DOWN-REGULATION OF CYTOCHROME P450 2C8
BY 3-METHYLCHOLANTHRENE
IN HUMAN HEPATOCELLULAR CARCINOMA CELL LINES

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Pharmacology and Toxicology
University of Toronto

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ABSTRACT

Down-regulation of cytochrome P450 2C8 by 3-methylcholanthrene in human hepatocellular carcinoma cell lines. Master of Science, 2012. Rucha Utgikar, Department of Pharmacology and Toxicology, University of Toronto.

3-Methylcholanthrene (MC) is a model polycyclic aromatic hydrocarbon that induces cytochrome P450 1A1 (CYP1A1) expression. This laboratory has shown previously that aromatic hydrocarbons, which are important environmental toxicants, down-regulate the expression of rat liver CYP2C11. Recent observations also suggested that CYP2C8, a human enzyme that metabolizes antineoplastic and antidiabetic drugs, among others, is down-regulated in response to aromatic hydrocarbon exposure in primary human hepatocytes. I examined the regulation of CYP2C8 at the mRNA level by MC in two human hepatocellular carcinoma cell lines, HepG2 and HepaRG. MC down-regulated CYP2C8 mRNA levels in HepG2 cells at 24 hours and in HepaRG cells at 48 hours. CYP1A1 mRNA was induced by MC in both cell lines and HepaRG cells appeared to be more sensitive than HepG2 cells to MC-induced cytotoxicity. Further studies are warranted to define the mechanisms and functional impacts of the modulation of this important human CYP by environmental toxicants.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>α-minimum essential medium</td>
</tr>
<tr>
<td>βNF</td>
<td>β-naphthoflavone</td>
</tr>
<tr>
<td>ΔΔCt</td>
<td>comparative threshold cycle method</td>
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<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
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<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
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<td>AIP</td>
<td>AHR-interacting protein</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>androgen receptor</td>
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<td>AHR-associated protein 9</td>
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<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<td>atrA</td>
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<td>AUC</td>
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<td>bHLH/PAS</td>
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<td>FBS</td>
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<td>growth hormone</td>
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<td>glucocorticoid receptor</td>
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<td>GRE</td>
<td>glucocorticoid response element</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
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<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HED</td>
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<td>HIF</td>
<td>hypoxia-inducible factor</td>
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<td>HNF</td>
<td>hepatic nuclear factor</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia response element</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>Hsp</td>
<td>heat shock protein</td>
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<td>I3C</td>
<td>indole-3-carbinol</td>
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<td>I3S</td>
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<td>ICZ</td>
<td>indole-[3,2-b]-carbazole</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of NF-κB</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LOX</td>
<td>lipoxygenase</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LTA₄</td>
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<td>MC</td>
<td>3-methylcholanthrene</td>
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<tr>
<td>miRNA</td>
<td>micro ribonucleic acid</td>
</tr>
<tr>
<td>MM</td>
<td>maintenance and metabolism medium</td>
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<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NC2</td>
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<td>NCoR</td>
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<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
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<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>NO</td>
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<td>negative regulatory element</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PAI-2</td>
<td>plasminogen activator inhibitor 2</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
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<td>PCDD</td>
<td>polychlorinated dibenzo-α-dioxin</td>
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<td>PCDF</td>
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<td>PCR</td>
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<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
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<td>RNA-induced silencing complex</td>
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<tr>
<td>ROR</td>
<td>retinoid-related orphan receptor</td>
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<td>RQ</td>
<td>relative fold change</td>
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<td>reverse transcription</td>
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<td>retinoid X receptor</td>
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<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
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<td>SMRT</td>
<td>silencing mediator for retinoic and thyroid hormone receptors</td>
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<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethlenediamine</td>
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<tr>
<td>TFIIA</td>
<td>transcription factor IIA</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>TNT</td>
<td>20 mM Tris, pH 7.6/137 mM NaCl/0.1% (v/v) Tween-20</td>
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<td>thaw/seed/general purpose medium</td>
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<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
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<td>UPS</td>
<td>ubiquitin-proteasome system</td>
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<td>ultraviolet</td>
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<td>hepatitis B virus X-associated protein 2</td>
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<tr>
<td>XME</td>
<td>xenobiotic-metabolizing enzyme</td>
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SECTION 1: INTRODUCTION

1.1 STATEMENT OF THE RESEARCH PROBLEM

Aromatic hydrocarbons are ubiquitous environmental contaminants that are found in cigarette smoke, automobile exhaust, and other by-products of incomplete combustion reactions, as well as in dietary sources. They are known to exert transcriptional effects on many genes, including the cytochromes P450 (CYPs). CYP1A1 induction following aromatic hydrocarbon exposure is a well-characterized response. However, our laboratory has also shown the capacity of aromatic hydrocarbons to suppress the expression of certain genes, such as that encoding the rodent hepatic enzyme CYP2C11. Recent observations in primary human hepatocytes indicated that 3-methylcholanthrene (MC), a model polycyclic aromatic hydrocarbon (PAH), down-regulates the expression of human CYP2C8, a primarily hepatic drug-metabolizing enzyme responsible for the disposition of clinically relevant drugs including antineoplastic and antidiabetic agents. A down-regulation of the transcription of this enzyme could have important functional consequences for the metabolism of drugs with narrow therapeutic indices, such as paclitaxel, an anticancer drug. In an attempt to identify cell models that may be more amenable to mechanistic investigation of CYP2C8 down-regulation than primary human hepatocytes, the current work examined the suppression of human CYP2C8 in two hepatocellular carcinoma cell lines after exposure to MC.

1.2 PHASE I AND PHASE II REACTIONS IN DRUG METABOLISM

Human beings are exposed to many xenobiotics through dietary sources, therapeutic agents, and sources of environmental contamination (Nebert and Russell,
The fate of xenobiotics in the body is primarily determined by the function of the liver. Metabolism of drugs and other compounds in the liver is part of first-pass clearance, the process by which xenobiotics are modified before entering the general circulation. Upon entering the body via its route of exposure, a drug will be absorbed, distributed to various tissues in the body, metabolized and subsequently excreted. The liver is the primary organ in which drugs are metabolized. Other important sites include the gastrointestinal tract, skin, lung and kidney. Drug metabolism is often considered to occur in two stages, Phase I and Phase II, although not all xenobiotics will undergo both phases or in this order. In general terms, Phase I reactions produce metabolites of the parent compound bearing chemical functional groups allowing for conjugation reactions in Phase II that will facilitate excretion. As such, Phase I is often thought of as an activating phase, while Phase II may be thought of as a detoxification phase. Phase I reactions include oxidation, reduction, and hydrolysis and are catalyzed by CYPs, flavin-containing monooxygenases, reductases, esterases, amidases and peptidases, among others. Phase II reactions include the conjugation of parent drugs or Phase I metabolites to glucuronic acid, glutathione, sulphate, and methyl or acetyl groups. Phase II enzymes include UDP-glucuronosyltranferases (UGTs), glutathione S-transferases (GSTs), sulfotransferases, N-acetyltransferases and methyltransferases (reviewed by Riddick, 2007).

1.3 CYTOCHROMES P450

CYPs are hemoproteins found in the smooth endoplasmic reticulum membrane (microsomal) and mitochondria of cells in various tissues. They are expressed in all life
forms ranging from bacteria to humans and are responsible for the catalysis of primarily oxidation reactions, which are quantitatively the most important Phase I reaction. Microsomal CYP oxidation activity depends on the presence of molecular oxygen ($O_2$) and the transfer of reducing equivalents from NADPH via the membrane-bound enzyme NADPH-cytochrome P450 oxidoreductase (POR) (Riddick, 2007). NADPH serves as a reducing cofactor that donates an electron to POR. POR then reduces the drug-bound CYP, which in turn binds molecular oxygen. One of the oxygen atoms is then transferred to the substrate (reviewed by Riddick, 2007). The overall balanced equation for the prototypical microsomal CYP monooxygenation reaction is shown below, where RH is the parent drug:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The 57 putatively functional human CYP isoforms are organized into families numbered 1-51 according to sequence homology. Those CYPs sharing over 40% sequence identity are said to belong to the same family; those demonstrating over 55% sequence identity are generally members of the same subfamily (A, B, C, etc.). CYPs are expressed primarily in the liver, although they are also found in lung, kidney, brain, heart, intestine and gonadal tissue, among others (Guengerich, 2005). The large number of CYP isoforms and the broad substrate selectivity they possess enable these enzymes to catalyze the biotransformation of hundreds of thousands of substrates (Nebert and Russell, 2002; Riddick, 2007).

**1.3.1 Functions of CYP Enzymes in Metabolism of Endogenous Substrates**

CYP enzymes have physiological activities unrelated to xenobiotic disposition, such as steroidogenesis and the metabolism of eicosanoids and vitamins A and D. These
activities are important in the maintenance of homeostasis (Nebert and Dieter, 2000; Guengerich, 2005). CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP17A2, and CYP19A1 are the six human CYPs responsible for steroidogenesis. Collectively, the activity of these enzymes contributes to the biosynthesis of pregenolone, 17-OH-pregnenolone, dehydroepiandrosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol, aldosterone, androstenedione, estrone and estradiol. Notably, the conversion of cholesterol to pregnenolone by CYP11A1 is the rate-limiting step in all steroidogenic reactions (reviewed by Miller and Auchus, 2011). CYP7A1, CYP8B1 and CYP27 are involved in the degradation of cholesterol into bile acids, another important endogenous function of CYP enzymes (reviewed by Akiyama and Gonzalez, 2003).

Other CYPs are involved in the metabolism of eicosanoids, a group of lipid molecules that play important roles in inflammatory signalling and arise from the metabolism of arachidonic acid. Eicosanoids include prostaglandins and leukotrienes, whose synthesis is catalyzed by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, while CYPs convert arachidonic acid into epoxyeicosatrienoic acids (EETs) or hydroxyeicosatetraenoic acids (HETEs). CYP2C and CYP2J enzymes act as epoxygenases to catalyze the production of EETs, while CYP4A and CYP4F enzymes act as ω-hydroxylases to catalyze the synthesis of HETEs, primarily 20-HETE. These eicosanoids are of biological significance because of their opposing activities on vasculature: 20-HETE is a vasoconstrictor while EETs have vasodilatory properties. Furthermore, these compounds are implicated in altered cell proliferation and angiogenesis, suggesting a role for the CYPs involved in their synthesis in the pathology of cancer (reviewed by Panigrahy et al., 2010).
1.3.2 Functions of CYP Enzymes in Xenobiotic Metabolism

Fifteen of the 57 putatively functional human CYPs are thought to be primarily involved in the metabolism of xenobiotics (Guengerich, 2005). Most of these drug-metabolizing enzymes are members of the CYP1, CYP2 and CYP3 families. Many xenobiotic-metabolizing CYPs are also able to oxidize endogenous substrates in a limited capacity (reviewed by Nebert and Russell, 2002; Guengerich, 2005). Of the drug-metabolizing CYPs, CYP3A4, CYP2D6 and CYP2C9 are responsible for the metabolism of ~75% of all drugs (Guengerich, 2005). Although there are numerous exceptions, metabolism of exogenous substrates usually results in their detoxification (Nebert and Russell, 2002).

The CYP1 gene family consists of three members whose expression is up-regulated by the activated aryl hydrocarbon receptor (AHR). CYP1A1 and CYP1B1 metabolize PAHs, while CYP1A2 preferentially metabolizes aryl and heterocyclic amines. Endogenous substrates include estrogen and melatonin, but knockout mice with targeted disruption of individual Cyp1 genes have proven to be viable, suggesting that their role in the metabolism of endogenous compounds is likely not critical for life (Nebert and Russell, 2002). Although CYP1 family enzymes can bioactivate certain compounds, such as benzo[a]pyrene, into genotoxic metabolites that are associated with carcinogenesis (Ma and Lu, 2007), the protective roles of these enzymes in detoxification are also substantial. For example, studies using Cyp1 knockout mouse models demonstrated that the induction of Cyp1a1 is important in the detoxification of benzo[a]pyrene. (Nebert and Russell, 2002; Uno et al., 2004, 2006).
1.3.3 Polymorphism of CYP Genes

Genes encoding several of the CYPs are polymorphic; in general terms, CYP families 1, 2, and 3 are more frequently polymorphic than CYPs in families 4-51. The former group is mainly involved in xenobiotic metabolism while the latter is mainly implicated in the metabolism and synthesis of endogenous compounds (reviewed by Johansson and Ingelman-Sundberg, 2010). CYP polymorphism may affect the rate of drug disposition, as ultra-rapid, extensive and poor metabolizing phenotypes have been documented in humans. Such phenotypic differences may relate to unexpected drug toxicity, if the rate of formation of a toxic metabolite is increased (in the case of a rapid metabolizer) or if the parent compound is toxic (in the case of a poor metabolizer). In contrast, phenotypic differences may relate to therapeutic failure if an active parent compound is metabolized too quickly (in the case of a rapid metabolizer) or the biotransformation to an active metabolite is deficient (in the case of a poor metabolizer). Important pharmacological consequences have been observed in individuals with CYP polymorphisms with respect to drugs such as nicotine (CYP2A6), warfarin (CYP2C9), mephenytoin (CYP2C19), and codeine and tamoxifen (CYP2D6), along with many others (reviewed by Johansson and Ingelman-Sundberg, 2010).

1.3.4 Regulation of CYP Expression

Expression of the CYP genes is regulated by a complex interplay among various nuclear factors, transcription factors and hormonal signals. Hormones that regulate CYP expression include glucocorticoids, estrogen, testosterone and growth hormone (GH). The hormonal regulation of these genes may explain some sex-specific differences in expression and activity levels.
Glucocorticoids are hormones released under control of the hypothalamic-pituitary-adrenal axis. Synthetic glucocorticoids are prescribed for the treatment of inflammation, autoimmune diseases and certain types of cancer, while endogenous glucocorticoids are involved in the control of energy metabolism, immune function and cell cycle progression. Glucocorticoids exert effects on CYP transcription through the glucocorticoid receptor (GR) in humans and in rodent models (reviewed by Dvorak and Pavek, 2010). For example, studies in Gr-null mice have demonstrated that GR plays a role in the constitutive and inducible expression of CYP2B and CYP3A enzymes (Schuetz et al., 2000). Studies in primary human hepatocytes have demonstrated that human CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A5 are regulated by glucocorticoids, as is CYP2B2 in rat hepatoma H4IIE cells (Dvorak et al., 2002, 2003; Audet-Walsh et al., 2008). These effects may in some cases be mediated directly by the activation of a GR homodimer, which binds glucocorticoid response elements (GREs) in the 5’-flanking region of the gene; in other cases, GR activation may play a more indirect role by increasing the synthesis of other transcription factors such as constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), discussed below. The GR can also be involved in other indirect, trans-regulatory mechanisms. For instance, the synthetic glucocorticoid dexamethasone was shown to increase the binding of hepatic nuclear factor (HNF)-4α to its response element, causing an induction of gene expression in primary human hepatocytes (Onica et al., 2008).

Studies in female rats have shown that 17β-estradiol, the main female sex hormone, increases the expression of CYP1A2, CYP2C6, CYP2C7 and CYP3A9 (Choi et al., 2011). More subtle sex differences appear to be involved in the regulation of
human CYP expression, as the metabolism of certain drugs, mediated by specific CYP isoforms, appears to occur at different rates in human males and females. Elimination rates for substrates of CYP2A6, CYP2B6 and CYP3A4 are higher in females while the rate of elimination of CYP1A2 substrates is lower (reviewed by Anderson, 2008). Androgen-dependent regulation of CYPs has been identified in pigs, in which sex-specific differences in the constitutive expression of CYP1A1 and CYP1A2 suggest a suppressive function of testosterone in the pig liver (Kojima et al., 2010). Androgens have also been implicated in the regulation of hepatic and renal microsomal mouse CYPs displaying sex-specific patterns of expression or induction (Degawa et al., 1985, 1990).

The effects of GH represent another example of hormonal modulation of CYP expression. GH is continuously present in the plasma of female rats, while peak levels are achieved every three to four hours in male rats, with virtually undetectable levels during the inter-pulse interval. CYP2C11 is a male-specific enzyme in rats that is induced by the pulsatile secretion of GH, while CYP2C12 is a female-specific rat enzyme that is maintained by the continuous GH secretion pattern seen in adult females (Sundseth et al., 1992). GH signalling in hepatocytes via the Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5b (STAT5b) pathway is a major determinant of sex-dependent liver gene expression in rodents and also likely contributes to the more subtle sex differences seen in humans (Udy et al., 1997; Clodfelter et al., 2006; Waxman and O’Connor, 2006).

Liver-enriched transcription factors, including HNFs and DNA-binding protein (DBP) among others, are involved in the constitutive expression of CYP genes (reviewed by Akiyama and Gonzalez, 2003). HNF-1α is a transcription factor that up-regulates the
expression of *CYP1A2*, *CYP2E1*, *CYP7A1* and *CYP27* genes in vitro. HNF-4α is an orphan nuclear receptor that binds as a homodimer to responsive elements in the promoter region of target genes. These elements have been identified in *CYP2C9* and *CYP2D6*, and knockdown of HNF-4α has been shown to down-regulate these genes. HNF-4α knockdown also caused a down-regulation in the expression of *CYP2A6*, *CYP2B6*, *CYP3A4* and *CYP3A5* in cultured human hepatocytes. DBP is a transcription factor that is expressed according to a circadian rhythm and as such is thought to be implicated in the regulation of *CYP* genes that demonstrate circadian expression patterns (reviewed by Akiyama and Gonzalez, 2003).

Other factors such as PXR, CAR and AHR are key regulators of CYP expression. These receptors, after binding their ligands, recognize response elements in the promoter regions of various genes and effect changes in transcription. Ligands of PXR include pregnanes, glucocorticoids, and various xenobiotics. The classical CAR activator is phenobarbital. PXR and CAR each require heterodimerization with retinoid X receptor (RXR) to exert their effects on gene transcription, which include induction of members of the *CYP2A*, *CYP2B*, *CYP2C*, and *CYP3A* subfamilies (reviewed by Akiyama and Gonzalez, 2003). AHR is a cytosolic factor whose nuclear translocation after ligand binding effects changes in the transcription of many genes. The structure and function of AHR is discussed in detail in Section 1.4.

1.4 THE ARYL HYDROCARBON RECEPTOR

The AHR is a cytoplasmic receptor that is a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors. It is evolutionarily
conserved, expressed in all vertebrate species, and is ubiquitously expressed in nearly
every tissue in the body (Nebert et al., 2004). This family of transcription factors is
divided into two groups: Class I factors, which are unable to dimerize with other Class I
factors; and Class II factors, which act as heterodimerization partners for Class I factors.
Besides the AHR, Class I factors include HIF-1α, -2α and -3α, and Class II factors
include the AHR nuclear translocator (ARNT), ARNT2, and brain and muscle ARNT
like protein 1 (BMAL1) and BMAL 2 (reviewed by Furness and Whelan, 2009).

Like other members of this family, the AHR contains a basic region responsible
for DNA binding, a helix-loop-helix domain that forms part of the heterodimerization
interface, and two PAS domain repeats (A and B) that act to increase the specificity of
heterodimerization and to increase the strength of this interaction. The AHR is the only
ligand-activated transcription factor in the bHLH/PAS family. As a Class I factor, it
heterodimerizes with its class II binding partner, ARNT, and is involved in various
developmental, physiological and adaptive processes (reviewed by Esser et al., 2009;
Furness and Whelan, 2009; Fujii-Kuriyama and Kawajiri, 2010; Dietrich and Kaina,
2010).

1.4.1 Physiological Roles of the AHR

Given the evolutionary conservation of the AHR across all vertebrate species, it is
likely that it has a physiological role in addition to its role in regulating xenobiotic-
metabolizing enzymes (XMEs) (Fujii-Kuriyama and Kawajiri, 2010). Additionally, the
AHR gene battery includes many genes not involved in the response to xenobiotics, such
as those involved in cell cycle control (reviewed by Bock and Kohle, 2009). Evidence for
physiological roles of AHR arises from the observation that Ahr-null mice demonstrate
persistent fetal ductus venosus after birth, reduced liver size, altered immune function, and compromised female reproductive function. Ahr-null mice have also shown altered expression of hundreds of genes involved in various cell processes unrelated to xenobiotic metabolism (e.g. energy metabolism) (McMillan and Bradfield, 2007). Other physiological roles of the AHR, uncovered through studies of Ahr-null mice, are discussed below.

1.4.1.1 Roles in Development and Cell Cycle Control

The AHR is involved in the normal development of the liver, as Ahr-null mice were shown to have poorly developed livers and other hepatic deficiencies (Schmidt et al., 1996). Additionally, Ahr-null mice demonstrate cardiomyopathy, hypotension, abnormal vascular development (e.g. persistent fetal ductus venosus), and the development of lesions in the skin and gastrointestinal tract (Fernandez-Salguero et al., 1997). Other studies have shown a role for the AHR in hematopoiesis. One study found that hematopoietic stem cells (HSCs) in Ahr-null mice have a higher proliferative capacity than HSCs from wild-type animals, suggesting that the AHR is involved in the maintenance of stem cell quiescence in wild-type animals (Singh et al., 2011). This evidence suggests that in the absence of known exogenous ligands, the AHR is involved in normal development and physiological function.

1.4.1.2 Tumour Suppression

Ahr-null mice were found to have altered expression of genes involved in the ubiquitin-proteasome system (UPS), which mediates the degradation of proteins involved in cell growth and proliferation (Reyes-Hernandez et al., 2010). Additionally, studies in human cell lines have shown the AHR to function as a ligand-dependent E3 ubiquitin
ligase, which recruits E2 ubiquitin-conjugating enzymes to target proteins for proteasomal degradation. Targets include estrogen receptor (ER) α and androgen receptor (AR), suggesting another role for AHR in the regulation of sex hormone signalling (Ohtake et al., 2007). Furthermore, Ahr-null mice are prone to intestinal carcinogenesis and showed an abnormal accumulation of β-catenin. A mouse model of familial adenomatous polyposis was shown to be prone to intestinal carcinogenesis, but activation of the AHR by natural ligands in these mice was shown to result in degradation of β-catenin and decreased tumour formation in an AHR ligand-dependent manner (Kawajiri et al., 2009). These results, together with evidence that AHR is involved in the regulation of cell cycle progression and cell proliferation, suggest that the AHR has an important role in the suppression of intestinal carcinogenesis.

1.4.2 Ligands of the AHR

The AHR binds a wide variety of exogenous and endogenous compounds. Exogenous ligands may be further categorized as synthetic or naturally occurring. Exposure to synthetic ligands is generally associated with various toxic effects in humans and animals. The AHR preferentially binds planar, lipophilic molecules with maximum dimensions of 14 x 12 x 15 Å, and many ligands of the AHR are structurally related, but others have been shown to be structurally diverse, indicating that the ligand binding site of the AHR is likely promiscuous (reviewed by Denison and Nagy, 2003; Ashida et al., 2008). No definitive physiological ligand of the AHR has been identified to date, but the promiscuity of the AHR binding site suggests that a broad range of endogenous molecules may serve this function (Denison and Heath-Pagliuso, 1998). Several classical and recently discovered AHR ligands are shown in Figure 1.1.
Halogenated aromatic hydrocarbons:

- 2,3,7,8-Tetrachlorodibenzo-\( p \)-dioxin
- 2,3,7,8-Tetrachlorodibenzofuran

Polycyclic aromatic hydrocarbons:

- 3-Methylcholanthrene
- Benzo[\( a \)]pyrene

Non-classical AHR ligands:

- Indolo[3,2-b]carbazole
- Kynurenic acid
- Indoxyl-3-sulphate (potassium salt)

Figure 1.1. Classical and non-classical ligands of the AHR.
1.4.2.1 Synthetic Exogenous Ligands

The most extensively characterized synthetic exogenous ligands of the AHR are PAHs and halogenated aromatic hydrocarbons (HAHs). HAHs include the polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-\(p\)-dioxins (PCDDs), such as 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD). The most active HAHs and PAHs have relatively high binding affinity for the AHR, with \(K_d\) values in the picomolar to nanomolar range (reviewed by Denison et al., 2002; Denison and Nagy, 2003; Nebert et al., 2004).

TCDD is the most potent HAH known and has an \textit{in vivo} biological potency of at least 30,000 times that of MC, the prototypical PAH (Nebert et al., 2004). TCDD is a contaminant in the production of 2,4,5-trichlorophenoxyacetic acid, a component of Agent Orange, which was used as a defoliant in the Vietnam War (Poland and Knutson, 1982; Stellman et al., 2003). HAHs also arise as by-products in incomplete combustion reactions or in the synthesis of industrial chemicals, such as the fungicide pentachlorophenol (Poland and Knutson, 1982; Safe, 1990). PCDDs and PCDFs are formed as by-products of the burning of coal and wood, during waste incineration and in emissions from automobiles fuelled by leaded gasoline. They are ubiquitous environmental contaminants that have been detected in air, ash and sediment samples. They are also found in dietary sources such as butter, milk, eggs, fish, and animals domesticated for food purposes (Safe, 1990). The lipophilic nature of these ligands contributes to their concentration in adipose tissue and bioaccumulation in the food chain. Consequently, a major source of exposure for humans is through the consumption of meat, fish, shellfish and dairy products (reviewed by Marshall and Kerkvliet, 2010).
These compounds also bioaccumulate in fatty tissues and substances in humans, such as adipose tissue and human breast milk (Safe, 1990). They are metabolically stable and do not require bioactivation into reactive metabolites to exert their toxic effects (Kafafi et al., 1993; Denison and Nagy, 2003). Despite extensive research, the mechanisms by which TCDD exerts its toxic effects in humans as well as in laboratory animals remain unclear. Persistent alterations in gene expression are thought to contribute to most toxic effects of TCDD, although the identity of the key target genes remains elusive. These toxicities are generally attributed to the parent compound itself rather than a metabolite, as TCDD is highly resistant to metabolism (reviewed by Hankinson, 2005; Chopra and Schrenk, 2011).

In contrast, PAHs are metabolically activated by XMEs and exert at least some of their toxic effects through reactive intermediates (Kafafi et al., 1993). PAHs are exemplified by compounds such as benzo[a]pyrene and the model PAH carcinogen and laboratory reagent, MC, which have reasonably high AHR binding affinity but diminished biological potency because of rapid metabolism (Riddick et al., 1994). The majority of PAHs arise as a result of human processes. Compounds in this category include several chemicals found in cigarette smoke, such as benzo[a]anthracene, benzo[a]pyrene, and fluoranthene (Kitamura and Kasai, 2007). PAHs are found in the air, soil, sediment and water and can arise from the incomplete combustion and pyrolysis of fossil fuels and meat, or from industrial processes such as the generation of heat and power, waste incineration, and the production of rubber, aluminum and cement. Automobile exhaust is another important source of PAHs (reviewed by Guo et al., 2011). Part of their toxicity arises as a result of their activation into reactive intermediates that
are able to form DNA adducts. For example, the metabolism of benzo[a]pyrene into benzo[a]pyrene-7,8-diol-9,10-epoxide causes the formation of lesions at guanine residues and has been linked to mutations in the tumor suppressor gene p53. Other mutations caused by reactive intermediates likely interfere with the regulation of the cell cycle and stability of genetic material (reviewed by Alexandrov et al., 2010)

1.4.2.2 Naturally Occurring Exogenous AHR ligands

Human exposure to natural AHR ligands occurs primarily through dietary sources in the form of vegetables, fruits, herbs and teas (reviewed by Denison et al., 2002, Ashida et al. 2008; White and Birnbaum, 2009). Indoles, such as tryptophan and indol-3-carbinole (I3C, found in cruciferous vegetables) can be converted from their native forms, which have little activity as AHR agonists, to more potent forms like indolo-[3,2-b]-carbazole (ICZ), and 3,3’-diidolylmethane (DIM). ICZ has been shown to induce AHR-mediated gene expression in cell culture and DIM has been shown to induce expression of CYP1A1 and CYP1B1 (reviewed by Denison et al., 2002; Denison and Nagy, 2003; Bradshaw and Bell, 2009). Actions of these indole derivatives are AHR-dependent and suggest conversion of dietary substances into functional AHR ligands.

Naturally occurring flavonoids found in fruits, vegetables and teas have been shown to demonstrate AHR antagonist activity. Quercetin, kaempferol and luteolin inhibit the TCDD-activated AHR signalling pathway by competing for AHR binding, thereby decreasing the inductive effects of AHR agonists on gene expression. Curcumin, a polyphenol, has also been shown to act as an AHR antagonist and to inhibit gene induction by TCDD by inhibiting the association between AHR and ARNT in cultured mouse cells. Curcumin also suppressed benzo[a]pyrene-mediated AHR activation in
mouse lung and liver (reviewed by Ashida et al., 2008). However, these effects may be cell-specific or dose-dependent, as studies also showed an induction of genes in the AHR battery in MCF-7 cells in response to curcumin treatment (Ciolino et al., 1998). The wide variety of natural compounds that interact with the AHR suggests that dietary constituents may be important natural ligands.

1.4.2.3 Endogenous Ligands

The evolutionary conservation of the AHR and its implication in pathways ranging from neurodevelopment to immune function suggest that the AHR responds to endogenous ligands. Several classes of endogenous compounds have been shown to activate the AHR (Bradshaw and Bell, 2009).

Leukotriene A₄ (LTA₄) is a metabolite produced during the metabolism of arachidonic acid by LOX enzymes in the inflammatory response. 5,6-diHETEs, which are LTA₄ metabolism by-products, have been shown to bind directly to the AHR and to activate the AHR signalling pathway (Chiaro et al., 2008). Additionally, kynurenic acid, a metabolite of a pathway involved in the modulation of immune tolerance, was recently shown to act as an AHR agonist. It is capable of inducing CYP1A1 expression in HepG2 cells and CYP1A1-mediated metabolic activity in primary human hepatocytes. Treatment of MCF-7 cells with physiologically relevant concentrations of kynurenic acid (100 nM) caused an AHR-dependent induction of the expression of the inflammatory cytokine interleukin-6 (IL-6) when IL-1β was administered concomitantly. This evidence further suggests a role for AHR and endogenous ligands in the control of the immune system (DiNatale et al., 2010). The same group has identified 3-indoxyl sulphate (I3S), a toxin
present at high concentrations in humans with renal disease, as a potent endogenous activator of the AHR (Schroeder et al., 2010).

Additionally, UV irradiation has been found to activate the AHR signalling pathway through the generation of 6-formylindolo[3,2-b]carbazole (FICZ) from tryptophan in human melanocytes, leading to an increase in melanin production (Luecke et al., 2010). Other potential endogenous ligands have been identified, but their physiological relevance remains unclear, as normal concentrations of these compounds do not activate the AHR signalling pathway. Examples of these compounds include cyclic adenosine monophosphate, bilirubin, tryptophan, and tryptophan metabolites tryptamine and indole acetic acid (reviewed by Denison and Nagy, 2003; Nguyen and Bradfield, 2008; Bradshaw and Bell, 2009).

1.5 TOXIC EFFECTS OF EXPOSURE TO PAHs AND HAHs

Exposure to PAHs and HAHs has been linked to toxic effects in animal models and in humans. TCDD exposure, for example, causes a wasting syndrome in animals marked by dramatic weight loss. Thymic atrophy and immunosuppression are also observed in mice, rats and guinea pigs exposed to TCDD. Liver toxicity is another hallmark of TCDD exposure in rodents, including hepatomegaly and the appearance of liver lesions. Other toxicities observed in animals include hair loss, skin pathologies, an increased incidence of cancer and porphyria, and endocrine disruption resulting in lower fertility and smaller litter sizes (reviewed by Poland and Knutson, 1982). *In utero* exposure to TCDD has been associated with infertility and abnormal reproductive development in male and female rats (Gray and Ostby, 1995). Rodent exposure to PAHs
is also associated with an increased incidence of skin, lung, and blood cancers, among others (Shimizu et al., 2000; Wogan et al., 2004; Yu et al., 2006). Toxic effects of PAHs and HAHs are absent or greatly diminished in Ahr-null mice (Fernandez-Salguero et al., 1996; Shimizu et al., 2000; Vorderstrasse et al., 2001; Nakatsuru et al., 2004).

Much of what is known about the human response to TCDD-like chemicals is due to accidental poisonings of large populations. The use of TCDD-contaminated Agent Orange in the Vietnam War between 1961 and 1971 exposed millions of individuals to TCDD, and an explosion in Seveso, Italy in 1976 caused wide-scale dioxin exposure as well. Additionally, thousands of people were exposed to PCBs and PCDFs in Taiwan and Japan through the contamination of cooking oil (Ngo et al., 2006; Pesatori et al., 2009; Tsukimori et al., 2011; Yang et al., 2011). The hallmark of human exposure to high doses of TCDD-like chemicals is chloracne, a severe and disfiguring skin pathology (White and Birnbaum, 2009).

TCDD is a known human carcinogen and has been linked to an increased risk of birth defects in children born to parents who were exposed to Agent Orange in Vietnam (Ngo et al., 2006). Evidence from studies in Seveso suggests that residents exposed to TCDD are at a higher risk for lymphatic and blood cancers (Pesatori et al., 2009). Breast-fed sons born to mothers who were exposed to dioxin were found to have lower sperm counts and motility in adulthood compared to similar subjects who were not breast-fed, or compared to sons born to mothers with background levels of serum dioxins (Mocarelli et al., 2011). Additionally, infants born to mothers exposed to TCDD were more likely to have hypothyroidism, similar to what is seen in animal models of maternal dioxin exposure (Baccarelli et al., 2008). Finally, persons with Yusho or Yucheng disease (“oil
“disease” in Japanese and Chinese, respectively) present with irregularities in the menstrual cycle and lesions on the skin and eyes (reviewed by Aoki, 2001). These studies suggest a role for HAHs in carcinogenesis, reproductive toxicity and endocrine dysregulation in exposed persons.

Epidemiological evidence also suggests a correlation between aromatic hydrocarbon exposure and autoimmune disease, possibly through regulation of T helper cell (Th17) differentiation. AHR activation, in a manner that seems to depend on the nature of the ligand, increases Th17 cell proliferation and subsequently increases cytokine production, which is associated with psoriasis, Crohn's disease, multiple sclerosis and inflammatory bowel disease (reviewed by Esser et al., 2009). Most or all of the toxic effects of aromatic hydrocarbon exposure seem to be a result of activation of the AHR. For example, induction of the CYP1 family of enzymes via an AHR-dependent mechanism can result in the bioactivation of PAHs to their ultimate carcinogenic forms. For both PAHs and especially persistent HAHs, AHR activation may also cause endocrine and cell cycle alterations due to dysregulated gene expression patterns and crosstalk with other signalling pathways.

### 1.6 CANONICAL AHR SIGNALLING PATHWAY

In its unliganded state, the AHR resides in the cell cytoplasm bound by one molecule of AHR-interacting protein (AIP; also known as XAP2 or ARA9), one molecule of p23 and a dimer of the 90-kDa heat shock protein (Hsp90), which is thought to mask a nuclear localization signal (NLS). Upon ligand binding, the NLS is exposed and is recognised by the protein importin-β, which allows for translocation of the AHR.
complex to the nucleus. Subsequently the chaperone proteins are shed and the AHR-ligand complex heterodimerizes with ARNT. This complex then binds to dioxin-response elements (DREs) located upstream of target genes, resulting in altered gene expression. A schematic representation of this signalling pathway is shown in Figure 1.2.

A 5′-CGTG-3′ motif is the invariant core within the DRE required for the AHR:ARNT heterodimer to interact with DNA (reviewed by Whitlock, 1999; Denison et al., 2002; Beischlag et al., 2008; Furness and Whelan, 2009; White and Birnbaum, 2009; Fujii-Kuriyama and Kawajiri, 2010; Dietrich and Kaina 2010). Studies have suggested that by binding the DRE, the AHR:ARNT complex disrupts the nucleosomeal organization of target genes and recruits additional transcriptional co-activators, resulting in increased gene transcription (Hankinson, 2005).

1.6.1 Target Genes of the AHR

The most well-characterized target gene of the AHR is CYP1A1, a member of the CYP1 enzyme family. Other members of this family include CYP1A2 and CYP1B1. These enzymes, as discussed in Section 1.3.2, are involved in the response to xenobiotics. Other genes induced via AHR signalling include Phase II enzymes such as specific forms of UGT and GST. Induction of these enzymes by AHR ligands can also interfere with the metabolism of endogenous molecules, resulting in a disruption of homeostasis (reviewed by Riddick et al., 2003).

Other genes demonstrating patterns of up- or down-regulation in response to aromatic hydrocarbon exposure include plasminogen activator inhibitor 2 (PAI-2), the cytokine IL-1β, and transforming growth factors α and β (TGF-α and –β) (reviewed by
Figure 1.2. Signalling mechanism of the AHR pathway. In its unliganded state, the AHR exists in complex with AIP (also known as XAP2 or ARA9), p23 and a Hsp90 dimer in the cell cytoplasm. Lipophilic ligands of the AHR diffuse through the cell membrane and activate the AHR, causing a conformational change. This change allows shedding of chaperone proteins via incompletely understood mechanisms and the activated AHR translocates into the nucleus, where it heterodimerizes with ARNT. The AHR:ARNT complex then interacts with a 5’-CGTG-3’ invariant core motif in DRE sequences and alters the transcription of target genes.
Riddick et al., 2003). PAI-2 contributes to the regulation of cell growth and differentiation and was up-regulated by TCDD exposure in a human keratinocyte cell line (Sutter et al., 1991). TGF-α and TGF–β are factors that are involved in the regulation of cell growth and proliferation. TGF-α mRNA levels were increased and TGF–β2 transcription was decreased in a human keratinocyte cell line after TCDD treatment. However, the increase in TGF-α levels was notably not caused by an increased rate of transcription but rather by stabilization of TGF-α mRNA, suggesting a post-transcriptional regulatory mechanism (Gaido et al., 1992). IL-1β is a proinflammatory cytokine that is implicated in the suppression of CYP expression, as detailed in Section 1.8.2. It too was up-regulated in a human keratinocyte line by TCDD (Sutter et al., 1991). Other genes down-regulated by TCDD include estrogen-regulated genes such as cathepsin D, c-fos, and pS2 (reviewed by Riddick et al., 2003).

1.6.2 Induction of CYP1A1 by Aromatic Hydrocarbons

As described previously, recruitment of the AHR to DREs causes changes in the expression of target genes. The best characterized of these changes is the induction of CYP1A1, an enzyme involved in the oxidation of PAHs and a wide range of endobiotics and xenobiotics. While this oxidation reaction usually results in detoxification of the xenobiotic, it can also lead to the formation of reactive species capable of forming DNA and protein adducts, leading to carcinogenesis and cytotoxicity (Ma and Lu, 2007). Its basal expression levels are low in the liver and extrahepatically, but it is highly inducible in response to some of its substrates, including benzo[a]pyrene and MC. Exposure to aromatic hydrocarbons results in activation of the AHR signalling pathway described above and therefore results in the induction of CYP1A1 at the mRNA and protein levels.
Since many of the CYP1A1 inducers are also CYP1A1 substrates, the induction results in increased metabolism of the inducing chemical to less active metabolites; however, bioactivation can also occur, generating ultimate carcinogens (Whitlock, 1999; Ma and Lu, 2007). One example of this process is the activation of the procarcinogen benzo[a]pyrene into benzo[a]pyrene-7,8-diol-9,10-epoxide with the involvement of epoxide hydrolase. These reactive species, also known as bay region epoxides, form DNA and protein adducts. DNA adducts are considered to be molecular predictors of cancer risk, and benzo[a]pyrene is a well-characterized inducer of lung tumour formation (Shimada, 2006; Uppstad et al., 2010). As such, induction of CYP1A1 and subsequent increases in catalytic activity that result in increased levels of adduct-forming metabolites are highly relevant to the risk of carcinogenesis in individuals exposed to PAHs (Uppstad et al., 2010).

In the mouse hepatoma Hepa-1 cell line, benzo[a]pyrene treatment was shown to recruit RNA polymerase II to the promoter of the Cyp1a1 gene in an AHR-dependent manner. This recruitment required the displacement of a histone deacetylase 1/DNA methyltransferase 1 (HDAC1/DNMT1) complex from the Cyp1a1 promoter, resulted in specific histone modifications, and was associated with increased Cyp1a1 transcription (Schnekenburger et al., 2007a, 2007b). MC, TCDD and β-naphthoflavone (βNF) were later shown to induce similar histone modifications in Hepa-1 cells (Ovesen et al., 2011). These results shed light on the epigenetic mechanisms that may lead to the changes in expression of members of the AHR gene battery upon aromatic hydrocarbon exposure.

Cessation of canonical signalling occurs by two separate mechanisms. Activated AHR can be exported from the nucleus, ubiquitinated in the cytoplasm and then degraded
by the 26S proteasome, leading to termination of AHR signalling. As well, the AHR repressor (AHRR) is a nuclear bHLH/PAS protein that is expressed constitutively in a wide variety of tissues. Its expression is induced upon exposure to aromatic hydrocarbons. The activated AHRR is a transcriptional repressor that may act by competing with AHR for ARNT and DRE binding, decreasing the AHR-dependent induction of target genes. AHRR also acts as a repressor of AHR-mediated transactivation (reviewed by Hahn et al., 2009; Abel and Haarmann-Stemmann, 2010).

1.7 NON-CANONICAL AHR SIGNALLING PATHWAYS

The AHR is involved in crosstalk with various other signalling pathways. Non-canonical signalling pathways involving ER and JunD are detailed below as examples. Interaction with the nuclear factor-kappa B (NF-κB) pathway is detailed elsewhere.

1.7.1 Crosstalk of the AHR with ER

Many toxic effects of aromatic hydrocarbon exposure and AHR activation involve disrupted regulation of the reproductive system. Studies in female rodents have demonstrated anti-estrogenic effects of AHR activation by TCDD, including decreased fertility and decreased incidence of estrogen-dependent uterine and mammary tumour formation (reviewed by Matthews and Gustafsson, 2006; Furness and Whelan, 2009). ERα and ERβ are ligand-activated transcription factors that, upon ligand binding, homodimerize and bind to estrogen response elements (EREs) in the promoter regions of target genes, inducing their transcription. ERα ligands include 17β-estradiol (E2). ERα and AHR likely interact directly, and AHR has been shown to inhibit E2-inducible gene expression. It is thought that this inhibition may be due to the recruitment of ERα by
AHR away from ER target genes, thereby resulting in a reduction in estrogen signalling (reviewed by Matthews and Gustafsson, 2006). Additionally, ERα is thought to be involved in mediating AHR-dependent gene induction, as ERα knockdown decreases TCDD-induced $CYP1A1$ induction (Matthews et al., 2005). Estrogens are also metabolized by TCDD-inducible CYP1A1, CYP1A2 and CYP1B1 enzymes, therefore lowering circulating estrogen levels and resulting in an indirect anti-estrogenic effect (Lee et al., 2003). Additionally, the AHR has been implicated in the co-regulation of ER signalling, as MC exposure has been shown to cause activated AHR to associate with unliganded ERα (Ohtake et al., 2003). The AHR/ERα complex is then recruited to EREs to modulate gene expression. As such, it is clear that AHR is involved in estrogen signalling via a non-canonical mechanism and that modulation of the AHR signalling pathway may be co-regulated by ER.

1.7.2 Role of AHR in JunD Signalling

The AHR is also implicated in cell cycle regulation. Exposure to TCDD was found to increase S-phase specific enzyme activity via the transcription factor JunD in an ARNT-independent manner. JunD then associates with partner protein ATF2 and induces cyclin A. Once activated, cyclin A allows for the cell to enter the S-phase from G1 arrest and results in a loss of contact inhibition, which is an indicator of abnormal cell proliferation and is likely involved in tumorigenesis (reviewed by Dietrich and Kaina, 2010).
1.8 DOWN-REGULATION OF CYP EXPRESSION

Environmental exposures or pathological conditions may result in the suppression of CYP expression (reviewed by Riddick et al., 2003, 2004). Hypoxia, inflammation or infection, and exposure to environmental toxicants reduce the expression of some CYP mRNAs and/or proteins by various mechanisms, as outlined below.

1.8.1 Cellular Response to Hypoxia

Hypoxemia, or a low partial pressure of oxygen in the blood, is a clinically relevant condition that presents in patients with respiratory illnesses such as chronic obstructive lung disease or severe asthma. It is associated with a higher risk of adverse drug-related events in these patients due to altered drug biotransformation. For example, patients with severe asthma demonstrate a reduction in theophylline clearance of as much as 75% compared to healthy subjects. Studies in rabbits have also shown decreased metabolism of drugs such as theophylline and phenytoin in models of hypoxemia. This decreased drug clearance is due in part to the altered expression of XMEs, nuclear factors involved in XME regulation, alterations in various signalling pathways and increased activation of the UPS (reviewed by du Souich and Fradette, 2011).

The cellular response to oxygen deprivation is dependent on HIF. Hypoxia increases the levels of HIF-1α protein, which is unstable and rapidly degraded under normoxic conditions via the action of proline hydroxylases. Upon oxygen depletion, HIF-1α is stabilized and translocates to the nucleus, where it binds ARNT (also called HIF-1β). The heterodimer then binds to hypoxia response elements (HREs) in regulatory regions of various target genes to alter their expression (reviewed by du Souich and Fradette, 2011; Miyata et al., 2011). Changes in gene expression include the up-
regulation of various genes involved in glycolysis, erythropoiesis, angiogenesis and cell proliferation and survival (reviewed by Miyata et al., 2011). However, part of the hypoxic response entails the down-regulation of various genes, including some encoding XMEs. Hypoxia causes the activation of NF-κB and its subsequent nuclear translocation (Oliver et al., 2009). The RelA subunit of NF-κB interacts with the AHR to prevent binding of the AHR:ARNT heterodimer to DREs and preventing up-regulation of the CYP1 family of genes. Additionally, competition between AHR and HIF-1α for their shared obligate binding partner, ARNT, contributes to the inhibition by hypoxia of the induction of CYP1 in response to aromatic hydrocarbon exposure (Chan et al., 1999). Importantly, AHR and ARNT are down-regulated in response to hypoxia (reviewed by du Souich and Fradette, 2011). Additionally, hypoxia alters the expression levels of various nuclear receptors that contribute to the regulation of CYP expression. For instance, CAR was shown to be upregulated in response to hypoxia in rabbits, while PXR expression remained unchanged. GR expression may also be altered depending on tissue type; in human muscle cells and renal tubule epithelial cells, GRα was upregulated after acute hypoxia, whereas a down-regulation was observed after 72 hours of hypoxia in human airway cells (Leonard et al., 2005; Huang et al., 2009). Differential expression of these receptors may influence the expression of downstream CYP genes.

Hypoxia increases protein degradation by inducing expression of genes involved in the UPS, such as ubiquitin B, ubiquitin-conjugating enzymes and ubiquitin ligases. AHR, ARNT, CAR, PXR, GR and HNF-4 proteins, as well as CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C19 and CYP2E1 proteins are targeted by this pathway for degradation during hypoxia (reviewed by du Souich and Fradette, 2011).
1.8.2 Effects of Cytokines on CYP Regulation

In addition to hypoxia, inflammation or infection can also cause a down-regulation of CYP expression. For example, exposure to bacterial endotoxins or induction of sepsis has been shown to cause down-regulation of CYP mRNA and protein levels in rodent models (Crawford et al., 2004; Ghose et al. 2009; Kinloch et al., 2011). Proinflammatory cytokines such as IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) are implicated in this down-regulation in mouse models of inflammation (Ghose et al., 2009; Kinloch et al., 2011). The response to infection in mice includes a down-regulation of CYP4A10, CYP4A14, CYP3A11, CYP3A25, and CYP3A41 mRNA levels and a decrease in CYP2B, CYP2C and CYP3A protein levels in a TNF-α-dependent manner (Kinloch et al., 2011). IL-1β has been shown to cause a decrease in CYP2B and CYP3A protein levels in rat primary hepatocyte cultures (Lee et al., 2009). Proinflammatory cytokines are also implicated in the down-regulation of various nuclear factors, including CAR, PXR and RXRα, all of which are necessary for the regulation of CYP expression. A decrease in levels of these factors may be responsible at least in part for the downstream suppression of CYP expression (Ghose et al., 2009).

The mechanisms of CYP down-regulation in response to proinflammatory cytokines may be transcriptional or post-transcriptional. For example, transcription of the rat CYP2C11 gene is inhibited by exposure to the bacterial endotoxin lipopolysaccharide (LPS) (Wright and Morgan, 1990). The CYP2C11 promoter contains an NF-κB binding site in very close proximity to the transcription start site. NF-κB is activated by proinflammatory cytokines, and the mutation of its binding site within the CYP2C11 promoter results in an ablation of CYP2C11 suppression in response to infectious or
inflammatory stimuli (Baldwin, 1996; Iber et al., 2000). Down-regulation may also occur at the protein level. Protein levels of the rat enzyme CYP2B1 are down-regulated via nitric oxide-dependent and –independent mechanisms. CYP2B1 degradation after exposure to IL-1β was attenuated by inhibition of nitric oxide (NO) synthase 2, which is normally induced in response to infection or inflammation. Additionally, inhibition of the proteasome diminished CYP2B1 protein degradation, suggesting a post-translational mechanism for protein down-regulation (Lee et al., 2008). CYP3A protein was also shown to be down-regulated in an NO-dependent manner in rat hepatocytes after 9 hours of IL-1β treatment; interestingly, protein levels were persistently low after 24 hours of treatment and this more prolonged suppression appeared to be NO-independent. This suggested that two mechanisms may contribute to the overall decrease in CYP3A expression in response to IL-1β (Lee et al., 2009).

1.8.3 Down-regulation of CYPs by Aromatic Hydrocarbons

In response to aromatic hydrocarbon exposure, CYP enzymes have shown patterns of down-regulation in mouse, rat and human. Mouse CYP3A is markedly decreased at the protein level after exposure to a single dose of MC. Additionally, mouse Cyp2d9 is down-regulated at the mRNA, protein, and catalytic activity levels by the same dose of MC (Lee et al., 2006). Like rat CYP2C11, mouse Cyp2d9 is a male-specific hepatic gene whose expression is regulated by pulsatile GH secretion in a manner that is at least partly dependent on JAK2-STAT5b signalling. Rat CYP2C11 is also down-regulated at the mRNA, protein, and catalytic activity levels by in vivo exposure to MC (Yeowell et al., 1987; Shimada et al., 1989; Jones and Riddick, 1996). CYP2C11 mRNA levels were decreased after a single dose of MC in rats, and this down-regulation was
shown by nuclear run-on analysis to be mediated at least in part by a decrease in gene transcription rate (Jones and Riddick, 1996; Lee and Riddick, 2000). Treatment of cultured primary rat hepatocytes with various PAHs caused a decrease in CYP2C11 protein levels, and the degree of suppression correlated with the affinity of a given PAH for the AHR and the ability of the PAH to activate the AHR, suggesting that the mechanism of down-regulation involves the AHR (Safa et al., 1997). Further work with primary rat hepatocytes showed that the down-regulation of CYP2C11 mRNA by TCDD occurred without a change in the half-life of the CYP2C11 mRNA, providing further support for a transcriptional mechanism (Bhathena et al., 2002). Binding of activated AHR to DREs within the proximal 2.4-kb region of the CYP2C11 5’-flank was characterized but luciferase reporter plasmids harbouring these regions were not down-regulated by TCDD in hepatoma cells or primary rat hepatocytes (Bhathena et al., 2002). Further studies examined an extended stretch of the CYP2C11 5’-flanking region, up to 10 kb. Luciferase reporter constructs under control of these larger regulatory regions showed a paradoxical induction in response to TCDD and MC that was AHR-mediated, DRE-dependent and unique to human HepG2 cells (Sawaya and Riddick, 2008a). Expression of these extended CYP2C11-luciferase reporter plasmids was down-regulated by MC in rat liver, following the use of high volume tail vein injection to deliver plasmid to the liver of live rats (Sawaya and Riddick, 2008b). Thus, the negative transcriptional response of the CYP2C11 gene to MC exposure may be mediated by a mechanism more complex than that governing the induction of CYP1A1 in response to the same stimulus. The in vivo context seems to be critical for observing transcriptional suppression of CYP2C11-luciferase constructs by aromatic hydrocarbons. Additionally, the response of
CYP2C11 to PAH exposure appears to be tissue- or organ-specific, as the down-regulation is not observed in cardiac tissue from rats exposed to MC or benzo[a]pyrene (Aboutabl et al., 2009).

Several mechanisms have been reviewed by Clark and Docherty (1993) and Ogbourne and Antalis (1998) to explain direct mechanisms of negative regulation of genes in general terms. Suppression may occur via silencer elements upstream of target genes that are capable of binding specific transcription factors. An example of this type of down-regulation is the regulation of dorsal switch protein, which binds a specific site in the promoter region of Drosophila zernullt genes. In doing so, it displaces transcription factor IIA (TFIIA), which is required for the recruitment of RNA polymerase to the promoter. This results in a suppression of gene expression (reviewed by Ogbourne and Antalis, 1998). Down-regulation may also occur via negative regulatory elements (NREs). These may be regions that flank other regulatory sequences, recruit transcription factors and block the interaction of other regulatory elements that are required for transcription. They may also recruit factors or repressors that hinder the recruitment of critical transcription factors or RNA polymerase, by either binding physically to a regulatory site or by introducing bends into the DNA structure that block recognition of relevant proteins (Ogbourne and Antalis, 1998). Clark and Docherty (1993) also review the following proposed mechanism of down-regulation, termed “squelching”. This is a process in which the transcription factors required to initiate transcription are sequestered by other factors to prevent signalling.

Any of these potential mechanisms may play a role in the down-regulation of genes such as rat CYP2C11 by aromatic hydrocarbons. Two silencer regions upstream of
the gene have been identified, and it was suggested that induction of CYP2C11 expression by pulsatile GH may involve derepression of these silencers (Strom et al., 1994). GH-activated STAT5b binds to a response element in the CYP2C11 5’-flank and likely regulates CYP2C11 expression as part of a cascade of transcription factors including HNF-3β (Park and Waxman, 2001). Our laboratory showed that MC interferes with the ability of GH to stimulate CYP2C11 expression in hypophysectomised rats (Timsit and Riddick, 2000). Combining in vivo rat studies with experiments in H4IIE rat hepatoma cells, our laboratory showed that MC does not alter GH-stimulated STAT5b phosphorylation, nuclear uptake and DNA binding (Timsit and Riddick, 2002). Further studies are required to elucidate the mechanisms and requisite transcription factors and promoter elements that may contribute to the observed down-regulated expression of CYP2C11 and other CYPs by PAHs and HAHs.

Interestingly, two independent research groups recently published findings suggesting that the human enzyme CYP2C8 is also down-regulated by exposure to a PAH. Studies in primary human hepatocytes demonstrated a down-regulation of CYP2C8 mRNA after treatment with MC (8 µM) for 24 hours (Ning et al., 2008) or MC (5 µM) for 72 hours (Richert et al., 2009), although CYP2C8 expression did not seem to be affected by AHR agonists such as βNF, MC and TCDD in HepG2 cells (Westerink and Schoonen, 2007). However, these incidental observations were not explored, quantified or discussed. The importance of CYP2C8 and the importance of a potential down-regulation in its expression are discussed in detail in Section 1.9.
1.9 CYP2C8

The human CYP2C subfamily consists of four members: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Together, these enzymes contribute to the metabolism of 20-30% of all clinically prescribed drugs (reviewed by Totah and Rettie 2005; Chen et al., 2009; Lai et al., 2009). CYP2C8 comprises 7% of the total CYP content of the liver (Shimada et al., 1994). CYP2C8 mRNA has also been detected in the brain, kidney, uterus, mammary gland, ovary, adrenal gland, duodenum and endothelium (Klose et al., 1999; Xu et al., 2011). The CYP2C8 gene is 31 kilobases long and consists of nine exons (Klose et al., 1999).

1.9.1 Regulation of CYP2C8 Expression

CYP2C8 is expressed constitutively at low levels in primary human hepatocytes, although it is inducible by a range of xenobiotics, which effect transcriptional change via various transcription factors (Raucy et al., 2002; Ferguson et al., 2005; Chen et al., 2009). These transcription factors include PXR, CAR, HNF-4α, GR and retinoid-related orphan receptor α (RORα) (Ferguson et al. 2005; Chen et al. 2009; Rana et al., 2010). A schematic representation of the structure of the promoter and 5′-flanking region of the CYP2C8 gene is shown in Figure 1.3.

Constitutive expression is thought to be mediated largely by HNF-4α. This transcription factor is involved in the expression of liver-specific genes and xenobiotic detoxification, among other processes. Knockdown of HNF-4α in primary human hepatocytes results in a reduction of constitutive CYP2C8 mRNA levels and diminished induction in response to the CYP2C8 inducer rifampicin (Rana et al., 2010). In addition, luciferase constructs containing the CYP2C8 promoter showed increased activity after
Figure 1.3. Known regulatory response elements in the 5'-flanking region of the human CYP2C8 gene. Sites shown are known to be functional response elements. Other putative elements have been identified and have been shown to interact with cognate transcription factors, but these elements have been shown to be non-functional by mutation analysis. Response element sequences are as follows: **CAR/PXR**: 5'-TCAAACCTtgaTGACCC-3' ; **ROR**: 5'-TGACCCACATTT-3' ; **GR**: 5'-TTAAACTggaTGGTTTT-3' ; **HNF-4α**: -181: 5'-GGACAAaAGAACA-3'; -152: 5'-GGGCCaAGTCCA-3'. The transcription start site is shown at +1. Adapted from Chen and Goldstein, 2009.
HNF-4α transfection in HepG2 cells. Two functional HNF-4α binding sites have been identified in the CYP2C8 promoter (Chen and Goldstein, 2009; Rana et al., 2010).

RORs may also be involved in the regulation of constitutive CYP2C8 levels (Chen et al., 2009). RORα and RORγ are nuclear factors whose ligands include cholesterol and its derivatives. Transfection of RORα and RORγ caused induction of CYP2C8 mRNA in primary human hepatocytes and increased the activity of CYP2C8-luciferase constructs in HepG2 cells. Knockdown of RORα and RORγ resulted in a decrease of endogenous CYP2C8 mRNA levels in HepG2 cells. Endogenous ROR ligands may therefore have an effect on the expression and downstream functionality of CYP2C8 (Chen et al., 2009).

CYP2C8 is also inducible by xenobiotics, such as rifampicin, phenobarbital, dexamethasone and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO), which are activators of various nuclear factors. PXR/CAR- and GR-responsive elements have been identified as the sites in the 5’-flanking region of CYP2C8 through which these compounds exert their inductive effects. Rifampicin is a ligand of PXR, phenobarbital and CITCO are CAR activators, and dexamethasone is a ligand of GR. HNF-4α- and ROR-responsive elements have also been identified (Ferguson et al., 2005; Chen et al., 2009; Rana et al., 2010).

While HNF-4α and RORs appear to be involved in the regulation of CYP2C8 in both primary human hepatocytes and HepG2 cells, induction responses are not necessarily preserved across cell types. For example, rifampicin, phenobarbital and low doses of dexamethasone induce CYP2C8 expression in primary human hepatocytes via PXR, CAR and GR, respectively (Gerbal-Chaloin et al., 2001). CITCO also induces
CYP2C8 expression via CAR in these cells (Ferguson et al., 2005). Luciferase constructs containing the CYP2C8 promoter were also induced in response to rifampicin and CITCO in primary human hepatocytes. In contrast, CYP2C8-luciferase constructs were not induced in HepG2 cells under the same treatment conditions, while mild induction was observed after GR transfection and dexamethasone treatment (Ferguson et al., 2005). These results suggest that CYP2C8 expression in HepG2 cells is not inducible by PXR and CAR ligands, despite their effectiveness as inducers in primary human hepatocytes. The differences in induction patterns between cell models suggest that HepG2 cells may not adequately express the factors required for induction by all xenobiotics (Ferguson et al., 2005).

At this time, there are no known reports in the literature addressing any potential relationship between the AHR and CYP2C8 expression.

1.9.2 Endogenous Substrates of CYP2C8

The activity of CYP2C8, along with CYP2C9, is important for the conversion of an endogenous substrate, arachidonic acid, to biologically active EETs, primarily 11,12-EET and 14,15-EET. These molecules are involved in various cell processes that include the activation of ion channels and transcription factors, induction of the COX-2 pathway, and vasodilation (reviewed in Xu et al., 2011). The inhibition of renal CYP activity increases blood pressure in rats that are given a high-salt diet, and endothelial expression of human CYPs lowers blood pressure in hypertensive mice (Makita et al., 1994; Lee et al., 2010). The extrahepatic expression of CYP2C8 in these tissues suggests a key role for this enzyme and its metabolites in the regulation of blood pressure. Additionally,
EETs are known to be important in the processes of hepatic glycogenolysis, peptide hormone secretion, and the inflammatory response (reviewed by Lai et al., 2009).

CYP2C8 is also involved in the conversion of all-trans-retinoic acid (atRA), the active form of vitamin A, into 18-hydroxyretinoic acid, 4-oxoretinoic acid and 5,6-epoxyretinoic acid (Marill et al., 2000). The metabolites of atRA are involved in cell differentiation, apoptosis, and cell growth inhibition and may therefore be important in the prevention of tumour formation (reviewed by De Luca, 1991; Gudas et al., 1994; Bushue and Wan, 2010).

1.9.3 Drug Substrates of CYP2C8

CYP2C8 contributes to the metabolism of 5-8% of all drugs cleared by Phase I reactions (Klose et al., 1999). It is a minor contributor to the metabolism of drugs such as carbamazepine, clozapine, phenytoin and tamoxifen and a moderate contributor to the metabolism of ibuprofen, fluvastatin, morphine and methadone. However, CYP2C8 is a major contributor to the metabolism of several clinically important drugs, such as the antimalarial drugs amodiaquine and chloroquine (reviewed by Totah and Rettie, 2005; Lai et al., 2009). An understanding of the metabolism of these drugs is critical for the avoidance of drug-drug interactions or adverse events. For instance, the contribution of CYP2C8 to the metabolism of repaglinide, an antidiabetic agent, is estimated to be 60-90% (Bidstrup et al., 2003; Lai et al., 2009). Inhibition of CYP2C8 was shown to increase the area under the concentration-time curve (AUC) of repaglinide, enhancing the lowering of blood sugar levels, thereby increasing the risk of hypoglycaemia in vivo (Niemi et al., 2004; Kajosaari et al., 2006). Other antidiabetic agents, such as rosiglitazone and pioglitazone, are also primarily metabolized by CYP2C8 (Baldwin et
al., 1999; Jaakkola et al., 2006). Inhibition of CYP2C8 was shown to elevate the AUC of these agents \textit{in vivo}, thereby potentially increasing the risk of dose-related adverse events (Hruska et al., 2005; Jaakkola et al., 2005).

The antineoplastic agent paclitaxel (taxol) is also primarily metabolized by CYP2C8 (Rahman et al., 1994). It is a therapeutic agent that is used against a variety of malignant tumours in the breast, lung, and ovary among others. Side effects of paclitaxel are dose-dependent and may include serious adverse events such as neuropathy and neutropenia. However, the incidence and severity of these adverse effects vary considerably from patient to patient (Henningsson et al., 2005). Its metabolic pathway in humans yields 6α-hydroxytaxol, a compound that is 30-times less cytotoxic than the parent compound, and as such is considered pharmacologically inactive (Harris et al., 1994; Rahman et al., 1994). The formation of 6α-hydroxytaxol is considered to be the prototypical probe for CYP2C8 enzyme activity, as formation of this metabolite does not arise through other metabolic pathways. Adverse effects of paclitaxel therapy include myalgia, peripheral neuropathy, nausea, vomiting and alopecia. The incidence of these toxicities is related at least in part to plasma concentration of the parent drug and as such, any changes in CYP2C8 expression or activity could have clinical importance with respect to paclitaxel-related toxicity (Nakajima et al., 2005).

1.9.4 Pharmacological Inhibition of CYP2C8

Inhibition of CYP2C8 enzyme activity has clinically relevant consequences. It is inhibited by various compounds, including the antiasthmatic agent montelukast and the lipid-lowering drug gemfibrozil, a drug that is commonly administered to patients with Type II diabetes or high cholesterol (reviewed by Totah and Rettie, 2005; Jacobson,
Gemfibrozil was found to increase the AUC of both rosiglitazone and repaglinide separately *in vivo* (Niemi et al., 2003a, 2003b), as well as statins such as atorvastatin (Whitfield et al., 2011). Importantly, coadministration of gemfibrozil was shown to dramatically inhibit the CYP2C8-mediated metabolism of cerivastatin in human liver microsomes as well as *in vivo*; the AUC of cerivastatin was shown to increase when gemfibrozil was administered concomitantly, to a maximum increase of 10-fold (Wang et al., 2002; Backman et al., 2002). Cerivastatin was removed from the market after the incidence of adverse events was found to be too high. Nearly 500 adverse events were reported in four years, including 52 deaths, and many of these events were associated with concomitant gemfibrozil therapy (Farmer, 2001; Lai et al., 2009).

Recently, *in vivo* studies have shown that in healthy volunteers, a single dose of gemfibrozil is sufficient to decrease the clearance of repaglinide as little as one hour after the gemfibrozil dose (Honkalammi et al., 2011). It is therefore clear that down-regulation of CYP2C8 may have clinical consequences for patients using prescription drugs with potential for adverse effects or toxicity, as inhibition of the activity of this enzyme has been shown to result in altered pharmacokinetics leading to adverse events, toxicities, or death.

**1.9.5 Pharmacogenetics of CYP2C8**

The human CYP2C8 gene is polymorphic, with at least 14 alleles identified to date (Gao et al., 2010). The functional activity of human CYP2C8 demonstrates a high degree of interindividual variation with respect to the metabolism of important therapeutic substrates (Rowbotham et al., 2010). Studies have estimated the range of mRNA expression among human livers to be 44-fold and the difference in protein levels
to be 33-fold (Naraharisetti et al., 2010). These differences in expression and activity are important when evaluating interpatient differences in drug efficacy and toxicity, particularly for those drugs with a narrow therapeutic index (Henningsson et al., 2005). For example, 38-fold variability in disposition has been observed among patients receiving paclitaxel therapy (Gao et al., 2010) Given the differences in mRNA expression, protein levels and catalytic activity, the polymorphisms that occur were suggested to affect the metabolism of CYP2C8 substrates.

Although 14 alleles have been identified, the most commonly studied alleles are the *2, *3, and *4 alleles. Of these, the *2 allele is most commonly found in African-American populations at a frequency of 18%, while the *3 allele is present in Caucasians at a frequency of 13% and in African-Americans at a frequency of 2%. The *4 allele is present mostly in African-Americans but does present in Caucasian populations with a frequency of 4% (Gao et al., 2010). Studies have been carried out in various systems in vitro as well as in vivo to determine the effects, if any, of CYP2C8 genotype on its catalytic activity, but such studies have yielded contradictory and inconclusive results.

The *3 allele was associated with decreased metabolism of paclitaxel and arachidonic acid in vitro (Dai et al., 2001; Soyama et al., 2001; Bahadur et al., 2002; Gao et al., 2010) but this decrease in functional activity was not observed in an in vivo study examining heterozygous *3 carriers (Henningsson et al., 2005). However, the impact of *3 allele homozygosity may not have been representative in this study due to the scarcity of homozygous carriers; therefore, it is possible that with a greater sample size a functional defect could be observed in *3 homozygotes (Henningsson et al., 2005).
In vivo studies are also inconclusive on the effect of the *3 allele on the clearance of other drugs, such as repaglinide. Repaglinide is an antidiabetic agent that is metabolized by CYP2C8 whose AUC increased 700% after inhibition of CYP2C8 with gemfibrozil (Tomalik-Scharte et al., 2011). For instance, the *3 allele was found to increase repaglinide clearance in vivo in heterozygous carriers while other groups identified no differences in repaglinide clearance in either heterozygous or homozygous *3 carriers (Niemi et al., 2003c; Bidstrup et al., 2006, Tomalik-Scharte et al., 2011). As such, the effects of this allele on drug clearance have not yet been clearly identified.

In vitro studies have also identified differences in the metabolism of endogenous CYP2C8 substrates, such as the conversion of arachidonic acid to 11,12-EET and 14,15-EET, compounds with critical roles in glycogenolysis, hormone secretion and vascular health. It was found in vitro that CYP2C8*3 demonstrated a 26-45% lower rate of arachidonic acid metabolism into 11,12-EET, 14,15-EET and HETEs compared to the wild-type CYP2C8*1. However, an in vivo study of hypertensive patients found no association between presence of a *3 allele and a tendency towards hypertension, a cardiovascular disease in which altered production of EETs may play a role (Teh et al., 2010).

As such, any association between CYP2C8 genotype and functional activity remains unclear. Further studies are required to elucidate this relationship, if any exists. If so, CYP2C8 genotyping may be of benefit in developing individualized therapeutic strategies, particularly with respect to agents associated with a narrow therapeutic index.
1.10 RESEARCH HYPOTHESIS

The preceding information demonstrates the importance of the human enzyme CYP2C8 in the metabolism of therapeutic agents and endogenous substrates that are important regulators of human health. Studies have shown that inhibition of CYP2C8 at the catalytic level may lead to drug-related toxicity. The observations by Ning et al. (2008) and Richert et al. (2009) indicate that the hepatic expression of CYP2C8 may be down-regulated in response to exposure to aromatic hydrocarbons. This could have implications for the metabolism of important endogenous substrates and clinically relevant drugs in individuals exposed to aromatic hydrocarbons through cigarette smoke or industrial sources, for example. The following hypothesis formed the framework for the experimental studies outlined in this thesis.

Hypothesis: Aromatic hydrocarbons down-regulate CYP2C8 expression at the mRNA and protein levels in human hepatocellular carcinoma cell lines.

1.11 SPECIFIC AIMS/OBJECTIVES

This study has four main objectives:

1) To determine the sensitivity of HepG2 and HepaRG cells to the cytotoxic effects of MC.

2) To demonstrate the induction of CYP1A1 mRNA by MC in HepG2 and HepaRG cells as a positive control response.

3) To compare the relative expression levels of CYP2C8 mRNA in HepG2 and HepaRG cells, in comparison to human liver.
4) To determine if MC down-regulates CYP2C8 at the mRNA and protein levels in HepG2 and HepaRG cells.

1.12 EXPERIMENTAL APPROACH AND RATIONALE

My experimental approach is outlined schematically in Figure 1.4.

The potential down-regulation of human CYP2C8 was examined in two human hepatocellular carcinoma lines, HepG2 and HepaRG. Both cell lines are known to have a functional, intact AHR signalling pathway that can be activated by aromatic hydrocarbons (Labruzzo et al., 1989; Roberts et al., 1990; Le Vee et al., 2010; Dere et al., 2011). HepG2 is a well characterized continuous human cell line. Although constitutive levels of CYPs are known to be dramatically decreased in this cell line compared to primary human hepatocytes (Hart et al., 2010), Chen et al. (2009) successfully quantified a down-regulation of CYP2C8 mRNA expression in response to knockdown of ROR-α and ROR-γ using HepG2 cells. In my studies, HepG2 cells were first used as a simple cell culture model in which to attempt the detection and quantitation of CYP2C8 mRNA and protein and any down-regulation in response to MC. As a continuous culture cell line, HepG2 cells represented a simple model for troubleshooting technical issues related to CYP2C8 mRNA detection and quantitation. HepaRG cells were used subsequently to examine the effects of aromatic hydrocarbon exposure on CYP2C8 mRNA levels. HepaRG is a terminally differentiated, non-continuous cell line that is currently being examined as a convenient and cost-effective alternative to primary human hepatocytes, which serve as the current gold standard model for drug metabolism studies. HepaRG cells, which cannot be subcultured, retain higher levels of constitutive CYP expression.
Experimental Approach

Treatment of human hepatocellular carcinoma cell lines with MC (1 or 5 μM) or vehicle (DMSO) for 24 or 48 hours

- Total RNA Isolation (HepG2 and HepaRG)
  - CYP2C8 and CYP1A1 mRNA quantitation via real-time PCR

- Microsome Preparation (HepG2 only)
  - CYP2C8 attempted protein detection via immunoblotting

Figure 1.4. Schematic representation of experimental design.
than HepG2 cells, are more highly differentiated, and are therefore more liver-like, potentially making them a more ideal substitute for primary human hepatocytes than the more convenient continuous HepG2 cell line (Kanebratt and Andersson, 2008). HepaRG cells have an intact AHR gene battery induction response. CYP2C8 expression levels in HepaRG cells are 1-20% of primary human hepatocyte levels and are inducible in response to rifampicin and phenobarbital (Kanebratt and Andersson, 2008; Turpeinen et al., 2009; Antherieu et al., 2010; Dumont et al., 2010; Hart et al., 2010; Lubberstedt et al., 2011).

CYP1A1 and CYP2C8 mRNA levels in HepG2 and HepaRG cells were quantitated using real-time RT-PCR. I attempted to assess CYP2C8 protein levels in HepG2 microsomes using an anti-CYP2C8 antibody via immunoblotting. In all experiments, the induction of CYP1A1 was used as a positive control to confirm intact AHR signalling responses following MC treatment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization of mRNA levels.

The experimental approach is described in detail in Section 2. Briefly, HepG2 cells in the log phase of growth, during which their metabolic activity would be highest, were incubated for 24 hours or 48 hours with a vehicle control, or with a low or high concentration of MC, followed by analysis of CYP1A1 mRNA levels and CYP2C8 mRNA and protein levels. HepaRG cells were also treated with vehicle control or with a low or high concentration of MC for 24 hours or 48 hours after a 7-day culture period designed to maintain differentiated status and maximal basal CYP levels. Changes in mRNA levels of CYP1A1 and CYP2C8 were subsequently examined. Protein levels
were not examined in HepaRG cells due to practical constraints regarding the number of cells required for microsome preparation.

Real-time RT-PCR assays were conducted using gene expression assay kits (Taqman) manufactured by Applied Biosystems Inc. The use of fluorescent SYBR Green I dye is another method of real-time PCR detection and quantitation. According to Applied Biosystems Inc., SYBR Green I offers more sensitive (albeit less specific) detection of gene expression than the proprietary Taqman assay kits. My initial work with HepG2 cells involved attempts to use SYBR Green I for detection of CYP2C8 mRNA, but inconsistent results due to the low constitutive levels of CYP2C8 mRNA in this cell line prompted me to adopt the Taqman probe approach. After attempting CYP2C8 quantitation with the gene expression assay kit (Taqman) from Applied Biosystems, it became clear that the accuracy and precision needed for our experimental purposes would be better achieved using this method, and this is the approach presented in this thesis.

Two methods are commonly used for analysis of real-time PCR data. Data may be analysed with the use of a standard curve relating C_t values to the quantity of input cDNA, or by the use of the comparative C_t (ΔΔCt) method, which requires that the two genes being compared are amplified with similar efficiency. Optimization of the real-time PCR assays outlined in Section 2 included a determination of the efficiency of the real-time RT-PCR reaction for each gene of interest compared to the housekeeping gene. After determining amplification efficiencies and finding them to be highly similar between each gene of interest and GAPDH, the ΔΔCt method was used for data analysis.
and the calculation of relative changes in CYP2C8 and CYP1A1 mRNA expression after different treatments.

MC was selected for use in these experiments since it is a strong AHR activator and CYP1A1 inducer, it has been used extensively to study CYP2C11 down-regulation in rats, and two recent reports of CYP2C8 suppression in primary human hepatocytes used MC as the ligand (Ning et al., 2008; Richert et al., 2009). MC concentrations of 1 and 5 µM were selected on the basis of known CYP1A1 induction activity in HepG2 cells (Labruzzo et al., 1989; Roberts et al., 1990), CYP2C8 suppression in human hepatocytes (Ning et al., 2008; Richert et al., 2009), and my assessment of cytotoxicity conducted in HepG2 cells that demonstrated high viability after 48 hours of treatment with 5 µM MC.

MC treatment at times of 24 and 48 hours were selected in order to maximize the opportunities to capture both CYP1A1 induction and CYP2C8 suppression. CYP1A1 induction in HepG2 cells is pronounced at 18-24 hours after PAH exposure (Labruzzo et al., 1989; Roberts et al., 1990) and the 24 to 48 hour window is consistent with the experiments reporting CYP2C8 suppression in primary human hepatocytes (Ning et al., 2008; Richert et al., 2009).
SECTION 2: MATERIALS AND METHODS

2.1 SOURCE OF CHEMICALS AND REAGENTS

MC, ß-mercaptoethanol, thimerasol, Ponceau S stain, Trypan blue dye, and TRI reagent were obtained from Sigma-Aldrich (St Louis, MO). Ethidium bromide was obtained from Invitrogen Life Technologies (Carlsbad, CA). RNeasy spin columns were obtained from Qiagen Inc. (Valencia, CA). Dimethyl sulfoxide (DMSO) was obtained from Caledon Laboratories Ltd. (Georgetown, ON).

Cell culture materials were obtained from the following sources. α-Minimum essential medium (α-MEM), trypsin, Williams E medium, GlutaMAX medium supplement and fetal bovine serum (FBS) were Gibco products obtained from Invitrogen Life Technologies (Carlsbad, CA). Phosphate-buffered saline (PBS) was obtained from University of Toronto Media Preparation Services. Supplements for the seeding and maintenance of HepaRG cells were obtained from BioPredic International (Overland Park, KS). HepaRG cells were plated on 24-well collagen I-coated plates obtained from BD Biosciences (Bedford, MA).

Reagents used in RT-PCR were obtained from the following sources. Deoxyribonuclease (DNase) I was obtained from GE Life Sciences (Baie d’Urfe, QC). An independent DNase I, Oligo d(T)$_{18}$, RNase inhibitor, and a dNTP set (100 mM each) were obtained from Fermentas Inc. (Glen Burnie, MD). Moloney murine leukemia virus (MMLV) reverse transcriptase, 5x first strand synthesis buffer, 100 mM dithiothreitol (DTT), Taq polymerase, and 10x PCR buffer were obtained from Invitrogen Life
Technologies (Carlsbad, CA). Primers for GAPDH were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Real-time PCR assays were carried out using Taqman Gene Expression Assay kits and gene expression master mix from Applied Biosystems, Inc. (Foster City, CA). Nuclease-free water was a Gibco product obtained from Invitrogen Life Technologies (Carlsbad, CA).

Polyclonal anti-human CYP2C8 antibody was obtained from GeneTex, Inc. (Irvine, CA). Anit-rabbit IgG horseradish peroxidise conjugate, enhanced chemiluminescence detection reagents and Hybond ECL nitrocellulose membrane were obtained from GE Life Sciences (Baie d’Urfe, QC).

Human liver microsomes and human liver RNA samples were generously provided by Dr. Rachel Tyndale (Department of Pharmacology and Toxicology, University of Toronto).

2.2 HepG2 CELLS

2.2.1 Continuous Culture of HepG2 Cell Line

The human hepatocellular carcinoma HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA). All cell culture was carried out under sterile conditions in the SterilGard Class II hood (The Baker Company, Inc., Sanford, ME). Cells were cultured in monolayer in 75 cm² (T-75) flasks obtained from BD Falcon (Bedford, MA). HepG2 cells were cultured in α-MEM medium supplemented with 10% FBS (α-MEM/10% FBS) and were maintained at 37°C in a humid atmosphere composed of 95% air and 5% CO₂ in a Forma Series II Water Jacketed CO₂ incubator.
from Thermo Electron Corporation (Merlette, OH). The cells were subcultured at a ratio of 1:5 – 1:10 as follows. Medium was aspirated and cells were rinsed twice with 10 mL warm sterile PBS. Approximately 2 mL of 0.25% trypsin was added to each flask and cells were allowed to detach for ~5 minutes before the trypsinization reaction was stopped by adding ~8 mL of α-MEM/10% FBS. A specific volume (1-2 mL) of the resulting cell suspension was then added to the appropriate volume of fresh α-MEM/10% FBS medium in new T-75 flasks. Medium was replaced twice weekly and cells were subcultured weekly upon reaching ~95% confluence.

2.2.2 Plating of HepG2 Cells for Subsequent MC Treatment

After reaching ~95% confluence in T-75 flasks, cells were plated into 55 cm² (P-100) cell culture plates as follows. The flask medium was aspirated and cells were rinsed twice with warm sterile PBS, after which ~2 mL of 0.25% trypsin was added. Cells were allowed to detach for ~5 minutes after which the reaction was stopped with the addition of ~8 mL of α-MEM/10% FBS. A small aliquot of the cell suspension was removed for cell counting in a hemocytometer to determine the total number of cells. The remaining cell suspension was centrifuged at 1,200 rpm (225 x g) for 10 minutes at 18°C to form a cell pellet in a Beckman Coulter Allegra X-15R (tabletop) centrifuge (Palo Alto, CA). After aspirating the medium, the cell pellet was resuspended in the appropriate volume of α-MEM/10% FBS to reach a final concentration of $10^6$ cells/mL. Cells ($10^6$ in 1 mL) were then dispensed into a series of P-100 plates containing 9 mL of fresh α-MEM/10% FBS.
2.2.3 Assessment of Cytotoxic Effects of MC in HepG2 Cells

To determine the effects of MC treatment on cell viability, a Trypan blue dye exclusion test was performed on HepG2 cells. Cells were plated as described in Section 2.2.2. After culturing for approximately 96 hours, when cells were in the log phase of growth, cells were treated with fresh α-MEM/10% FBS containing MC (0.1, 1, 2.5, 5, or 10 µM) or the vehicle DMSO. A MC dilution series was prepared from a 2 mM stock solution prepared in DMSO. The final DMSO concentration in all plates was 0.5% v/v. After 48 hours of treatment, the treatment medium was aspirated and cells were washed twice with ~10 mL of warm PBS. Approximately 1 mL of 0.25% trypsin was then added for ~3 minutes. Trypsinization was stopped with the addition of 4 mL of α-MEM/10% FBS and the cells were centrifuged at 1,000 rpm (200 x g) for 8 minutes at 18°C. The supernatant was aspirated from each sample and the cell pellet was resuspended in 5 mL of α-MEM/10% FBS. Cell suspension (100 µL) was added to 100 µL of 0.2% Trypan blue dye in PBS. The mixture was incubated for two minutes at room temperature before dead (blue) and viable (unstained) cells were counted in a hemocytometer under light microscopy.

2.2.4 Treatment of HepG2 Cells with MC for Subsequent RNA and Microsome Isolation

After plating, cells were allowed to reach ~60% confluence, estimated by viewing plates under a Nikon TMS-F light microscope, at which time the cells were in the log phase of growth. The medium was aspirated and 10 mL of α-MEM/10% FBS containing DMSO (vehicle control) or MC (1 or 5 µM MC) were added to begin timed treatment of HepG2 cells. The treatment period was 24 or 48 hours. Each treatment experiment was
conducted on three independent occasions. Cells were digitally photographed at each time point.

2.2.5 Isolation of Total RNA from HepG2 Cells

All equipment used in the isolation of RNA was cleaned with RNase Zap from Ambion Inc. (Austin, TX) and 70% ethanol to prevent RNA degradation and contamination of samples by RNase and exogenous DNA. Pipette tips were certified to be nuclease-free, but were autoclaved as an additional precaution. Reagents used were of appropriate purity to be used for RNA experiments.

After 24 or 48 hours of treatment with DMSO or MC (1 or 5 µM), total RNA was isolated from HepG2 cells using the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Treatment medium was aspirated from each P-100 plate and cells were lysed by the addition of 1.5 mL of TRI reagent to each plate. Cells were scraped from the plate and homogenized by passing the lysate through a pipette until the viscosity of the lysate decreased. Cell homogenates were transferred to 2-mL microfuge tubes and 300 µL of chloroform was added to each tube. The samples were mixed by 30 vigorous inversions of the tubes. The samples were incubated at room temperature for five minutes, after which the tubes were centrifuged at 12,000 rpm (12,000 x g) at 4°C for 15 minutes in an Eppendorf 5415C microcentrifuge, resulting in the separation of RNA (upper aqueous phase) from protein (lower organic phase) and DNA (interphase). The aqueous phase containing the RNA was carefully removed by pipette and transferred to new 2-mL microfuge tubes, after which 750 µL of isopropanol was added to each tube to precipitate the RNA. The samples were mixed by 30 vigorous inversions of the tubes. The samples were incubated at room temperature for
five minutes and were centrifuged at 12,000 rpm (12,000 x g) at 4°C for 15 minutes. The supernatant was then removed, leaving an RNA precipitate. The precipitate was washed by the addition of 1.5 mL of 70% ethanol per sample and by gently inverting each tube ten times. The samples were centrifuged at 10,000 rpm at 4°C for five minutes. The ethanol wash was carefully aspirated and the RNA pellets were air-dried for approximately ten minutes. Each pellet was then resuspended in 50 µL of diethylpyrocarbonate (DEPC)-treated water. The tubes were then incubated at 55°C for 15 minutes to solubilize the RNA pellets. DNA contamination was removed by treating each sample with ~140U of DNase I from GE Life Sciences at 37°C for 20 minutes, after which the enzyme was inactivated by sample incubation at 55°C for 15 minutes.

The purity of the resulting RNA was determined by measuring the absorbance of each sample at 260 nm and 280 nm in duplicate using a Beckman DU-65 spectrophotometer (Fullerton, CA). An $A_{260}/A_{280}$ ratio between 1.6 and 1.8 was considered to be acceptable. The RNA yield was calculated from the average $A_{260}$ value for each sample, obtained from duplicate measurements. The RNA samples were stored at -80°C until further use.

2.2.6 Assessment of HepG2 RNA Integrity

The integrity of RNA isolated from HepG2 cells was assessed by agarose gel electrophoresis. Total RNA (1 µg) from each sample was mixed with an appropriate volume of water and 6x RNA loading dye. Each sample was loaded into one lane of a gel consisting of 1% (w/v) agarose and 40 mM Tris-acetate/1 mM EDTA, pH 8.0 (TAE) containing 0.0005% (v/v) ethidium bromide to allow visualization of RNA under ultraviolet (UV) light. The gel was submerged in TAE buffer and connected to the Bio-
Rad Model 200/2.0 Constant Voltage Power Supply set at 65V for 30 minutes to resolve total RNA by size. After resolution, the gel was examined under UV light using the UVP BioDoc-It UV transilluminator and the samples were considered to have adequate integrity if two distinct bands were present, corresponding to the ethidium bromide-stained 28S and 18S ribosomal RNA species.

2.2.7 Reverse Transcription of HepG2 RNA

HepG2 RNA was reverse transcribed to cDNA according to the following procedure. Oligo d(T)$_{18}$ primers (2 µg) were annealed to the mRNA template strands by incubating 1 µg of RNA at 60°C for five minutes using the Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, CT). Primer extension was carried out in a final reaction volume of 40 µL containing RNase inhibitor (80 U), 1x first strand synthesis buffer (50 mM Tris-HCl, pH 8.3/75 mM KCl/3 mM MgCl$_2$), dNTPs (1 mM each), DTT (25 mM), and MMLV reverse transcriptase (400 U). The primer extension was carried out at 37°C for 60 minutes, after which samples were centrifuged to collect condensation and were subsequently stored at -80°C.

2.2.8 Preparation of Microsomal Fraction from HepG2 Cells

HepG2 cells were plated at a density of 10$^6$ cells/P-100 in 10 mL of a-MEM/10% FBS and treated with DMSO or MC (1 or 5 µM) as described in Section 2.2.4. Ten P-100 plates were prepared for each treatment group. Following the appropriate treatment time of 24 or 48 hours, the cells were harvested and microsomes were prepared for use in immunoblotting as follows.

Medium was aspirated and each plate was washed twice with warm PBS. Each plate was incubated with ~1 mL of 0.25% trypsin for ~5 minutes to facilitate cell
detachment. Trypsinization was halted by the addition of ~1 mL of warm α-MEM/10% FBS medium. Cells were collected by pipetting into a cold 50-mL polystyrene tube kept on ice. Cold PBS (~10 mL) was added to the first plate in the treatment group to allow collection of the remaining trypsinized cells, and this solution was used for sequential washes for the remaining nine plates of the treatment group. This process of cell collection was repeated for each treatment group. The final wash solution was added to the pooled cells.

The cells were harvested by centrifugation at 1,200 rpm (225 x g) for 10 minutes at 4°C, after which the supernatant was removed and the pellet was resuspended in ~30 mL of cold PBS. Cells were centrifuged at 1,200 rpm (225 x g) for 10 minutes at 4°C for a second time and the supernatant was removed. The resulting pellet was resuspended in 3 mL of cold HED buffer (25 mM HEPES/1.5 mM EDTA/1 mM DTT, pH 7.4) and kept on ice for 15 minutes to allow the cells to swell. The cells were then homogenized with the use of a Polytron 1200 homogenizer (Kinematica AG, Switzerland) fitted with a 7-mm probe until a smear of the homogenate showed ~90% cell breakage with minimal damage to cell nuclei. Three mL of cold HED2G (25 mM HEPES/1.5 mM EDTA/1 mM DTT/20% glycerol, pH 7.4) buffer was then added to the cell homogenate and the cells were transferred to precooled centrifuge tubes. After balancing, the tubes were loaded into a JA-17 rotor and transferred to a Beckman J2-21M centrifuge (Palo Alto, CA). The cell homogenate was centrifuged at 11,000 rpm (9,000 x g) for 20 minutes at 4°C.

Following this centrifugation, the supernatant was removed and transferred to clean, precooled centrifuge tubes. The supernatant was centrifuged at 40,000 rpm (106,000 x g) for 70 minutes at 4°C in a Beckman L-80 ultracentrifuge. The supernatant
was removed and the resulting pellet was suspended in 300 µL of TGE buffer (10 mM Tris/20% (v/v) glycerol/1 mM EDTA, pH 7.4) and transferred to a 5-mL glass homogenizer tube. The suspension was homogenized ~10 times with a glass pestle and then was transferred into cryotubes, frozen in liquid nitrogen, and stored at -80°C for future use.

2.2.9 Determination of HepG2 Microsomal Protein Content

Protein content was determined using the protocol of Lowry et al. (1951). In brief, a series of bovine serum albumin standards was created, ranging in concentration from 0 mg/mL to 0.4 mg/mL. Each microsome sample (10-20 µL) was added to 480-490 µL of water to make up a final volume of 500 µL. An equal volume of 0.5N NaOH was added at timed intervals, and the samples and standards were incubated at room temperature for 30 minutes. Following incubation, 5 mL of Lowry solution (made up of 100 parts of 2% sodium carbonate, one part of 1% cupric sulphate, and one part of 2% sodium potassium tartrate) was added at timed intervals to all samples and standards. Following a 10 minute incubation at room temperature, 500 µL of 1.0N Folin-Ciocalteu phenol reagent was added to each tube and the tubes were incubated at room temperature for 30 minutes. The absorbance of each standard was read at 670 nm on a Beckman DU-65 spectrophotometer to create a standard curve, after which the absorbance of each sample was read. Final protein concentration was determined by interpolation from the standard curve and by using the dilution factor for each sample.

2.2.10 Immunoblotting of HepG2 Microsomes

HepG2 microsomal proteins were separated by size via sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were cast in the Bio-Rad Mini
Protean II gel apparatus with 0.75 mm combs. The separating gel consisted of 10% combined N,N’-methylenebisacrylamide (Bis) and acrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% SDS; 0.05% ammonium persulfate; and 0.1% N,N,N’,N’-tetramethylethylene-diamine (TEMED). The stacking gel consisted of 4% combined Bis and acrylamide; 0.125 M Tris-HCl, pH 6.8; 0.1% SDS; 0.05% ammonium persulfate; and 0.1% TEMED. A human liver microsome sample was used as a positive control.

HepG2 microsomal samples were diluted to a final concentration of 2.5 µg/µL in TGE buffer and 2x sample buffer (0.125 M Tris-HCl, pH 6.8/20% glycerol/4% SDS/10% β-mercaptoethanol/0.002 % Bromophenol blue) and boiled for four minutes. The human liver microsome sample was prepared in the same manner to a final concentration of 0.5 µg/µL. The gel was fitted into the apparatus and the electrophoresis tank was filled with running buffer (0.025 M Tris-HCl, pH 8.3/0.192 M glycine/0.1% SDS). After cooling samples to room temperature, 10 µL of each sample was loaded into the gel, and the cell was connected to the Bio-Rad Model 200/2.0 Constant Voltage Power Supply and run at 180V for 45 minutes.

Following protein separation, the samples were transferred as follows to a nitrocellulose membrane (Hybond ECL) for the purpose of immunoblotting. The gel was removed from the electrophoresis apparatus and allowed to equilibrate in transfer buffer (25 mM Tris-HCl, pH 8.3/0.192 M glycine/20% (v/v) methanol) for 15-30 minutes. Nitrocellulose membrane, filter paper, and sponges were also allowed to soak in transfer buffer for this time period. A sandwich containing the gel, nitrocellulose membrane, filter paper and sponges was assembled in a locking cassette. This cassette was placed into the Bio-Rad Mini Trans-Blot transfer cell, and the cell was filled with cold transfer buffer.
and an ice pack. The apparatus was connected to the Bio-Rad Model 200/2.0 Constant Voltage Power Supply and was run at 100V for one hour to transfer protein from the gel to the nitrocellulose membrane. Proteins on the nitrocellulose membrane were then visualized using 0.2% Ponceau S stain, which was then removed from the membrane using several rinses of water and TNT buffer (20 mM Tris, pH 7.6/137 mM NaCl/0.1% (v/v) Tween-20). The membrane was blocked overnight with a 5% Blotto solution (5.0 g skim milk powder in 100 mL of TNT) with gentle shaking at 4°C.

Following blocking, the membrane was washed for 45 minutes in several rinses of TNT at room temperature with gentle shaking. The membrane was then incubated with rabbit polyclonal anti-human CYP2C8 antibody, diluted to 1:1000 in 10 mL of Blotto, for two hours at room temperature with shaking. CYP2C8 antibody was removed by washing for 45 minutes in several rinses of TNT at room temperature with gentle shaking, after which secondary antibody (donkey anti-rabbit IgG horseradish peroxidase conjugate) was added at a dilution of 1:1000 in 10 mL of Blotto. The membrane was incubated for one hour at room temperature with shaking. Secondary antibody was removed by washing the membrane for 45 minutes in several rinses of TNT at room temperature with gentle shaking. Protein was then visualized using enhanced chemiluminescence solution (ECL) in an autoradiography cassette. Equal volumes of ECL Detection reagents 1 and 2 were mixed and ~6.5 mL of the mixture was added to each membrane. Incubation with ECL reagents was conducted for one minute. The membrane was then drained of excess reagent, wrapped in Saran wrap, and exposed to Bioflex Scientific Imaging film in a Fisher Scientific autoradiography cassette for five seconds to thirty minutes. The film was developed using the Kodak M35A X-omatic processor.
2.3 HepaRG CELLS

2.3.1 Seeding and Maintenance of HepaRG Cell Line

The human hepatocellular carcinoma-derived HepaRG cell line was obtained from Biopredic International (Overland Park, KS) in a cryopreserved format. Upon thawing at 37°C, cells were resuspended in 5 mL of Williams E medium supplemented with Glutamax and the provided thaw/seed/general purpose medium supplement (TSGP medium). The cells were centrifuged at 368 x g for 2 minutes at 20°C in a tabletop centrifuge to form a pellet. The medium was aspirated and the cells were resuspended in 5 mL of TSGP medium. Cells were seeded at a density of 480,000 cells per well in 24-well collagen I-coated plates in 500 µL of TSGP medium. The medium was replaced with fresh Williams E/Glutamax medium supplemented with the provided maintenance/metabolism supplement (MM medium) one, four, and six days after seeding. On the seventh day following seeding, cells were treated as described below. Cells were digitally photographed throughout the culture and treatment periods.

2.3.2 Treatment of HepaRG Cells with MC

Existing medium was aspirated from HepaRG cells and was replaced with 500 µL of MM medium containing MC (1 or 5 µM) or the vehicle DMSO. A MC dilution series was prepared from a 2 mM stock solution prepared in DMSO. The final DMSO concentration in all wells was 0.5% (v/v). Treatment durations were 24 and 48 hours.

2.3.3 Assessment of Cytotoxic Effects of MC on HepaRG Cells

To determine the effects of MC treatment on cell viability, a Trypan blue dye exclusion test was performed on HepaRG cells. Cells were treated as described in Section 2.3.2. At each time point, the treatment medium was aspirated and cells were
washed twice with warm PBS. A 0.5% trypsin solution (500 µL) was added to each well and the cells were incubated at 37°C for 10 minutes. The plates were tapped gently to complete cell detachment, and trypsinization was stopped with the addition of 500 µL of MM medium containing 20% FBS. The cells were then centrifuged at 170 x g for 5 minutes at 4°C using a tabletop centrifuge. Supernatant was removed and the cells were resuspended in 500 µL of MM medium and mixed thoroughly by pipetting. Each cell suspension (100 µL) was mixed with 400 µL of 0.1% Trypan blue solution in PBS. Cells were then counted in a hemocytometer under light microscopy, and the number of dead (blue) and viable (unstained) cells was determined for each sample. Cell counts were performed in duplicate for each sample. Each treatment was carried out in duplicate.

2.3.4 Isolation of Total RNA from HepaRG Cells

Due to the limited number of cells available, HepaRG total RNA was isolated using the Qiagen RNeasy Mini kit, designed to recover RNA from 10 – 10^7 cells. All buffers used were supplied by Qiagen and all centrifugations were carried out using the Eppendorf 5415C microcentrifuge. All equipment used was treated with RNase Zap to prevent sample degradation by RNase. All pipette tips were certified to be DNase- and RNase-free but were autoclaved as an additional precaution.

After 24 or 48 hours of treatment with DMSO or MC (1 or 5 µM), the treatment medium was aspirated. Cells were lysed in 175 µL of lysis buffer and homogenized by passing 10 times through a 1-mL syringe with a 21-gauge needle. Cell lysates from two wells belonging to the same treatment group were pooled. A 350 µL volume of 70% ethanol was added to each pooled sample to facilitate the binding of RNA to the RNeasy spin column. Each pooled sample (700 µL) was loaded into RNeasy mini spin columns.
The columns were centrifuged at 10,000 rpm (7,500 x g) at room temperature for 15 seconds. The eluent was discarded and 700 µL of Buffer RW1 was added to remove protein contamination. The columns were centrifuged at 10,000 rpm (7,500 x g) at room temperature for 15 seconds. The eluent was discarded and the wash step with RW1 was repeated. After centrifugation, the eluent was discarded and 500 µL of Buffer RPE was loaded onto each column. The columns were centrifuged at 10,000 rpm (7,500 x g) for 15 seconds to remove salt contamination. The eluent was discarded and 500 µL of Buffer RPE was again loaded onto each column and centrifuged at 10,000 rpm (7,500 x g) at room temperature for two minutes to remove trace contamination. The eluent was discarded and the columns were centrifuged at 14,000 rpm (14,000 x g) for one minute to remove any remaining traces of buffer. RNase-free water (30 µL) was loaded onto each column and the columns were centrifuged at 10,000 rpm (7,500 x g) for one minute to elute RNA.

2.3.5 Quantitation and Assessment of HepaRG RNA Purity

HepaRG RNA was quantitated using the Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Purity of the RNA was assessed by measuring the absorbance at 260 nm and 280 nm in duplicate to determine the amount of RNA and protein, respectively. An A$_{260}$/A$_{280}$ ratio between 1.8 and 2.1 was considered to be acceptable, as noted in the Qiagen RNeasy spin column protocol. The RNA yield was calculated from the average A$_{260}$ value for each sample, obtained from duplicate measurements. The RNA samples were stored at -80°C until further use.
2.3.6 DNase I Treatment of HepaRG RNA

Due to discontinuation of the DNase I from GE Life Sciences that I had used in my HepG2 experiments, DNase I from Fermentas was used for treatment of HepaRG samples. All pipettes and equipment were cleaned with RNase Zap and 70% ethanol prior to use to avoid contamination of samples by RNase and exogenous DNA. Pipette tips were certified to be DNase- and RNase-free, but were autoclaved as an additional precaution.

Microfuge tubes containing 220 ng of HepaRG RNA, 1x DNase I buffer (10 mM Tris-HCl, pH 7.5/2.5 mM MgCl₂/0.1 mM CaCl₂), DNase I (1 µL at 1 U/µL) made up to 10 µL with DEPC water were incubated at 37°C for 30 minutes. DNase I was inactivated by adding 1 µL of 50 mM MgCl₂ to the reaction mixture and incubating at 65°C for 10 minutes. Due to the limited yield of HepaRG RNA, the RNA integrity was not routinely assessed after DNase I treatment as was done for HepG2 RNA.

2.3.7 Reverse Transcription of HepaRG RNA

HepaRG RNA was reverse transcribed to cDNA according to the following procedure. Oligo d(T)₁₈ primers (4.5 µg) were annealed to the mRNA template strands by incubating 200 ng of RNA at 60°C for five minutes in a Perkin-Elmer DNA Thermal Cycler 480. A negative control was incorporated by carrying out the reverse transcription reaction using DEPC water and oligod(T)₁₈ primer alone, excluding RNA. Primer extension was carried out in a final reaction volume of 40 µL containing RNase inhibitor (80 U), 1x first strand synthesis buffer (50 mM Tris-HCl, pH 8.3/75 mM KCl/3 mM MgCl₂), dNTPs (1 mM each), DTT (25 mM), and MMLV reverse transcriptase (400 U). A second negative control was incorporated by carrying out the reverse transcription
reaction using RNA from vehicle-treated cells, excluding the MMLV reverse transcriptase in the primer extension stage. Primer extension was carried out at 37°C for 60 minutes, after which samples were briefly centrifuged to collect condensation and subsequently stored at -80°C. The same procedure was used in the reverse transcription of RNA (200 ng) obtained from each of three human liver samples.

2.4 POLYMERASE CHAIN REACTION (PCR)

2.4.1 Conventional PCR Analysis of cDNA

PCR was carried out for all HepG2, HepaRG and human liver cDNA samples to qualitatively confirm successful reverse transcription. cDNA (1 µL from reverse transcription reaction) was added to a reaction mixture consisting of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 9 mM MgCl₂, 1.6 mM of each dNTP, 0.20 µM of forward primer, 0.20 µM of reverse primer, and 2.5 U of Taq DNA polymerase made up to 40 µL with double-distilled autoclaved water. Primers were used to amplify a 141-bp region of the human GAPDH gene and were designed by Gresner et al. (2009). Primer sequences and PCR cycling parameters are shown in Table 2.1. Primer specificity was verified by BLAST analysis and thermodynamic properties were evaluated using online tools available through Integrated DNA Technologies (Coralville, IA). Primers spanned an intron-exon junction to avoid amplification of genomic DNA. A negative control consisted of the reaction mix as indicated above, excluding the input cDNA. A positive control was established using 1 µL of cDNA previously generated from RNA isolated from vehicle-treated HepG2 cells. Reactions were carried out in thin-walled 0.2-mL PCR tubes (Axygen) using the PTC-100 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Cycling Parameters</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-CAT GGG TGG AAT CAT ATT GGA -3’</td>
<td>Initial denaturation: 95°C, 3 minutes</td>
<td>141 base pairs</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- GAG TCA ACG GAT TTG GTC GT -3’</td>
<td>Denaturation: 94°C, 30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing: 56°C, 30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension: 72°C, 45 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 repetitions</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final extension: 72°C, 7 minutes</td>
<td></td>
</tr>
</tbody>
</table>

(Gresner et al., 2009)
The PCR cycler lid was heated to 92°C before samples were placed in the cycler and the reaction commenced. After the required number of cycles was completed, the products were cooled to 4°C and were briefly centrifuged to collect condensation. Subsequently, an appropriate amount of 6x DNA loading dye was added to each amplification product to result in a final 1x DNA dye solution. Each diluted sample (10-12 µL) and a 100-bp gene ladder were loaded into individual wells of a 1% agarose gel containing 0.0005% ethidium bromide and samples were run in a BioRad Mini Sub Cell in TAE buffer, connected to a Bio-Rad Model 200/2.0 Constant Voltage Power Supply at 65V for 30 minutes. PCR products stained with ethidium bromide were visualized under a UV light source. Confirmation of product size was carried out by visually estimating band position in relation to the 100-bp gene ladder.

2.4.2 Real-Time PCR Analysis of cDNA

CYP2C8, CYP1A1 and GAPDH were quantitated in cDNA samples originating from HepG2 and HepaRG cells that had been treated with DMSO or MC (1 or 5 µM) for 24 or 48 hours using a real-time PCR assay as follows.

Taqman probe kits were selected from Applied Biosystems to minimize the amplification of genomic DNA. Taqman assays contain forward and reverse primers, Taq polymerase and target-specific probes. The detection of gene expression is based on the following chemistry. A probe that is highly specific to the gene of interest is labelled with a fluorescent reporter dye at the 5’-end and a quencher dye at the 3’-end. When the probe is intact, no fluorescence is emitted from the reporter dye. However, after denaturation of cDNA strands, the primers and probe anneal to their target sequences and Taq polymerase activity amplifies the target sequence. The 5’-nuclease activity of Taq
polymerase cleaves the reporter dye from the probe, causing an emission of fluorescence. As such, increased fluorescent signal indicates a greater abundance of the target mRNA for which the labelled probe is specific.

Real-time PCR reaction conditions were optimized by using various quantities of cDNA with each gene probe to determine which conditions yielded the most ideal threshold cycle ($C_t$) value. $C_t$ values between 20 and 31 were considered ideal. Real-time PCR assays were carried out in 8-tube strips (Applied Biosystems, Foster City, CA). Reactions were carried out in the 7500 Real Time PCR System and analysis of the results was performed using 7500 System SDS Software, v.1.3.1, from Applied Biosystems (Foster City, CA).

In the quantitation of CYP2C8 and CYP1A1 mRNA levels, samples were normalized to mRNA levels of the housekeeping gene GAPDH. The optimized amount of cDNA was added to a reaction mixture containing 1x gene-specific probe, 1x gene expression master mix (Applied Biosystems), and 5 µL of nuclease-free water. cDNA from vehicle-treated cells was used as a normalizing calibrator sample.

In order to assess the relative amount of CYP2C8 mRNA across a variety of sample types, a real-time PCR assay was conducted using three samples each of human liver, HepaRG and HepG2 cDNA.

Reactions were run under standard conditions (Table 2.2). All real-time PCR assays for all samples were conducted in triplicate for each of the three genes and were repeated to ensure consistency. Standard curves were generated for each gene using serial dilutions of cDNA ranging from 0.001 to 25 ng per well. Upon demonstrating that
<table>
<thead>
<tr>
<th>Gene</th>
<th>Applied Biosystems Product Number</th>
<th>Cycling Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8</td>
<td>Hs00946140_g1</td>
<td>Stage 1: 50°C, 2 minutes</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Hs00153120_m1</td>
<td>Stage 2: 95°C, 10 minutes</td>
</tr>
<tr>
<td>GAPDH</td>
<td>4333764F</td>
<td>Stage 3: Step 1 - 95°C, 15 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Step 2 - 60°C, 1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 repetitions</td>
</tr>
</tbody>
</table>
calculations of relative expression performed using both the ΔΔC<sub>t</sub> method and by the relative standard curve method were comparable, the ΔΔC<sub>t</sub> method was used to determine the relative amount of mRNA expression. According to the ΔΔC<sub>t</sub> method, C<sub>t</sub> values for each sample were normalized to GAPDH mRNA (ΔC<sub>t</sub>), and the calibrator sample (ΔΔC<sub>t</sub>). Relative fold change (RQ) was calculated as 2<sup>-ΔΔCt</sup> and the mean RQ for each treatment group was expressed as a percentage of the 24 hour vehicle group.

2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the computer program InStat 3.1a for Macintosh (GraphPad, San Diego, CA). Data are expressed as the mean ± standard deviation (SD). All statistical tests were performed using raw data rather than the percent control data shown in the figures. Comparison across groups was conducted using a repeated-measures design one-way analysis of variance (ANOVA), followed by a post-hoc Newman-Keuls test. A result was considered to be statistically significant if p < 0.05.
SECTION 3: RESULTS

3.1 HYPOTHESIS: Aromatic hydrocarbons down-regulate CYP2C8 expression at the mRNA and protein levels in human hepatocellular carcinoma cell lines.

The effects of aromatic hydrocarbons on human CYP2C8 expression at the mRNA level was examined by exposing HepG2 and HepaRG human hepatocellular carcinoma cell lines to MC. Cytotoxicity studies were conducted to determine MC treatment concentrations that would allow HepG2 cells to retain high viability after 48 hours of treatment. Appropriate concentrations were then utilized in HepG2 experiments assessing CYP2C8 mRNA levels. The induction of CYP1A1 mRNA was measured as a positive control. Similar experiments were conducted in the more highly differentiated HepaRG cell line. Results from these studies demonstrated a down-regulation of mRNA expression and suggested that an examination of CYP2C8 at the protein level should be conducted. As such, a similar experiment was carried out in HepG2 cells to determine any effects of MC on CYP2C8 protein levels in this cell line. In all experiments, cell morphology was assessed via light microscopic examination. The results from these experiments are presented below.

3.2 CELL MORPHOLOGY AND VIABILITY AFTER MC EXPOSURE

Using light microscopy, I monitored the effects of the MC treatment on the appearance and morphology of HepG2 and HepaRG cells at the 24 hour and 48 hour timepoints. MC had minimal effects on the appearance of HepG2 cells as all treatment
groups displayed the typical epithelial morphology of this cell line (Figure 3.1A).
HepaRG cell monolayers displayed a hepatocyte-like cell organization in clusters, and the main observable effect of MC was to decrease the number of attached cells, particularly at the 5 µM concentration (Figure 3.1B). An assessment of the effects of MC on HepG2 cell viability was then conducted to determine concentrations at which cytotoxicity would be minimized while still producing changes in gene expression. This assessment was conducted using the Trypan blue dye exclusion test to determine cytotoxicity after exposure of HepG2 cells to vehicle or MC concentrations of 0.1 µM, 1 µM, 2.5 µM, 5 µM and 10 µM for 48 hours. The results are shown in Figure 3.2A. Viability of cells at each treatment concentration is shown as a percentage of the viability of time-matched vehicle-treated control cells. Treatment of HepG2 cells with 0.1 µM, 1 µM, 2.5 µM, 5 µM and 10 µM resulted in a decrease in viability of 1%, 21%, 34%, 25% and 57%, respectively. Based on this estimation, MC concentrations of 1 µM and 5 µM were chosen for subsequent experiments, as cell viability of at least 75% was maintained after 48 hours of treatment.

A similar assay was conducted by exposing HepaRG cells to vehicle or MC concentrations of 1 µM or 5 µM for 24 hours and 48 hours. The results of this assay are shown in Figure 3.2B. Vehicle-treated cells at 48 hours showed a decrease in viability of 35% compared to vehicle-treated cells at 24 hours. Relative to the vehicle-treated cells at 24 hours, MC (1 µM) decreased viability to 61% and 44% of control at 24 hours and 48 hours of treatment, respectively, whereas MC (5 µM) decreased viability to 32% and 34% of control at 24 hours and 48 hours of treatment, respectively (Figure 3.2B).
Figure 3.1. Light microscopic assessment of HepG2 and HepaRG cell appearance and morphology after MC exposure. Cells were treated with vehicle (0.5% DMSO) or MC (1 μM or 5 μM) for 24 hours or 48 hours. Cells were examined using a light microscope at 400x magnification. (A) HepG2 cells were cultured in P-100 plates and treatment medium consisted of α-MEM/10% FBS containing the indicated MC concentration. (B) HepaRG cells were cultured in 24 well collagen I-coated plates and treatment medium consisted of MM medium (Williams E medium supplemented with Glutamax and additives supplied by Biopredic International) containing the indicated MC concentration.
Figure 3.2. Assessment of cytotoxicity in MC-treated HepG2 and HepaRG cells. Cells were treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Trypan blue dye exclusion was used to determine the number of viable cells remaining. (A) HepG2 cells were treated in the log phase of growth with the indicated concentrations of MC for 48 hours. Each treatment group consisted of three P-100 plates and cell counts were performed in duplicate for each plate. The results are expressed as a percentage of the mean of the 48 hour vehicle control and represent the mean of six determinations in a single experiment. (B) HepaRG cells were treated after 7 days of culture with the indicated concentrations of MC for 24 or 48 hours. Each treatment group consisted of two wells in a 24-well plate and cell counts were performed in duplicate for each well. The results are expressed as a percentage of the mean of the 24 hour vehicle control and represent the mean of four determinations in a single experiment.
3.3 REAL-TIME PCR ANALYSIS OF GENE EXPRESSION

The effect of MC treatment on CYP2C8 and CYP1A1 mRNA levels in HepG2 and HepaRG cells was examined via real-time quantitative PCR using Taqman gene expression assays from Applied Biosystems, Inc. The integrity of the total RNA isolated from these cell lines was assessed by determining the presence of two distinct 28S and 18S ribosomal RNA bands on ethidium bromide-stained agarose gels under UV light. A photograph demonstrating the integrity of RNA obtained from one representative experiment for each cell line is shown in Figure 3.3.

Total RNA for each treatment group from both cell lines was reverse transcribed into cDNA for real-time quantitative PCR analysis. Conventional PCR with GAPDH primers was performed to confirm successful reverse transcription and amplification of the predicted 141-bp GAPDH product. PCR products were resolved on ethidium bromide-stained agarose gels and were visualized under UV light. RNA samples from all HepG2 and HepaRG treatment groups generated a GAPDH product that migrated to a position consistent with the predicted size of 141 bp, as demonstrated by the qualitative assessment shown in Figure 3.4.

Real-time quantitative PCR analysis was carried out on cDNA samples from each treatment group for both cell lines. CYP2C8 mRNA level was normalized to the mRNA level of the housekeeping gene GAPDH. Normalized CYP1A1 mRNA level was also measured as a positive control for the activation of the AHR following MC treatment. The amount of cDNA input was optimized for each gene in each cell line to minimize variation in Ct values for a given amount of starting material. cDNA input of 100 ng, 10 ng, and 1 ng proved to be optimal for the quantitation of CYP2C8, CYP1A1 and
Figure 3.3. Verification of sample integrity for total RNA isolated from HepG2 and HepaRG cells. Total RNA was isolated from cells treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Samples were run on a 1% agarose gel containing ethidium bromide for 30 minutes at 65V. (A) Total RNA (1 µg) isolated from HepG2 cells by the guanidinium thiocyanate-phenol-chloroform extraction method was examined by electrophoresis. Distinct 28S and 18S rRNA bands are clearly visible and there are no smears indicative of RNA degradation. (B) Total RNA (0.5 µg) isolated from HepaRG cells using Qiagen RNeasy mini spin columns was examined by electrophoresis. The 28S rRNA band is clearly visible, but the 18S rRNA is barely detectable under these photographic conditions because of the smaller amount of total RNA run on the gel. There are no smears indicative of RNA degradation.
Figure 3.4. Conventional RT-PCR analysis of GAPDH mRNA in HepG2 and HepaRG cells.
Total RNA was isolated from cells treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Following reverse transcription, PCR was performed using GAPDH primers according to the conditions outlined in Table 1 and PCR products were resolved on ethidium bromide-stained agarose gels. Lane 1 contains a 100-bp DNA ladder. Lane 2 is a positive control. Depicting the PCR product generated from cDNA derived from 25 ng of RNA previously isolated from HepG2 cells treated with vehicle for 24 hours. (A) HepG2 cDNA derived from 25 ng of RNA was used for PCR amplification. (B) HepaRG cDNA derived from 10 ng of RNA was used for PCR amplification. The GAPDH PCR product migration was consistent with the expected product size of 141 bp.
GAPDH mRNA respectively, in HepG2 samples. cDNA input of 10 ng was optimal for
CYP2C8 and CYP1A1 mRNA quantitation and cDNA input of 1 ng was optimal for
GAPDH mRNA quantitation in HepaRG samples. Negative controls were established in
each real-time PCR reaction by including reactions in which cDNA input was replaced
with nuclease-free water to ensure that all reagents and equipment used were free of
DNA contamination. All real-time quantitative PCR assays consisted of 40 cycles. Assay
parameters are detailed in Table 2.2.

The relative quantitation of gene expression levels was calculated as follows. The
cycle number at which target gene amplification (CYP2C8 or CYP1A1) became
exponential ($C_t$) was normalized to that at which GAPDH amplification became
exponential ($\Delta C_t$). This normalized value was in turn normalized to the normalized value
of a vehicle-treated calibrator sample ($\Delta \Delta C_t$). Data analysis was performed on raw data to
determine the mean relative expression (RQ value) and standard deviation of each target
gene across treatment groups. For graphical representation, the RQ value of the target
gene for each sample was then expressed as a percentage of mean RQ value for the 24
hour vehicle-treated control sample.

The results of quantitative assessments of CYP2C8 and CYP1A1 mRNA levels in
HepG2 cells are summarized in Figure 3.5. CYP1A1 mRNA levels were shown to be
significantly induced after treatment with MC at 5 µM. A 424-fold induction was
observed after 24 hours of treatment and a 247-fold induction was maintained after 48
hours of treatment when compared to time-matched vehicle-treated control ($p<0.001$).
CYP2C8 mRNA levels were decreased by 78% in HepG2 cells after treatment for 24
Figure 3.5. Real-time quantitative PCR analysis of CYP1A1 and CYP2C8 mRNA levels after MC treatment of HepG2 cells. Total RNA was isolated from HepG2 cells treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Following reverse transcription, real-time PCR was performed according to the conditions outlined in Table 2.2. (A) CYP1A1 mRNA levels were normalized to GAPDH. The results are expressed as the fold-change over the mean of the 24 hour vehicle control and represent the mean ± SD of three independent experiments. (B) CYP2C8 mRNA levels were normalized to GAPDH. The results are expressed as a percentage of the mean of the 24 hour vehicle control and represent the mean ± SD of three independent experiments. Statistical analysis was performed using a repeated-measures design ANOVA followed by a post-hoc Newman-Keuls test. **p<0.01, ***p<0.001, significantly different from time-matched vehicle control.
hours with MC at 5 µM (p<0.01). This suppression was no longer apparent after 48 hours of treatment with MC at 5 µM.

The results of quantitative assessments of CYP2C8 and CYP1A1 mRNA levels in HepaRG cells are summarized in Figure 3.6. In contrast to HepG2 cells, CYP1A1 mRNA levels in HepaRG cells were induced 75-fold and 67-fold after treatment for 24 hours with MC at 1 µM and 5 µM, respectively (p<0.01). CYP1A1 mRNA levels were induced 59-fold and 72-fold after treatment for 48 hours with MC at 1 µM and 5 µM, respectively (p<0.05). CYP2C8 mRNA levels were not significantly affected by treatment for 24 hours with MC at 1 µM or 5 µM in HepaRG cells, although there was a strong trend for suppression at 5 µM. Basal CYP2C8 mRNA levels in vehicle-treated cells were higher at 48 hours compared to 24 hours (p<0.01). CYP2C8 mRNA levels were decreased by 98% in HepaRG cells after treatment for 48 hours with MC at 5 µM compared to time-matched vehicle control (p<0.001).

When using cell lines, it is informative to compare the level of expression of a gene of interest to that seen in the relevant target organ, in this case human liver. I used real-time PCR to compare CYP2C8 mRNA levels (normalized to GAPDH) in vehicle-treated HepG2 and HepaRG cells to three samples of human liver. cDNA input of 15 ng was used for CYP2C8 quantitation and cDNA input of 1 ng was used for GAPDH quantitation. The results of this comparison are shown in Figure 3.7. CYP2C8 mRNA levels are expressed as a percentage of the mean level measured in human liver. HepaRG samples were found to have CYP2C8 mRNA levels of 16% compared to human liver and HepG2 samples were found to have CYP2C8 mRNA levels of less than 1% compared to human liver.
Figure 3.6. Real-time quantitative PCR analysis of CYP1A1 and CYP2C8 mRNA levels after MC treatment of HepaRG cells. Total RNA was isolated from HepaRG cells treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Following reverse transcription, real-time PCR was performed according to the conditions outlined in Table 2.2. (A) CYP1A1 mRNA levels were normalized to GAPDH. The results are expressed as the fold-change over the mean of the 24 hour vehicle control and represent the mean ± SD of three independent experiments. (B) CYP2C8 mRNA levels were normalized to GAPDH. The results are expressed as a percentage of the mean of the 24 hour vehicle control and represent the mean ± SD of three independent experiments. Statistical analysis was performed using a repeated-measures design ANOVA followed by a post-hoc Newman-Keuls test. *p<0.05; **p<0.01; ***p<0.001, significantly different from time-matched vehicle control. ††p<0.01; †††p<0.001, significantly different from 24 hour vehicle control.
Figure 3.7. Real-time quantitative PCR analysis of CYP2C8 mRNA levels in hepatocellular carcinoma lines in comparison to human liver. Total RNA was isolated from HepG2 and HepaRG cells treated with vehicle (0.5% DMSO). These RNA samples and RNA samples from three different human livers were reverse transcribed and real-time PCR was performed according to the conditions outlined in Table 2.2. CYP2C8 mRNA levels were normalized to GAPDH. The results are expressed as a percentage of the mean for the human liver group and represent the mean ± SD of determinations from three independent samples.
3.4 IMMUNOBLOT ANALYSIS OF CYP2C8 PROTEIN IN MICROSONES FROM HEPG2 CELLS

The down-regulation of CYP2C8 in HepG2 cells was demonstrated at the mRNA level and I therefore subsequently attempted to examine this response at the protein level via immunoblot analysis (Figure 3.8). A human liver microsome sample was used as a positive control, for which an immunoreactive band was observed that migrated to a position consistent with the expected molecular weight of 56 kilodaltons (kDa). In microsomal samples from vehicle- and MC-treated HepG2 cells, the most prominent immunoreactive band displayed a molecular weight that was higher than expected and did not align with the band observed in the human liver microsome sample (Figure 3.8A). Extended film exposure revealed that the anti-CYP2C8 antibody recognized several proteins in HepG2 microsomes (Figure 3.8B). For these reasons, it was not possible to determine CYP2C8 protein levels in HepG2 cells and this analysis was not attempted with HepaRG because of the low cell numbers.
Figure 3.8. Immunoblot analysis of CYP2C8 protein in microsomes from HepG2 cells. Microsomes were harvested from HepG2 cells treated with vehicle (0.5% DMSO) or the indicated concentrations of MC for 24 hours or 48 hours. Each microsomal protein sample (30 μg) was separated by SDS-PAGE and then transferred to nitrocellulose. After incubation of the membrane with primary and secondary antibodies, CYP2C8 immunoreactive protein was detected by enhanced chemiluminescence (ECL). Film was exposed for a period of 2 minutes (A) or 10 minutes (B). Lane 1 contains human liver microsomes (2.5 μg) as a positive control. Numbers on the left side of the blots indicate the size of molecular weight markers.
SECTION 4: DISCUSSION

4.1 SUMMARY OF MAIN FINDINGS

Real-time PCR analysis of gene expression revealed that the treatment of human hepatocellular carcinoma cell lines with MC at 5 µM decreases CYP2C8 mRNA levels. Suppression by 78% was observed in HepG2 cells after 24 hours of treatment, while suppression by 98% was observed in HepaRG cells after 48 hours with a strong trend towards suppression at 24 hours that did not reach statistical significance. CYP1A1 was induced in both cell lines, although the magnitude of induction was lower in HepaRG cells than HepG2 cells. Maximal CYP1A1 induction in HepaRG cells was 75-fold, while that in HepG2 cells was 424-fold. MC appeared to be more toxic to HepaRG cells than HepG2 cells, as established by a Trypan blue dye exclusion assay. HepaRG cells are a more liver-like cell model than HepG2 cells, as CYP2C8 mRNA levels were approximately 16% of levels in human liver, whereas HepG2 cells showed CYP2C8 mRNA levels of less than 1% of human liver. CYP2C8 protein could not be detected in HepG2 microsomes.

4.1.1 Suppression of CYP2C8

Real-time PCR analysis of gene expression requires normalization of the target gene to a housekeeping or reference gene. GAPDH is commonly used as a housekeeping gene in real-time PCR, as is β-actin, microtubulin, or 18S ribosomal RNA. The ideal housekeeping gene is one whose expression does not vary across treatments or time points, such that expression levels of target transcripts are corrected for inter-sample variation and any differences in the values of normalized target genes represent true
differences in target transcript concentration (Pohjanvirta et al., 2006). However, the expression of housekeeping genes has been shown to vary across species and tissue types. For example, TCDD and βNF have been shown to up-regulate GAPDH mRNA levels in mouse hepatoma cells as well as in vivo (Reyes-Hernandez et al., 2009). TCDD was also shown to up-regulate GAPDH mRNA in cultured human keratinocytes (McNulty and Toscano, 1995). In contrast, Pohjanvirta et al. (2006) demonstrated that GAPDH expression in TCDD-treated rats appeared to stay at constitutive levels, while β-actin, glucose-6-phosphate dehydrogenase and aminolevulinic acid synthase 1 mRNA levels were significantly altered. It therefore appears that cell type, treatment conditions and duration may affect the inducibility of GAPDH.

In my studies, GAPDH mRNA levels were used in the normalization of CYP2C8 and CYP1A1 mRNA levels. Fluctuations in GAPDH expression over treatments or time points would result in misleading data regarding the expression of those target genes. For example, an inductive effect of MC on GAPDH mRNA would cause the normalized value of relative CYP2C8 mRNA levels to be lower than expected. However, GAPDH C_t values remained remarkably consistent between treatments and time points across experiments within a given cell type. Importantly, cell death due to the cytotoxicity of MC did not appear to have an effect on the expression of GAPDH in living cells of either cell line studied, suggesting that the suppression observed after MC treatment at 5 µM represents a true down-regulation of CYP2C8 mRNA levels.

4.1.2 Comparison of Effects of MC on HepG2 and HepaRG Cells

The results of real-time PCR analysis on different cell and tissue types demonstrated that HepaRG cells have higher basal levels of CYP2C8 mRNA than HepG2
cells. While both HepG2 and HepaRG cell lines are capable of metabolizing PAHs (Sassa et al., 1987; Le Vee et al., 2010), studies have shown that HepaRG cells express higher basal mRNA levels for CAR, PXR, AHR and several CYPs (including CYP2C8), among other genes (Aninat et al., 2006; Hart et al., 2010). In addition, the metabolic activities of various CYPs, including CYP1A1 and CYP2C9, were shown to be inducible in HepaRG cells by various compounds, including MC and rifampicin, which are ligands of the AHR and PXR, respectively (Aninat et al., 2006). Of note, CYP2C8 mRNA expression was also shown to be modestly up-regulated in response to rifampicin and phenobarbital in HepaRG cells. An induction of CYP2C8 mRNA expression in response to rifampicin is also observed in primary human hepatocytes and in vivo in humans, but no up-regulation is observed in primary human hepatocytes upon phenobarbital exposure (Kanebratt and Andersson, 2008; Antherieu et al., 2010). CYP2C8 activity has been measured in HepaRG cells, while no known reports exist of determinations of endogenous CYP2C8 activity in HepG2 cells (Antherieu et al., 2010). The higher metabolic activity of HepaRG cells may partially explain the lower magnitude fold-induction of CYP1A1 when compared to HepG2 cells; it is possible that MC was metabolized more rapidly in HepaRG cells and that these cells were more sensitive to the toxic effects of MC treatment.

It is possible that the cytotoxicity of MC in HepaRG cells contributed to the difference in magnitude of CYP1A1 induction between HepG2 and HepaRG cells. NF-κB is capable of interacting with AHR and decreasing activation of AHR target genes (Tian et al., 1999; Tian et al., 2003). As detailed in Section 4.3, it is possible that NF-κB was activated to a larger extent in HepaRG cells than HepG2 cells, which could lead to
increased association with AHR. By doing so, it may have interfered with the induction of CYP1A1.

4.2 POTENTIAL TRANSCRIPTIONAL MECHANISMS OF CYP2C8 DOWN-REGULATION

The decrease of CYP2C8 mRNA levels suggests that MC may exert its effects via a transcriptional or post-transcriptional mechanism. A transcriptional mechanism would imply that MC treatment causes a decrease in the rate of transcription of the CYP2C8 gene, while post-transcriptional regulation of the gene could imply that CYP2C8 mRNA stability is decreased after MC treatment, leading to more rapid degradation and lower overall levels of CYP2C8 transcript detection. Several possible mechanisms of down-regulation are detailed below, although many more could be proposed.

4.2.1 Potential Contributions of Epigenetic Modifications

The down-regulation of CYP2C8 could occur via an epigenetic mechanism. Histone proteins are responsible for the organization of DNA into nucleosomes. The epigenetic modification of histone proteins results in activation or suppression of gene transcription (reviewed by Strahl and Allis, 2000). HDAC complexes are responsible for the deacetylation of chromatin and are critical in the silencing of gene transcription, as acetylation of chromatin is associated with gene activation (reviewed by Hayakawa and Nakayama, 2011). As such, activation of HDAC complexes may be a potential mechanism through which CYP2C8 mRNA expression is directly suppressed.

Studies have shown that TNF-α mediates the suppression of phosphoenolpyruvate carboxykinase (PEPCK) expression by recruiting HDAC and silencing mediator for
retinoic and thyroid hormone receptors (SMRT) in an NF-κB-dependent manner (Yan et al., 2007). This recruitment and down-regulation was shown to be mediated by the RelA subunit of NF-κB (Yan et al., 2010). Recruitment of nuclear receptor co-repressor 1 (NCoR) alongside HDAC and SMRT has also been implicated in the down-regulation of gene expression (Suzuki et al., 2010). As outlined in Section 4.3, NF-κB is activated in response to oxidative stress or the release of inflammatory mediators, and both HepG2 and HepaRG cells express certain proinflammatory cytokines. In accordance with the putative mechanism outlined above, either or both of these mediators (TNF-α and NF-κB) may therefore be involved in CYP2C8 suppression via a potential epigenetic silencing mechanism. TNF-α, IL-1 and IL-6 have been shown to be involved in the down-regulation of rodent CYP2C11 and Cyp2c29 (Chen et al., 1995; Sewer and Morgan, 1997; Ashino et al., 2004). It is possible that any cytokines released in HepG2 or HepaRG cells due to MC treatment may activate NF-κB, which could translocate to the nucleus and recruit HDAC and SMRT to inhibit transcription directly. Other possible roles of NF-κB in the transcriptional regulation of CYP2C8 are detailed in Section 4.3.

It is also possible that AHR activation plays a direct role in epigenetic silencing, if such silencing occurs in the CYP2C8 promoter. As demonstrated by Papoutsis et al. (2010), AHR activation and recruitment to the promoter of BRCA-1 results in decreased histone acetylation, increased histone methylation and epigenetic silencing of BRCA-1 mRNA expression. AHR has also been shown to recruit HDAC to the BRCA-1 promoter (Hockings et al., 2006). It is therefore possible that such a mechanism involving activated AHR could be in place in the regulation of CYP2C8 expression as well.
4.2.2 Potential Involvement of AHR and HNF-4α

Transcriptional down-regulation could potentially occur by the following direct, AHR-mediated mechanism. Analysis of the 10-kb region upstream of the CYP2C8 coding region using the MatInspector online software program revealed one putative binding site for AHR:ARNT heterodimer or an AHR-related factor, while simple scanning of this region yielded several sequences matching the invariant core sequence required for AHR:ARNT binding. Activation of the AHR by MC could result in recruitment of AHR:ARNT to putative DREs and subsequent recruitment of co-repressors like negative cofactor 2 (NC2), which directly inhibit the formation of the machinery required to initiate transcription. Hypoxia has been shown to induce the binding of NC2 to the promoters of several genes, resulting in a down-regulation of mRNA levels (Denko et al., 2003). The exact mechanism by which this occurs is unclear. However, it is possible that stressful conditions altering cellular homeostasis could trigger the recruitment of NC2 to the promoter regions of genes. Therefore, it is possible that co-repressors may be involved in the suppression of CYP2C8 in response to aromatic hydrocarbon exposure.

A direct interaction of AHR:ARNT with the CYP2C8 promoter could also disrupt the interaction between the transcription factor HNF-4α and its consensus DNA sequence. HNF-4α is thought to be critical for the constitutive expression of CYP2C8 (Rana et al., 2010). As such, interference with this signalling pathway could result in a decrease in transcription of the gene, leading to lower mRNA levels. It is also possible that MC exerts an indirect effect by altering the transcription of the gene encoding HNF-4α. This transcription factor does not appear to be activated by any ligands, but the promoter
region of the \textit{HNF-4\textalpha} gene may contain responsive elements that allow for down-regulation of mRNA expression, leading to lower protein levels and less constitutive transcription of target genes. MicroRNA (miRNA) regulation may also play a role in controlling the activity HNF-4\textalpha. The mechanism of miRNA regulation is detailed in \textbf{Section 4.4}; in brief, miRNAs can decrease the mRNA stability or protein translation of certain transcripts. Studies in human embryonic stem cell-derived mesenchymal stem cells have shown that the let-7 family of miRNAs may target HNF-4\textalpha (Koh et al., 2010). However, the effects of miRNA are cell-type specific and no studies to date have examined any potential targeting of HNF-4\textalpha by miRNA in liver cells.

\textbf{4.3 POTENTIAL MECHANISM OF CYP2C8 DOWN-REGULATION MEDIATED BY NF-\kappaB}

The following sections describe potential signalling pathways through which NF-\kappaB may alter the expression of \textit{CYP2C8} in response to MC. NF-\kappaB is a transcriptional regulator that is induced in response to oxidative stress and proinflammatory cytokines, among other stimuli. It is involved in the regulation of many genes, including those encoding proinflammatory cytokines, and is composed of a heterodimer of members of the Rel family of proteins, which consists of p50, p52, p65 (RelA), RelB and c-Rel. The most common form of the NF-\kappaB heterodimer is p50-RelA (reviewed by Kabe et al., 2005).

In its inactive form, NF-\kappaB is retained in the cell cytoplasm by one of several members of the family of inhibitors of NF-\kappaB (I\kappaB), which masks a NLS. When phosphorylated in response to a stimulus, I\kappaB is ubiquitinated and degraded, allowing the
NF-κB to translocate to the nucleus to alter the transcription of target genes. One response involves the induction of IκB genes, which helps to terminate NF-κB signalling. The activation of NF-κB up-regulates NOS expression, thereby increasing NO production. NO inhibits NF-κB DNA-binding activity, representing a second mechanism by which NF-κB signalling is terminated (reviewed by Kabe et al., 2005).

4.3.1 Relationship Between Oxidative Stress and Metabolism of PAHs

PAHs are metabolized by CYP1A1, CYP1A2, CYP1B1, CYP3A4 and epoxide hydrolases to form reactive electrophilic species such as diol-epoxides, which then cause damage by forming stable DNA and protein adducts. Additionally, some PAH metabolites, such as o-quinones (formed through the activity of dihydrodiol dehydrogenases or aldo-ketoreductases), are associated with the production of reactive oxygen species (ROS) (Flowers et al., 1997; Penning et al., 1999; Bolton et al., 2000; Shimada, 2006; Song et al., 2011). ROS are markers of oxidative stress and are involved in the stimulation of various signalling pathways, including the inflammatory pathway (Wong et al., 2011). PAH metabolism can therefore cause toxicity by various mechanisms.

4.3.2 Oxidative Stress and Activation of NF-κB

Of particular relevance to the findings of this thesis is the fact that PAH metabolism causes oxidative stress. Exposure to PAHs as a component of fine particulate matter, found in air pollution, has been associated with inflammation and oxidative stress in vitro in a human bronchial cell line and in vivo in mouse lung. Responses included lipid peroxidation, endogenous ROS production and the induction of CYP enzyme and proinflammatory cytokine expression (Nakayama Wong et al., 2011; Riva et al., 2011).
Damage induced by oxidative stress also includes protein oxidation and DNA damage, thereby contributing to mutagenesis, carcinogenesis and cell membrane damage (reviewed by Sies, 1993). As outlined below, the generation of ROS and oxidative stress represents a possible mechanism through which CYP2C8 expression was suppressed in the current study. ROS can be generated directly as a result of PAH metabolism, or indirectly through the induction of proinflammatory cytokines, which stimulate ROS production.

Cellular ROS are involved in the activation of NF-κB by proinflammatory stimuli, such as interleukins and mitochondrial ROS themselves can activate cytoplasmic NF-κB. However, some groups also report a decrease in NF-κB signalling due to ROS (Janssen-Heininger et al., 2009). Nonetheless, H$_2$O$_2$ has been shown to activate protein kinase D, which subsequently phosphorylates IκB kinase, leading to degradation of IκB and activation of NF-κB in HeLa cells (Storz and Toker, 2003; Storz et al., 2004). ROS have also been shown to activate NF-κB in human and rat hepatoma cell lines, as well as murine primary hepatocytes (Lluis et al., 2007; Xiao et al., 2011). DNA damage may also activate NF-κB (Mercurio and Manning, 1999). Activation of NF-κB by other stimuli, such as proinflammatory cytokines, has been shown to cause down-regulation of various rodent CYPs, including rat liver CYP2C11 (Iber et al., 2000; Ashino et al., 2004). It is therefore possible that by inducing oxidative stress via ROS production, MC may activate a similar signalling pathway that results in the down-regulation of human CYP2C8.

Activated NF-κB also induces the expression of various proinflammatory cytokines. Therefore, it is possible that the activation of NF-κB may be implicated in the down-regulation of CYP2C8 by multiple mechanisms. NF-κB may directly interact with
the CYP2C8 promoter region as a negative regulator. For instance, the p65 subunit of NF-κB was shown to interfere with the binding of PXR/RXR to DNA response elements, preventing the transcriptional activation of the CYP3A4 gene in response to the PXR ligand rifampicin (Gu et al., 2006). It is possible that NF-κB, activated by PAH-mediated oxidative stress, may also act as a transcriptional repressor for the CYP2C8 gene.

MC may also exert its suppressive effects indirectly by inducing cytokine production. Both HepG2 and HepaRG cell lines express various proinflammatory cytokines, including TNF-α and IL-6 (Stonans et al., 1999; Lambert et al., 2009; Liu et al., 2011). These cytokines are implicated in the down-regulation of various genes, including rat CYP2C11 and mouse Cyp2c29 (Iber et al., 2000; Ashino et al., 2004). As such, proinflammatory cytokines could potentially also play a role in the down-regulation of CYP2C8. A summary of the potential NF-κB-dependent mechanisms by which CYP2C8 expression may be regulated by MC at the transcriptional level is depicted in Figure 4.1.

4.4. POTENTIAL POST-TRANSCRIPTIONAL REGULATION OF CYP2C8 mRNA LEVELS

CYP2C8 mRNA down-regulation may arise due to a decrease in mRNA stability following MC exposure. One mechanism through which this may occur involves miRNA. MiRNAs are short RNA sequences that associate with target mRNAs through base pairing and form part of the RNA-induced silencing complex (RISC), which can direct endonucleolytic cleavage of target mRNAs, resulting in decreased transcript abundance. The RISC may also promote the association of mRNA with decapping enzymes, which leads to increased transcript degradation (reviewed by Valencia-Sanchez et al., 2006).
Figure 4.1. Potential involvement of NF-κB in the suppression of CYP2C8 mRNA levels following PAH exposure. AHR ligands are metabolized by various CYPs and by dihydriodiol dehydrogenases and aldo-ketoreductases into genotoxic metabolites, and by other enzymes into reactive α-quinones. Genotoxic metabolites form stable DNA adducts and contribute to cytotoxicity and carcinogenesis. The metabolism of α-quinones results in the formation of ROS, which activates NF-κB (p50/RelA). DNA damage can also activate NF-κB. Activated NF-κB induces the expression of proinflammatory cytokines, which are also able to activate NF-κB. Activated NF-κB may be involved in inhibiting the transcription of the CYP2C8 gene, possibly by binding to its response element in a way that prevents the binding of a positive transcriptional regulator (left side) or by recruiting HDAC/SMRT (right side).
MiRNAs have been implicated in decreased CYP1B1 expression (Tsuchiya et al., 2006). In addition, miRNAs have been shown to decrease CYP3A4 expression, both directly by decreasing CYP3A4 mRNA and protein levels, as well as indirectly by decreasing mRNA and protein levels of a transcriptional regulator of CYP3A4, the vitamin D receptor (Pan et al., 2009). MiRNAs are encoded in the introns and exons of coding and non-coding RNA sequences, as well as intergenic regions. Different miRNAs can bind the same mRNA target and each miRNA can bind several mRNA targets. Since sequences encoding miRNAs can be located within protein-coding sequences, regulation of miRNA transcription may be dependent on the factors regulating the transcription of the gene it is found within (reviewed by Hudder and Novak, 2008). MiRNA expression can be up- or down-regulated in response to various stimuli, such as transcription factors like nuclear factor I/A, CCAAT/enhancer binding protein alpha, p53 and c-myc (reviewed by Hudder and Novak, 2008). The transcription factor PXR is also negatively regulated by miRNA (Takagi et al., 2008). TCDD and benzo[a]pyrene exposure only modestly alters miRNA expression patterns in rodent liver, while benzo[a]pyrene caused more dramatic changes in the expression of various miRNAs in mouse lung (Moffat et al., 2007; Halapannavar et al., 2011; Yauk et al., 2011). This suggests that regulatory mechanisms of miRNAs are cell-type specific and implies that modulation of miRNA levels is not likely to play an important role in dioxin-mediated down-regulation of liver gene expression. In contrast, both hypoxia and oxidative stress have been shown to induce the expression of many miRNAs and subsequently down-regulate mRNA levels of their target genes. However, these results were demonstrated in cell lines derived from human ear, colon, breast and esophagus and are therefore not necessarily directly relevant
to liver-derived cell lines (Chen et al., 2010; Wang et al., 2010). A very interesting preliminary report suggests that miRNAs 103 and 107 regulate the expression of CYP2C8, and that increased expression of these miRNAs may be responsible for the low expression of CYP2C8 protein in HepG2 cells compared to primary human hepatocytes (Zhang et al., 2011). Further studies at the miRNA level could be performed to determine what role, if any, these molecules play in the down-regulation of CYP2C8 mRNA levels by PAHs in human liver-derived cell lines.

4.5 POTENTIAL DOWN-REGULATION OF CYP2C8 PROTEIN AND CATALYTIC ACTIVITY

Although CYP2C8 mRNA was shown to be down-regulated in response to MC, no conclusions can be made about protein levels in either of the cell lines studied. A decrease in mRNA levels does not necessarily indicate a decrease in protein or catalytic activity levels, nor is the converse necessarily true. It is also possible that the magnitude of suppression of a specific CYP may vary at the mRNA, protein and activity levels. Work from our laboratory showed that MC caused a 28% down-regulation of CYP2D9 mRNA in mice, and this was accompanied by a 42% decrease in CYP2D9 protein but only a 27% decrease in catalytic activity (Lee et al., 2006). Further studies are required to determine what effects, if any, MC exerts on CYP2C8 protein and catalytic activity levels. These experiments are detailed Section 4.8.
4.6 CLINICAL RELEVANCE OF CYP2C8 DOWN-REGULATION

The down-regulation of CYP2C8 may have important clinical consequences, as demonstrated by several studies documenting the effects of pharmacological inhibition of enzyme activity on the metabolism of various therapeutic substrates. For instance, inhibition of CYP2C8 activity by gemfibrozil led to adverse effects and death in patients concomitantly treated with the CYP2C8 substrate cerivastatin (Farmer, 2001; Lai et al., 2009). Gemfibrozil has also been shown to decrease the metabolism of various other therapeutic substrates of CYP2C8 increasing the risk of dose-related toxicity (Niemi et al., 2004; Kajosaari et al., 2006). It is therefore clear that decreased CYP2C8 activity, which could potentially be brought about via decreased mRNA levels, decreased protein levels, and/or decreased catalytic activity, could have potentially serious consequences for patients using therapeutic agents that are substrates of this enzyme. From the current study, it is not possible to estimate the extent to which CYP2C8 activity could be altered in response to MC treatment. It is possible that protein levels or catalytic activity levels do not decrease to the same extent that CYP2C8 mRNA levels were decreased after MC treatment, perhaps due to compensatory mechanisms that could allow for maintenance of relatively normal functional activity. However, future in-depth exploration of this down-regulation at the mRNA, protein and catalytic levels could illuminate functional consequences of clinical importance.
4.7 LIMITATIONS OF THE CURRENT STUDY

4.7.1 Use of GAPDH as Housekeeping Gene for Normalization of Target Genes

As described previously, there exists much debate about the choice of a suitable reference gene for the normalization of target gene expression in real-time PCR. My selection of GAPDH for this purpose appears to be appropriate since substantial changes in expression were not observed over time or across treatment groups. Small variations in the expression of this GAPDH mRNA in HepG2 or HepaRG cells may occur upon treatment with MC. Given the sensitivity of the real-time PCR technique, this may have had some bearing on the results of these quantitative experiments. For example, a slight down-regulation of GAPDH after MC treatment would lead to an inaccurately low estimate of the magnitude of \textit{CYP2C8} suppression, whereas a slight up-regulation of GAPDH could result in a falsely high estimate of the magnitude of \textit{CYP2C8} suppression.

According to experiments conducted by Applied Biosystems, the manufacturer of the Taqman kits used for quantitation of gene expression in my studies, 18S ribosomal RNA is the best choice out of several endogenous control genes with respect to variability of expression across samples and tissue types. Others have suggested the use of several housekeeping genes in each experiment to ensure data validity (Cook et al., 2009; Cui et al., 2009). The use of GAPDH as a housekeeping gene is a potential limitation in the current study, as slight changes in expression levels may have affected the apparent magnitude of \textit{CYP2C8} suppression. Future studies could include statistical tests to validate the selection of a reference gene from the various genes commonly used for this purpose.
4.7.2 Concentrations of MC and Duration of Treatment

The use of only two time points for treatment presents another limitation of this study. Additional time points would have been helpful in determining the duration of MC’s suppressive effects on CYP2C8 mRNA levels. For example, it is possible that MC at 1 µM exerts a significant effect on CYP2C8 mRNA levels at times earlier than 24 hours in HepG2 cells. Furthermore, only two concentrations of MC were examined. A greater range of concentrations would have provided more information about exposures at which down-regulatory effects may be observed. The current study has demonstrated that MC at 5 µM down-regulates CYP2C8 mRNA levels, but it is not possible to conclude that lower concentrations do not also elicit this response, nor is it possible to draw conclusions regarding the time periods over which such changes occur.

Additionally, the pharmacological and toxicological relevance of the MC concentrations used is unknown. The concentrations used in this study were in accordance with other studies in which CYP1A1 induction was quantitated and in which the AHR activation response has been observed (Labruzzo et al., 1989; Roberts et al., 1990; Le Vee et al., 2010) but their relevance compared to human exposure scenarios for environmentally-relevant PAHs is not known. Experiments examining a wider range of MC concentrations and treatment time points, along with additional PAHs and HAHs, would add to our understanding of the down-regulation of CYP2C8 and could lead to a better estimate of human clinical relevance.

4.7.3 High Degree of MC-Induced Cytotoxicity in HepaRG Cells

The degree of cytotoxicity observed in HepaRG cells constitutes another study limitation. HepaRG cells retained only approximately 35% viability after treatment with
MC at 5 µM at both time points. In contrast, HepG2 cells retained approximately 75% viability under comparable conditions. Importantly, assays of cell viability were conducted only once and as such, no conclusions about standard error or statistical significance can be made. At least four measurements were taken of each treatment group for each cell line; however, these were taken on only one occasion. Additionally, the inclusion of untreated cell cultures could have provided an informative comparison in assessing possible effects of DMSO on cell viability and gene expression.

As outlined previously, there are many regulatory mechanisms that can result in a down-regulation of mRNA levels. It is possible that MC exerts its suppressive effects by different mechanisms or a combination of mechanisms in each cell line. Due to the difference in toxicity between these two cell lines, the suppression of CYP2C8 mRNA may have resulted from different signalling pathways and therefore may not be comparable to each other. For instance, CYP2C8 suppression was observed after 24 hours in HepG2 cells (which demonstrated high viability) and after 48 hours in HepaRG cells (which demonstrated low viability). Different regulatory responses may have been activated in response to different environmental conditions, and these responses may have different kinetics. It is therefore possible that suppression of the same gene could occur at different time points via different mechanisms.

No attempts were made in this study to determine if cells were dying via apoptosis or necrosis. However, these two pathways towards cellular death differ in certain ways. Apoptosis is a highly regulated, programmed cell death, while necrosis is an unregulated process of cell death that occurs under extreme stress, such as hypoxia, oxidative stress, toxicant exposure and ischemia. One of the processes involved in
necrosis is the random degradation of DNA and cell autophagy (reviewed by Syntichaki and Tavernarakis, 2002). Cells swell and disintegrate, releasing their contents into the cellular milieu. The release of proteolytic enzymes and other material is thought to lead to inflammation (reviewed by de Saint-Hubert et al., 2009). Exposure to aromatic hydrocarbons has been shown to induce apoptosis in cell culture. For instance, TCDD induced apoptosis in murine cerebellar cells (Sanchez-Martin et al., 2011). Benzo[a]pyrene and other PAHs were also shown to cause apoptosis in HepG2 cells (Staal et al., 2007). However, since necrosis and apoptosis share certain characteristics, such as the release of ROS and increased mitochondrial membrane permeability, it can be difficult to distinguish between the two mechanisms. The methods currently available cannot determine with absolute certainty the occurrence of one mechanism over the other (reviewed by Kepp et al., 2011). An investigation of the cytotoxicity caused by MC in HepG2 and HepaRG cells could be valuable in further elucidating the mechanism of PAH toxicity in different cell lines. For instance, if cells appear to undergo necrosis at a greater rate than apoptosis after MC treatment, their disintegration and the release of their contents may be implicated in the mechanism of down-regulation of CYP2C8.

4.8 FUTURE DIRECTIONS

4.8.1 Establishment of an Optimal Cell Model for the Study of CYP2C8 Suppression

The demonstration of CYP2C8 suppression in human hepatocellular carcinoma cell lines introduces many potential avenues of exploration for the study of the mechanism and clinical relevance of this phenomenon. However, neither of the cell models used in my work appear to be ideal for these studies.
Primary human hepatocytes are considered the gold standard for liver drug metabolism and toxicity studies, as CYP expression and activity in freshly isolated hepatocytes prior to culture is comparable to levels seen in human liver. However, primary human hepatocytes are expensive to purchase and demonstrate large interindividual differences in CYP activity. As well, the functional activity of primary human hepatocytes is only maintained for a short period of time after they are plated and cultured (reviewed by Lin et al., 2012). HepG2 cells can be cultured indefinitely and have an intact AHR signalling pathway, but drug effects and toxicities may be underestimated due to the low metabolic activity of this cell line (Westerink and Schoonen, 2007). On the other hand, HepaRG cells are more metabolically active and have been shown to have higher basal levels of CYP2C8 mRNA (approximately 16% of primary human hepatocyte levels, according to my data) and functional catalytic activity levels, but they reach their peak CYP catalytic activity after seven days of culture and cannot be subcultured as they are terminally differentiated. The large number of cells that would therefore need to be purchased on an ongoing basis make this cell line somewhat impractical for extensive functional and mechanistic investigations (Kanebratt and Andersson, 2008; Turpeinen et al., 2009; Antherieu et al., 2010). In contrast, treatment of the human hepatoma Huh7 cell line with DMSO has been shown to increase mRNA levels of various CYPs. CYP2C8 mRNA levels that are approximately 1% of primary human hepatocyte levels have been achieved under these conditions and these cells demonstrate an induction of CYP1A1 in response to treatment with MC at 1 µM (Choi et al., 2009). Furthermore, CYP activity levels have been shown to be significantly higher in Huh7 cells than in HepG2 cells, including CYP2C8/2C9 activity, through passages 2-6.
Drug metabolism activities were at their highest between passages 2-4 (Lin et al., 2012). While HepaRG cells express higher basal levels of CYP2C8 mRNA, Huh7 is a continuous cell line, which may make it a better alternative for mechanistic studies and work at the protein level. As outlined in the current study, CYP2C8 protein could not be reliably detected in HepG2 cells and the isolation of microsomes from HepaRG cells could not be undertaken due to financial constraints. The human hepatoma Huh7 line therefore presents possibilities for mechanistic studies, including an examination of CYP2C8 protein levels and functional activities. Future studies should include attempts to identify and quantitate CYP2C8 protein in this cell model.

Once a suitable cell culture model is established, many approaches could be employed to study potential mechanisms involved in the suppression of CYP2C8 by PAHs. Experiments could be performed using actinomycin D to inhibit mRNA synthesis, allowing a determination of the half-life of CYP2C8 mRNA and any changes thereof after MC treatment, thereby elucidating any effects of MC treatment on the stability of the CYP2C8 transcript. In combination with other studies examining the transcriptional regulation of CYP2C8, outlined below, these experiments could distinguish between transcriptional and post-transcriptional mechanisms of suppression.

The use of short interfering RNA (siRNA) in the knockdown of AHR expression or the use of a pharmacological antagonist of the AHR (e.g. CH-223191 and others) in cell culture experiments could address the involvement of the AHR, if any, in the down-regulation of CYP2C8 mRNA levels. Electrophoretic mobility shift assays or chromatin immunoprecipitation could be performed to determine whether AHR physically interacts with any part of the CYP2C8 promoter region and could help identify potential direct or
epigenetic effects of AHR on CYP2C8 expression. Additionally, luciferase reporter plasmids containing the promoter region of the CYP2C8 gene could be constructed and transfected into a suitable cell line. Treatment of these cells with an AHR ligand and quantitation of CYP2C8 promoter activation via measurement of luciferase activity could be used to determine effects of AHR ligands on the CYP2C8 promoter. These experiments could provide valuable insight into the mechanisms underlying the down-regulatory effect of MC that was established in the current study.

Finally, the catalytic activity of CYP2C8 should be carefully studied using high performance liquid chromatography to quantify changes in the 6α-hydroxylation of paclitaxel after MC treatment to determine whether aromatic hydrocarbon exposure has any impact on the functional activity of this enzyme. These studies will contribute to the mechanistic understanding of the regulation of this enzyme and will help to elucidate any functional consequences of down-regulation.

4.8.2 In Vivo Examination of CYP2C8 Suppression

In vivo studies would be valuable in elucidating the consequences of CYP2C8 suppression in the context of a whole animal, potentially providing insight into the physiological and pharmacological consequences of aromatic hydrocarbon exposure on the activity of this enzyme. CYP2C8 is a human-specific enzyme and there currently exists no animal model in which to study CYP2C8 in the liver, but researchers have recently created transgenic mice expressing human CYP2C18/CYP2C19 (Lofgren et al., 2008). The generation of a transgenic mouse expressing functional CYP2C8 under the control of the gene’s normal regulatory elements would be useful in developing an
understanding of the functional impact of aromatic hydrocarbon exposure on CYP2C8 activity.

A second intriguing *in vivo* model involves the use of mice whose livers have been populated with transplanted human hepatocytes. This model retains liver function and stability for at least two months and was designed for use in the study of hepatitis B. Human hepatocytes comprised 15% of the mouse liver (Dandri et al., 2001). More recently, another mouse model with humanized liver was generated in which the human hepatocyte content of the liver was as high as 96% in some animals; MC was shown to induce CYP1A1 and CYP1A2 mRNA expression in these mice (Tateno et al., 2004). This model was also used to study human CYP2C9 activity, demonstrating that the humanized mice are capable of metabolizing S-warfarin in a manner similar to humans (Inoue et al., 2008). CYP2C8 mRNA, protein and catalytic activity have also been detected in these mice, the extent of which depended on the degree of repopulation of the mouse liver with human cells (Katoh et al., 2004). These findings indicate that this model may be a promising *in vivo* system in which to study CYP2C8 expression and function.

Very recently, mice transplanted with human ectopic artificial livers were shown to maintain “humanized” drug metabolism activities for several weeks (Chen et al., 2011), providing another potential animal model in which to study *CYP2C8* suppression. Studies could be undertaken to examine changes in mRNA, protein and activity levels, as well as mechanisms behind these changes and potential cross-talk of signalling pathways involved in the down-regulation of *CYP2C8* following PAH exposure. As such, these whole animal models could provide invaluable insight into the functional impact of *CYP2C8* down-regulation.
4.8.3 Human Clinical and Epidemiological Studies

Little to no epidemiological data exist regarding the effectiveness and/or toxicity of therapeutic substrates that are metabolized by CYP2C8 in humans exposed to higher levels of aromatic hydrocarbons. One easily identifiable source of aromatic hydrocarbon exposure is cigarette smoke. Studies examining rates of incidence of adverse drug-related events in smokers versus non-smokers receiving therapy with paclitaxel, for instance, could shed light on a possible functional decrease in CYP2C8 activity. Paclitaxel, as previously described, is an anti-cancer agent with a narrow therapeutic index. Adverse events are related to the amount of drug circulating in the blood. One clinical study examined survival rates of smoking and non-smoking patients treated with paclitaxel/carboplatin therapy and found that smoking was associated with a lower survival rate two years after treatment (Itaya et al., 2007); of course, survival could be affected by the many negative aspects of smoking that are unrelated to paclitaxel metabolism. Another study examined current and former smokers and addressed paclitaxel-related toxicity in lung cancer patients, but did not stratify the incidence of adverse events by smoking category (Riely et al., 2009). As such, the effect of smoking on the clearance of the CYP2C8 substrate paclitaxel is unclear and should be studied in more detail. These studies could also be conducted using healthy volunteers, both smokers and non-smokers, receiving repaglinide as a CYP2C8 non-invasive probe drug (Honkalammi et al., 2011). Such studies could identify the relationship, if any, between smoking and the pharmacokinetics of a CYP2C8 substrate, potentially contributing to a preliminary understanding of clinically relevant effects of aromatic hydrocarbon exposure on CYP2C8 activity in humans. These studies would add to the body of
knowledge gleaned from studies of pharmacological inhibition of CYP2C8 in humans by

gemfibrozil and could add another dimension of clinical relevance to the findings

presented in this thesis.

4.9 CONCLUSIONS

This thesis describes the quantitation of a marked down-regulation of the mRNA

encoding human CYP2C8, an important drug-metabolizing enzyme, in human

hepatocellular carcinoma cell lines HepG2 and HepaRG in response to MC. My studies

have also confirmed some of the limitations of using these models in the study of

CYP2C8 and I have proposed models that may be better suited for the study of the

suppression of this enzyme.

The down-regulation of a human drug-metabolizing enzyme poses interesting

questions for future studies. Additional investigations should examine the mechanisms

involved in CYP2C8 down-regulation and in vivo and epidemiological studies should be

conducted to uncover the impacts of aromatic hydrocarbon exposure on the metabolism

of drugs and endogenous substrates by this enzyme. There are many pathways through

which CYP2C8 expression may be suppressed, some of which are outlined in preceding

sections. The findings presented here are not sufficient to provide evidence for any

potential mechanisms of CYP2C8 suppression. However, my work lays the basic

foundation for future studies that will investigate the mechanisms and functional

consequences of CYP2C8 down-regulation by aromatic hydrocarbon toxicants.
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