts, Sacramento, CA).

2.3. Real time PCR

Total RNA was obtained for reverse transcription using the RNeasy RNA isolation kit from Qiagen (Valencia, CA) according to the manufacturer's protocol. Two micrograms of total RNA was used for complementary DNA (cDNA) synthesis in a total reaction volume of 20 µl. Reverse transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen) and primed with random hexamers (Invitrogen) according to the manufacturer's instructions. Following cDNA synthesis, each reaction was diluted into a total of 200 µl of RNase-free water, which was then used as template for real-time PCR. SYBR Green real-time PCR was performed according to standard protocols. Quantities were normalized to those for the 36B4 ribosomal housekeeping gene.

2.4. RNA interference

siRNA duplexes targeting the 5'-AAGCTTCTGGAGCAAAATAT-3' and 5'-CAGACTCAACTGGAGGATCA-3' sequences of hGR and the 5'-CATGCTGACAGTCATTTA-3' and 5'-CACCACCATCTACAAGCCTT-3' sequences of hCYP2S1, as well as a scrambled control oligo (SXC) (sequence proprietary) were synthesized by Qiagen (Valencia, CA). A549 cells were transfected with 100 nM of each siRNA using the Oligofectamine Transfection Reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were treated with siGR and DEX at the start of transfection for a total of 48 h.

2.5. Actinomycin D treatment

A549 cells were grown to 80% confluency and treated with 5 µg/mL of actinomycin D or DMSO (as control). Cells were collected and the declines in CYP2S1 and 36B4 mRNAs were determined from the fraction of remaining RNA in treated cells as compared to control.

2.6. Western blotting

Cell homogenates were resolved in a 7–12% SDS mini-gel apparatus (Invitrogen, Carlsbad, CA) and transferred with a semi-dry apparatus (Thermo Scientific Pierce, Rockford, IL). After non-specific sites were blocked with 3% non-fat milk, the blots were incubated with antibodies to hGR at 1:500, CYP2S1 at 1:200 or GAPDH at 1:2000. The primary antibodies were subsequently localized with donkey anti-goat IgG or anti-mouse IgG conjugated with horseradish peroxidase (at 1:5000). Horseradish peroxidase was detected with a chemiluminescent kit (GE Healthcare, Piscataway, NJ).

2.7. Other analyses

Data are presented as mean ± the standard deviation. Representative graphs and blots of at least three separate experiments are depicted in the figures. Initial comparisons of means were accomplished using one-way Analysis of Variance (ANOVA). If the ANOVA results indicated significance at $p < 0.05$, a Tukey's pair-wise comparison was used for post-hoc determination of significant differences between groups. All statistical operations were carried out with Graph Pad Prism software (v5.0).

3. Results

3.1. Repression of CYP2S1 in A549 cells

We performed a dose–response curve for A549 cells treated with 1 nM–3 µM DEX. DEX treatment decreased the amount of CYP2S1 mRNA. The ED₅₀ for CYP2S1 mRNA repression by DEX was between 1 nM and 3 nM, and maximal repression was achieved after treatment for 48 h (Fig. 1A and B). We also examined the effect of DEX on CYP2S1 protein levels. Basal levels of CYP2S1 protein were

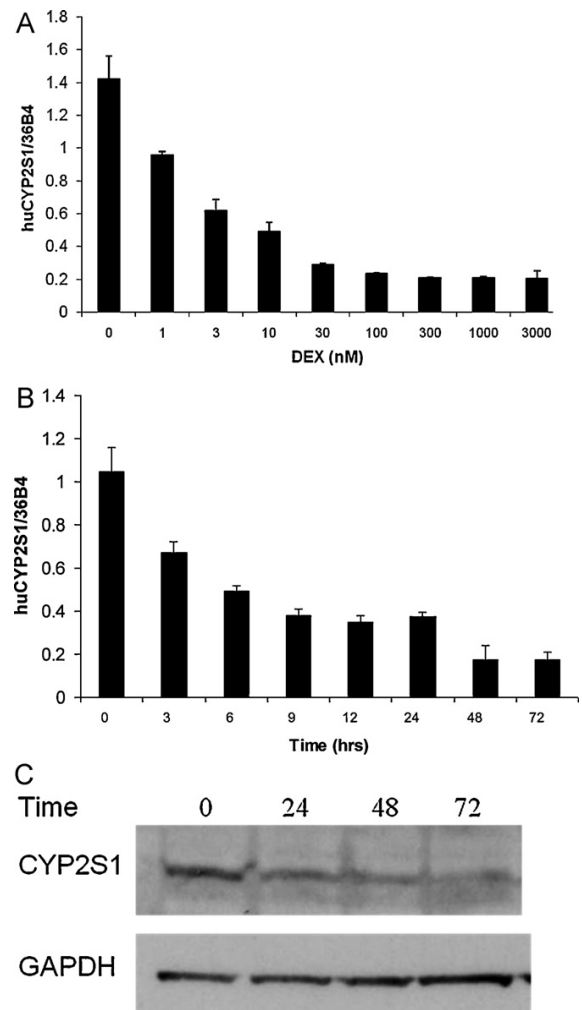


Fig. 1. Dexamethasone represses CYP2S1. (A) Dose–response curve of the effect of DEX on CYP2S1 mRNA levels in A549 cells treated for 48 h. (B) Time response of DEX repression of CYP2S1 mRNA after treatment with 100 nM DEX. All treatments with DEX resulted in a statistically significant decrease in gene expression ($p < 0.001$). (C) Western blot showing time response of DEX repression of CYP2S1.

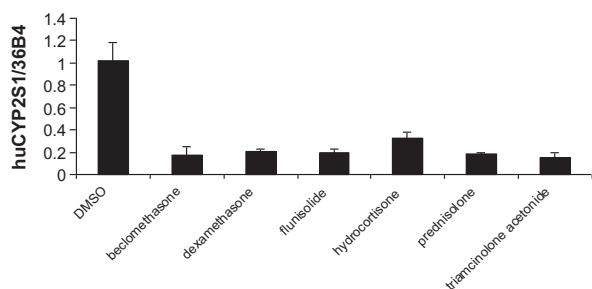


Fig. 2. A variety of corticosteroids repress CYP2S1 mRNA expression. A549 cells were treated with 100 nM of each glucocorticoid (except prednisolone, 1 μM) for 24 h. All treatments resulted in a statistically significant decrease in gene expression ($p < 0.001$).

significantly decreased within 24 h of treatment with DEX and the degree of repression continued for 72 h (Fig. 1C).

In order to determine the scope of glucocorticoid repression of CYP2S1 we also tested the effects of other corticosteroids, including beclomethasone, flunisolide, hydrocortisone, prednisolone and triamcinolone acetonide. We treated the cells with 100 nM of each corticosteroid, with the exception of prednisolone (1 μM), based on concentrations typically used in the literature. All agents tested significantly repressed CYP2S1 mRNA expression in A549 cells within 24 h (Fig. 2).

3.2. Repression of CYP2S1 by dexamethasone is dependent on the glucocorticoid receptor

In order to verify that the GR response was intact in our cells, we examined expression of a known GR target gene after treatment with DEX. The GR primary response target gene glucocorticoid induced leucine zipper (GILZ) (Wang et al., 2004) was significantly upregulated in a dose response manner after treatment with DEX for 48 h in A549 cells, confirming the presence of the GR response in these cells (Supplementary Fig. 1). We next sought to determine whether the repression of CYP2S1 by DEX is mediated by the GR. A549 cells treated with two different siRNAs targeted to the GR exhibited considerable reductions in GR protein levels (Fig. 3A). In cells treated with siGR and DEX, the repression of CYP2S1 that was produced by DEX treatment alone was abrogated, both at the protein (Fig. 3A) and mRNA (Fig. 3B) levels, indicating that the repressive effect is mediated by the GR. siGR did not affect the endogenous levels of CYP2S1 mRNA (Fig. 3B). As previously observed by others, treatment with DEX reduced both mRNA (data not shown) and protein (Fig. 3A) levels of the GR (Schaaf and Cidlowski, 2003).

3.3. The half-life of CYP2S1 mRNA is not decreased by DEX treatment

DEX can repress gene activation and basal transcription by destabilizing the corresponding mRNA (Gille et al., 2001; Henderson and Kefford, 1993). Actinomycin D (ActD) is an inhibitor of RNA polymerase II, and can be used to study the half-life of mRNA (Sobell, 1985). After 9 h of treatment of A549 cells with ActD, the CYP2S1 mRNA levels were at 50% of the original levels (Fig. 4A). Addition of DEX to the cells did not accelerate the loss of the mRNA, indicating that DEX does not decrease the stability of CYP2S1 mRNA (Fig. 4A). The mRNA levels of the ribosomal subunit 36B4 did not change significantly, consistent with the long half-life of this mRNA (Fig. 4B). These observations indicate that repression by DEX occurs via effects on CYP2S1 mRNA synthesis rather than on its degradation.

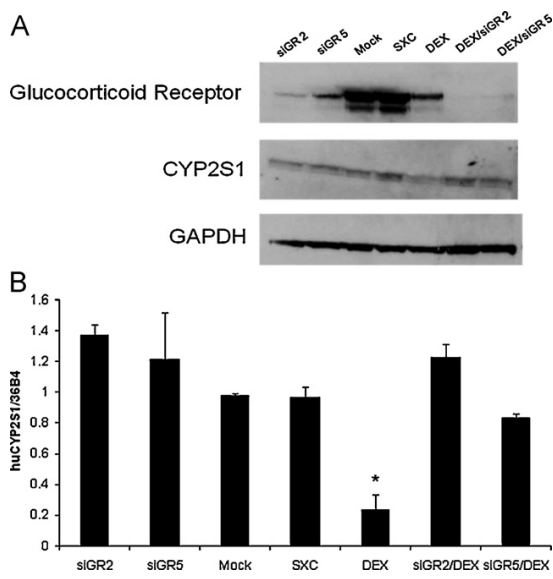


Fig. 3. Knockdown of glucocorticoid receptor inhibits repression of CYP2S1 by DEX. (A) Western blot of CYP2S1 expression in cells treated with 100 nM siGR (or SXC) and 100 nM DEX for 48 h. (B) CYP2S1 mRNA levels in cells treated with 100 nM siGR (or SXC) and 100 nM DEX for 48 h. * $p < 0.01$ (DEX vs. all treatments).

3.4. Repression of CYP2S1 by DEX is mediated by histone deacetylation

The mechanism of transrepression by GR can involve recruitment of histone deacetylases, which silence gene expression via removal of acetyl groups from histones (Newton and Holden, 2007;

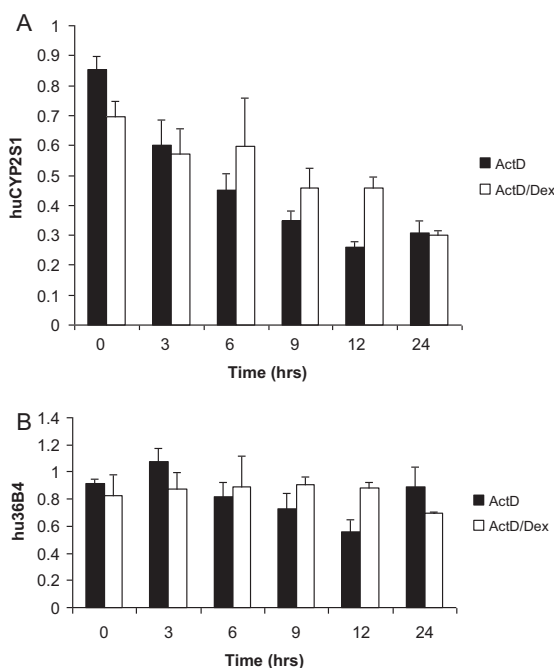


Fig. 4. DEX does not decrease the CYP2S1 mRNA half-life. (A) Expression of CYP2S1 after treatment with 5 ng/mL actinomycin D and 100 nM DEX. (B) Expression of the ribosomal subunit gene 36B4 after treatment with 5 ng/mL actinomycin D.

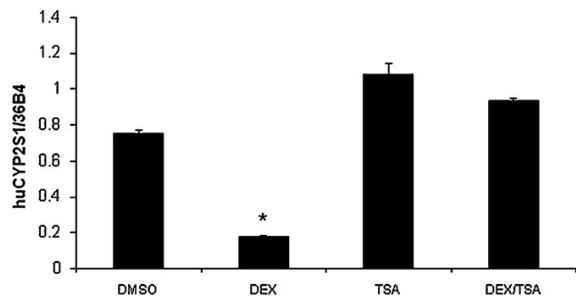


Fig. 5. Trichostatin A reverses repression of CYP2S1 mRNA expression by DEX. A549 cells were treated with 100 nM DEX and with 200 nM of the histone deacetylase inhibitor trichostatin A for 24 h. * $p < 0.001$ (DEX vs. all treatments).

Pujolsa et al., 2009; Urnov and Wolffe, 2001). In order to determine whether the repression of CYP2S1 by DEX occurred by such a mechanism, we utilized Trichostatin A (TSA), a histone deacetylase inhibitor. Treatment of cells with DEX in the presence of TSA, prevented DEX mediated repression of CYP2S1 (Fig. 5). Thus, repression of CYP2S1 by DEX is at least in part mediated by inhibition of histone acetylation.

4. Discussion

Glucocorticoids are widely used to treat various allergic, inflammatory and autoimmune disorders. They exert many of their effects via the glucocorticoid receptor (GR). In the absence of ligand, GR is localized in the cytoplasm as part of an inactive multi-protein complex. Ligand binding induces a conformational change and allows the transfer of the GR to the nucleus via a nuclear localization sequence found in its DNA binding domain (Cadepond et al., 1992).

The GR can regulate genes via transactivation or transrepression. Typically transcriptional activation involves binding of ligand to the GR, which initiates translocation into the nucleus followed by binding of a GR dimer at imperfect DNA palindromes, known as glucocorticoid response elements (GRE)s (Newton, 2000). Transrepression by liganded GR can occur via several mechanisms. The GR can repress transcription by binding to DNA directly, or by binding to NF- κ B or AP-1 and thereby disrupting transcriptional activation by these factors. Additionally, liganded GR can destabilize mRNA. In some genes a negative GRE (nGRE) sequence can be used as a docking site for GR; however this concept is controversial (Newton, 2000). In this regard, an analysis of the CYP2S1 proximal promoter and enhancer did not yield any nGREs or GREs.

Some of the aforementioned mechanisms of GR-mediated transrepression involve histone deacetylation (Adcock et al., 2005; Bilodeau et al., 2006; Ito et al., 2000). Our results suggest that CYP2S1 basal levels are repressed by DEX via histone deacetylation. These changes are mediated by the GR in A549 cells. Interestingly, we observed a similar repression of CYP2S1 expression by DEX in the human pancreatic cancer cell line AsPC1 (Supplementary Figs. 2 and 3).

Regulation of other cytochrome P450s by DEX and other glucocorticoids has been reported. CYP2C11 was shown to be upregulated and downregulated by DEX at low and high concentrations, respectively, in primary rat hepatocytes (Iber et al., 1997). Conversely, CYP2B1/2 was suppressed at low DEX levels and upregulated at high concentrations in primary rat hepatocytes (Kocarek et al., 1994). CYP3A1 mRNA and protein activity were significantly increased in primary rat hepatocytes, while *in vivo*, CYP3A1, CYP3A2 and CYP3A18 were also upregulated by DEX (Hoen et al., 2000). Furthermore, induction of CYP3A by DEX has been reported in human cultured hepatocytes (Pichard et al., 1992).

DEX reduced EROD activity of CYP1A1 and 1A2 in primary human hepatocytes and augmented CYP1A1 expression upon addition of the AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene (3MC) (Vrzal et al., 2009). Additionally, DEX is also able to suppress TCDD induced CYP1B1 expression in primary culture of rat mammary fibroblasts and rat embryo fibroblasts (Brake et al., 1998). This is the first time, however, that CYP2S1 has been reported to be regulated by DEX.

DEX can reduce the levels of prostaglandin E2 (PGE2), and cyclooxygenase 2 (COX 2) and the release of arachidonic acid (AA) (Seibert and Masferrer, 1994; Vigano et al., 1997; Yao et al., 1999). We have demonstrated that CYP2S1 can metabolize certain eicosanoids (Bui et al., 2011), therefore it is interesting that DEX, an anti-inflammatory agent, downregulates this gene. The consequences of CYP2S1 repression by DEX on eicosanoid metabolism have yet to be determined and warrant further study.

Recent reports suggest that DEX can regulate cytochrome P450s via the pregnane X receptor (PXR). However this is not relevant in our case since A549 cells do not contain PXR mRNA (Jahoor et al., 2008), and furthermore, we showed that DEX represses CYP2S1 via the GR. In conclusion, our study demonstrates repression of CYP2S1 by DEX in A549 cells. The ED₅₀ of this repression was found to occur with physiologically relevant concentrations (Balis et al., 1987; Braat et al., 1992). Repression of CYP2S1 by DEX is mediated by the GR in a process dependent upon histone deacetylation. The potential significance of the repression of CYP2S1 in the whole organism will be an interesting area for further study.

Conflict of interest statement

No conflict of interest declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2011.11.020.

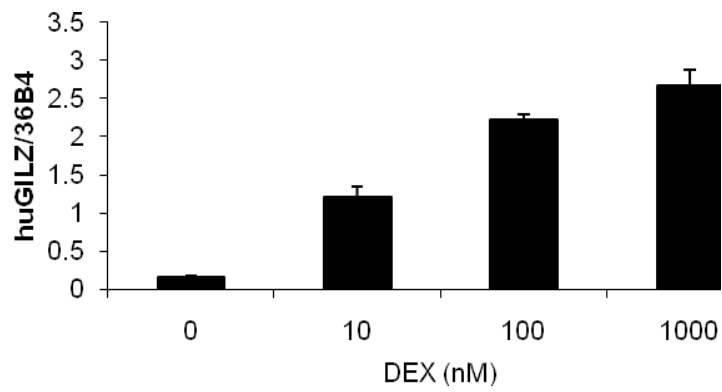
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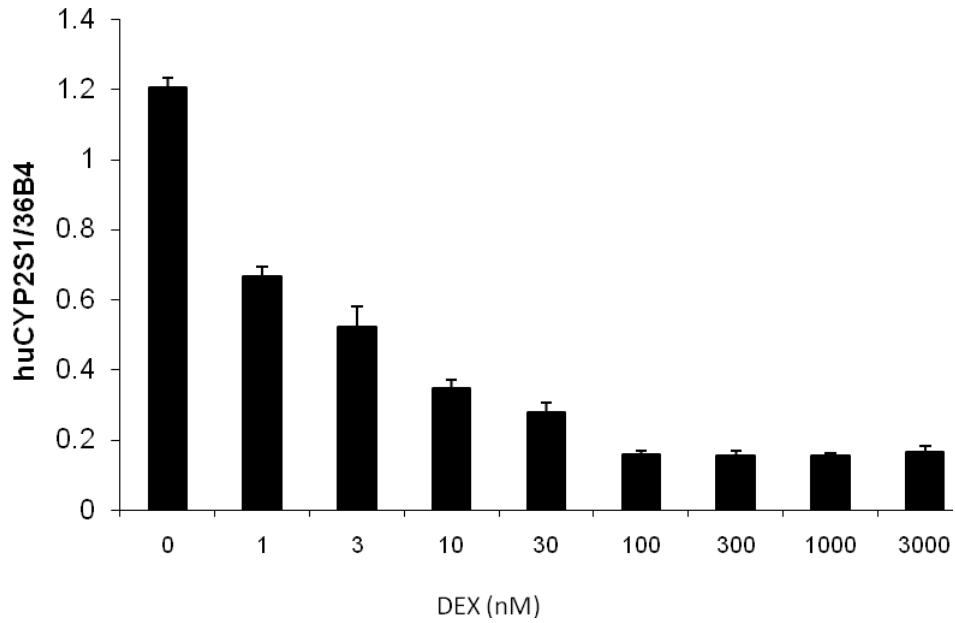
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SUPPLEMENTARY DATA

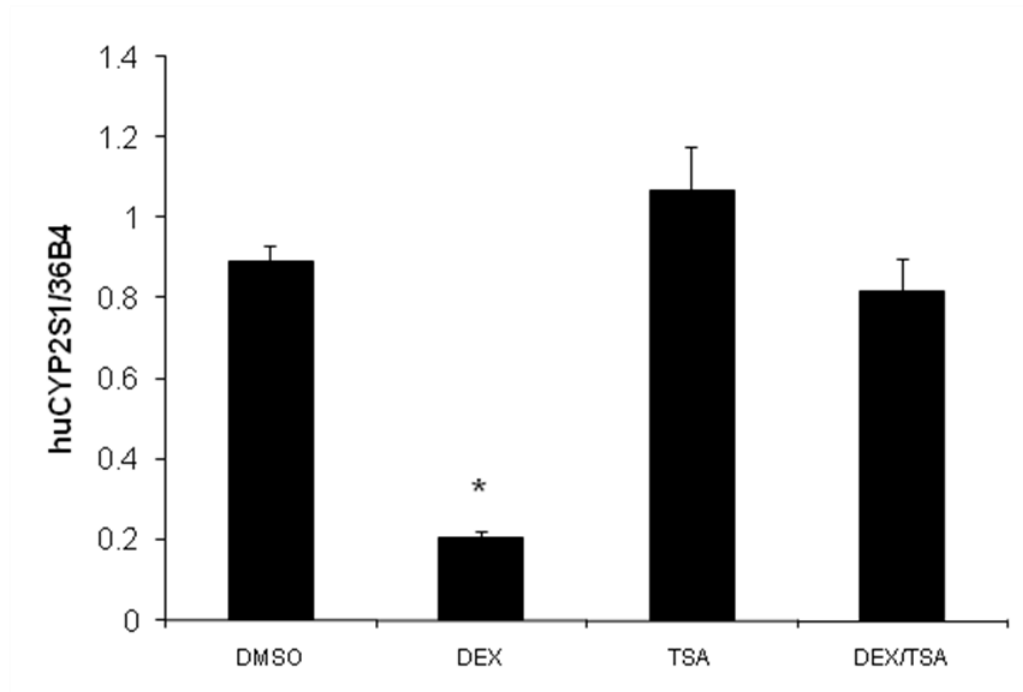
Supplementary Figure 1. DEX induces expression of GILZ. A549 cells were treated with DEX cells for 48 hours. All treatments with DEX resulted in statistically significant increases in gene expression ($p < 0.001$).



Supplementary Figure 2. DEX represses CYP2S1 in AsPC 1 cells. Dose response curve of DEX treated AsPC 1 cells for 48 hours. All treatments with DEX resulted in a statistically significant decrease in gene expression ($p < 0.001$).



Supplementary Figure 3. Trichostatin A reverses repression of CYP2S1 mRNA expression by DEX in AsPC1 cells. AsPC 1 cells were treated with 100 nM DEX and with 200 nM of the histone deacetylase inhibitor Trichostatin A for 24 hours. * $p < 0.001$ (DEX vs. all treatments)



Chapter 6

Conclusion to the Dissertation

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is an environmental toxicant that is one of the most toxic compounds known to man. Its diverse toxicological and biological effects, which are mediated by the aromatic hydrocarbon receptor (AHR), have made TCDD a focal point of scientific research for decades. In an effort to understand TCDD toxicity a substantial amount of this research has been focused on regulation of AHR target genes and its subsequent downstream events. The AHR pathway is activated upon exposure to TCDD, and other environmental pollutants such as benzo[a]pyrene (B[a]P), which leads to the induced expression of genes that can bioactivate procarcinogens and eicosanoids. Several cytochrome P450's (CYP) are AHR target genes and as such scientists investigating AHR toxicity are particularly interested in clarifying the mechanism of their induction, as well as in identifying bioactive metabolites of these enzymes.

The paradigm target gene of AHR encodes the metabolizing enzyme CYP1A1, and in Chapter 2 we utilized RNAi high throughput technologies to identify proteins that modulate the AHR-dependent induction of *Cyp1a1* gene expression. For this purpose we first developed methods for an RNAi screening in a 384-well format and looked at TCDD induced CYP1A1 EROD activity as a readout assay. To validate the results from our screening we also optimized methods for the construction and use of endoribonuclease-prepared siRNA (esiRNA) in a 96-well format. The in-house production of esiRNA proved to be an economical alternative method for screening validation and thereby enabled us to follow up on a much more substantial number of hits. From these screenings we successfully identified and confirmed 39 hits and next sought to determine the role each candidate hit played in induction of CYP1A1

expression. The expression of CYP1A1 after siRNA transfections was measured using quantitative PCR for mRNA, followed by western blot densitometry analysis for protein. From this study we found that expression of PDC, CD9, TMEM5, and Sin3A are necessary for CYP1A1 induction and that expression of Rbm5, ARMC8, Rab40C, Rad50, and Ube2i are necessary for both CYP1A1 induction and AHR expression. In summary, we optimized methods in this study for an RNAi high throughput screening to explore inducible gene expression of *Cyp1a1*. Utilizing our optimized methods we identified and are first to report 9 novel proteins necessary for the induction of CYP1A1 and/or for the expression of AHR.

In chapter 3 we performed additional studies on one candidate protein of particular interest, the transcription factor Sin3A, to define its role in CYP1A1 induction. Sin3A is known as a scaffolding protein that forms several diverse and specialized complexes to influence gene transcription, but HDAC1 and HDAC2 are the most recognized of these proteins. The deacetylation activity from the Sin3A/HDAC complex generally favors transcriptional repression, thus Sin3A is primarily identified as a co-repressor. In this study we quantitated CYP1A1 mRNA and protein expression in induced Hepa-1 cells after treatments with siSin3A. We found that Sin3A not only affects TCDD induced transcription of *Cyp1a1* independent of AHR and ARNT, but also that Sin3A physically associates with the 5'-flanking regulatory regions of *CYP1A1* in both human and mouse cell lines as shown by chromatin immunoprecipitation. These studies establish a novel role for Sin3A in the AHR-mediated induction of *Cyp1a1*, and provide additional evidence for Sin3A as a transcriptional activator of gene expression, which is a role for Sin3A that has scarcely been observed.

We next looked at the role of eicosanoids in AHR-dependent TCDD toxicity. CYP1A1 and other cytochrome P450s can metabolize arachidonic acid into a variety of bioactive eicosanoids which can play a significant role in the inflammatory response. The observation that the physiological roles of eicosanoids parallel those of AHR prompted us to determine if AHR toxicity by TCDD could be mediated through eicosanoids. In this study we measured arachidonic acid as well as up to 23 eicosanoids in different organs/tissues of control and treated wild-type and AHR null mice. TCDD increased the levels of the 4 dihydroxyeicosatrienoic acids (DHETs), 5,6- epoxyeicosatrienoic acid, and 18-, 19- and 20-hydroxyeicosatrienoic acids (HETEs) in the serum, liver, spleen and lungs. There were also increased levels of several metabolites in both their esterified and free forms that can be generated by cytochrome P450s which were found in the serum, liver and spleen. Moreover, these changes correlated with an increase in CYP1A1, CYP1B1, and CYP1A2 mRNA expression and were specific to wild-type mice. In summary, this study revealed that the synthesis of certain eicosanoids can occur only in wildtype mice that are expressing AHR and the CYP1 AHR target genes, which demonstrates that the effects we observed are mediated through the AHR.

Lastly, in chapter 5 we explored dexamethasone-mediated regulation of CYP2S1, a cytochrome P450 which also metabolizes eicosanoids and can thereby play a role in inflammation. CYP2S1 was first identified as a TCDD inducible gene, which was unique to the CYP2 family, and unlike most CYP's it is expressed mostly in extrahepatic tissues. From an initial screening looking for inhibitors of CYP2S1 we discovered that dexamethasone represses its expression in multiple cell lines. Additional investigation established that dexamethasone regulates CYP2S1 via the

glucocorticoid receptor (GR) and that this regulation occurs through the recruitment of histone deacetylases to the CYP2S1 promoter and enhancer. Dexamethasone is a GR ligand that is widely used to treat inflammatory diseases, and future studies will clarify if dexamethasone acts as an anti-inflammatory by negatively regulating CYP2S1 and subsequently decreasing the bioactivation of certain eicosanoids.

This work offers insight into several aspects of AHR-mediated toxicity. Understanding the mechanisms behind AHR provides valuable contributions to our current knowledge in this field, which can aid in the development of preventative or therapeutic measures for AHR-mediated toxicity.