1521-009X/47/3/279–282$35.00
DRUG METABOLISM AND DISPOSITION

https://doi.org/10.1124/dmd.118.084509
Drug Metab Dispos 47:279–282, March 2019

Short Communication

Suppression of Hepatic *CYP3A4* Expression and Activity by 3-Methylcholanthrene in Humanized PXR-CAR-CYP3A4/3A7 Mice

Received September 13, 2018; accepted December 19, 2018

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that activate the aryl hydrocarbon receptor, thereby triggering a range of biologic responses, exemplified by the induction of *CYP1A1*. PAHs can also regulate the expression of members of the *CYP3A* subfamily, with reports of mainly suppressive effects on mouse hepatic *Cyp3a11* expression, but paradoxically both inductive and suppressive effects on human hepatic *CYP3A4* expression. Understanding the regulation of *CYP3A4* expression by PAHs is important because of the widespread exposure of humans to these chemicals and the central role of the CYP3A4 enzyme in the metabolism of clinically important drugs and endogenous substances. The present study used 3-methylcholanthrene (MC) as a model PAH to characterize the in vivo regulation of *CYP3A4* expression and activity in humanized pregnane X receptor–constitutive androstane receptor-CYP3A4/3A7 mice. Adult mice were treated by intraperitoneal injection with MC (80 mg/kg), or corn oil vehicle, and euthanized 24 or 72 hours later. As a positive control response, pronounced induction of hepatic *Cyp1a1* by MC was confirmed at both time points in males and females at the mRNA, protein, and catalytic activity levels. Basal hepatic *CYP3A4* expression and activity were significantly higher in female versus male mice. MC treatment suppressed hepatic *CYP3A4* in female mice at 72 hours postdosing at the mRNA, protein, and catalytic activity levels. A similar response was observed in male mice, although the suppression of CYP3A4 protein levels did not achieve statistical significance. This mouse model will facilitate further studies of the mechanisms and consequences of *CYP3A4* suppression by PAHs.

Introduction

CYP3A4 is among the most important human drug-metabolizing enzymes, due to its abundant hepatic and gastrointestinal expression, broad substrate selectivity, and susceptibility to induction and inhibition (Guengerich, 1999). Although polycyclic aromatic hydrocarbons (PAHs), important carcinogenic constituents of cigarette smoke and other combustion sources, are known to induce multiple cytochrome P450s (P450s) such as *CYP1A1*, *CYP1A2*, and *CYP1B1* via activation of the aryl hydrocarbon receptor (AHR) (Nebert et al., 2004), the regulation of human *CYP3A4* by these chemicals is poorly characterized.

Studies of the regulation of the major *CYP3A4* counterpart in mice (*Cyp3a11*) by 3-methylcholanthrene (MC), a model PAH that is readily biotransformed by P450s (Riddick et al., 1994), have provided valuable insight. Our laboratory showed a pronounced loss of mouse hepatic Cyp3a11 protein triggered by MC treatment (Lee et al., 2006) and subsequently established that the suppression of Cyp3a11 mRNA and protein caused by MC is comparable in wild-type and liver *Cpr*-null mice that are nearly devoid of hepatic microsomal P450 activity due to hepatocyte-specific conditional deletion of NADPH-cytochrome P450 oxidoreductase (Lee et al., 2013a). Thus, MC appears to downregulate mouse hepatic *Cyp3a11* via a pretranslational mechanism that does not require hepatic microsomal P450-dependent activity. Using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin as an essentially nonmetabolized AHR agonist and *Ahr*-null mice, our laboratory demonstrated the AHR dependence of the suppression of hepatic Cyp3a11 mRNA levels (Lee and Riddick, 2012).

Smokers show decreased CYP3A4-mediated metabolism of some (Jokinen et al., 2001), but not all, substrates (He et al., 2006). MC treatment suppresses *CYP3A4* expression and activity in cultured primary human hepatocytes (Richert et al., 2009). Paradoxically, MC exposure increases CYP3A4 mRNA levels in the HepG2 human hepatocellular carcinoma cell line (Westerink and Schoonen, 2007). This induction response was reported to require activation of the human, but not mouse, pregnane X receptor (PXR) (Kumagai et al., 2012), a nuclear receptor with a central role in the transcriptional regulation of *CYP3A4* expression. Further in vitro studies showed that PAHs and their phase I metabolites can activate human PXR (Luckert et al., 2013). More recently, evidence has emerged for bidirectional inhibitory crosstalk between AHR and PXR. First, activated PXR can bind directly to AHR and inhibit AHR binding to, and activation of, specific target genes (Cui et al., 2017). Second, studies in primary human hepatocytes and HepaRG cells showed that AHR activation decreases *CYP3A4* expression and inducibility by PXR activators (Rasmussen et al., 2017).

To overcome limitations associated with wild-type mice and human continuous cell lines in studies of human *CYP3A4* regulation and to attempt to resolve confusion regarding the effects of PAHs on this key enzyme, we studied the regulation of *CYP3A4* expression and activity by MC in an in vivo context with an intact endocrine system using humanized PXR–constitutive androstane receptor (CAR)-CYP3A4/3A7 mice (Hasegawa et al., 2011). In this targeted replacement model, the mouse genes encoding the PXR and CAR are replaced with the human orthologous genes, and seven full-length mouse *Cyp3a* genes located in

This work was supported by the Canadian Institutes of Health Research [Grant MOP-142442].
https://doi.org/10.1124/dmd.118.084509.
This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; GH, growth hormone; MC, 3-methylcholanthrene; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PXR, pregnane X receptor.

a cluster on chromosome 5 (*Cyp3a11*, *16*, *25*, *41*, *44*, *57*, and *59*) are replaced with 125 kb of human genomic DNA comprising *CYP3A4*, *CYP3A7*, and their regulatory regions.

Materials and Methods

Treatment of Humanized PXR-CAR-CYP3A4/3A7 Mice. The University of Toronto Animal Care Committee approved all animal protocols and experiments, following the principles established by the Canadian Council on Animal Care (https://www.ccac.ca/en/standards/). Male and female humanized PXR-CAR-CYP3A4/3A7 mice (model 11585) were purchased from Taconic Biosciences (Hudson, NY), with arrival at the Division of Comparative Medicine, University of Toronto at 4–7 weeks of age. After a period of acclimatization to housing conditions with same-sex littermates (12-hour light-dark cycle with lights on from 7:00 AM to 7:00 PM, ad libitum access to water and food) experimentation commenced when mice were 8–9 weeks of age. Mice received a single intraperitoneal injection of MC (80 mg/kg), or corn oil vehicle, followed by euthanasia by cervical dislocation at 24 or 72 hours after dosing. Livers were processed for total RNA isolation and microsome preparation as described previously (Lee et al., 2006), with microsomal protein determination by the method of Lowry et al. (1951).

Analysis of mRNA Levels by Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction. Hepatic mRNA levels for all target genes, normalized to mouse β-actin as the internal reference standard, were determined in triplicate using the comparative threshold cycle method described previously (Lee and Riddick, 2012). Primer sequences were derived from published sources as follows: mouse Cyp1a1 (Xu and Miller, 2004), human CYP3A4 (Miyoshi et al., 2002), and mouse fibroblast growth factor 21 (Fgf21) (Woolsey et al., 2016).

Immunoblot Analysis. Nitrocellulose membranes containing resolved hepatic microsomal proteins were probed with the following primary antibodies: mouse monoclonal against rat Cyp1a1 (MAb 1-31-2; Dr. Harry Gelboin, National Cancer Institute, Bethesda, MD) (Park et al., 1982) used at a 1:5000 dilution, and mouse monoclonal against human CYP3A4 (MAb 275-1-2; Kristopher Krausz, National Cancer Institute, Bethesda, MD) (Gelboin et al., 1995) used at dilutions of 1:500 (male samples) or 1:1000 (female samples). For relative quantitation of CYP3A4 protein levels, films derived from enhanced chemiluminescence detection were scanned and subjected to densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD), with normalization to total protein as a loading control as determined using Revert Total Protein Stain (Li-Cor Biosciences, Lincoln, NE). Cyp1a1 immunoblots were assessed visually as a qualitative positive control response.

Catalytic Activity Assays. Using our previously reported methods (Lee et al., 2013a,b), modified such that each reaction contained 1 μg of hepatic microsomal protein, P450-Glo assays (Promega, Madison, WI) were used to assess mouse Cyp1a1 activity, with luciferin 6′-chloroethyl ether as substrate, and human CYP3A4 activity, with luciferin isopropyl acetal as substrate (Cali et al., 2012).

Statistical Analysis. The sample size of five mice per treatment group provides 80% power ($\beta = 0.20$) at a significance level of $\alpha = 0.05$, to detect cases where the ratio of the estimated S.D. to the minimum effect magnitude is 0.56 (e.g., a 45% increase or decrease in a measured outcome with a S.D. for that parameter equal to 25% of the mean). For CYP3A4 protein levels, where the marked difference in basal expression between sexes (female > male) necessitated the use of different experimental conditions for each sex, data were analyzed initially using randomized-design two-way analysis of variance to identify the effects of the two independent variables and their interaction (MC treatment; time; treatment × time interaction). All other data were analyzed initially using randomized-design three-way analysis of variance to identify the effects of the three independent variables and their interactions (MC treatment; time; sex; all pairwise two-factor interactions and three-factor interaction). Bonferroni-corrected post tests were performed for the planned comparisons to discern specific MC treatment, time, and sex effects. If Bartlett's test showed significant heterogeneity of variance, specific comparisons of interest were based on the nonparametric Mann-Whitney test.

Results and Discussion

Male and female humanized PXR-CAR-CYP3A4/3A7 mice were exposed to MC under conditions previously shown to downregulate hepatic *Cyp3a11* expression and activity in male wild-type C57BL/6 mice (Lee et al., 2006, 2013a). MC treatment had minimal effects on the liver to body weight ratio, with a 25% increase seen at 72 hours postdosing only in females (Supplemental Fig. 1). As a positive control for AHR activation, MC caused pronounced induction of hepatic *Cyp1a1* at both time points in females and males at the mRNA, protein, and catalytic activity levels (Fig. 1).

Basal hepatic *CYP3A4* expression was higher in female versus male mice at the mRNA, protein, and catalytic activity levels (Fig. 2); the sex difference in CYP3A4 protein levels was so pronounced that male and female samples could not be analyzed under the same conditions. The original publication describing the humanized PXR-CAR-CYP3A4/3A7 mouse model (Hasegawa et al., 2011) did not report a sex difference in hepatic *CYP3A4* expression; however, other *CYP3A4*-transgenic mouse models have consistently found higher *CYP3A4* expression in the liver of adult females versus males (Yu et al., 2005; Cheung et al., 2006; Kobayashi et al., 2017). This parallels the higher expression and activity of *CYP3A4* seen in female human liver (Wolbold et al., 2003) and is attributed to the stimulatory influence of the continuous growth hormone (GH) secretion pattern characteristic of females (Cheung et al., 2006). Several transcription factors are implicated in the female-predominant expression of *CYP3A4* driven by a continuous GH profile: signal transducer and activator of transcription-5 (Lamba et al., 2016); PXR and hepatocyte nuclear factor-4α (Thangavel et al., 2011); hepatocyte nuclear factor-6, CCAAT-enhancer binding protein-α, and retinoid X receptor-α (Li et al., 2015).

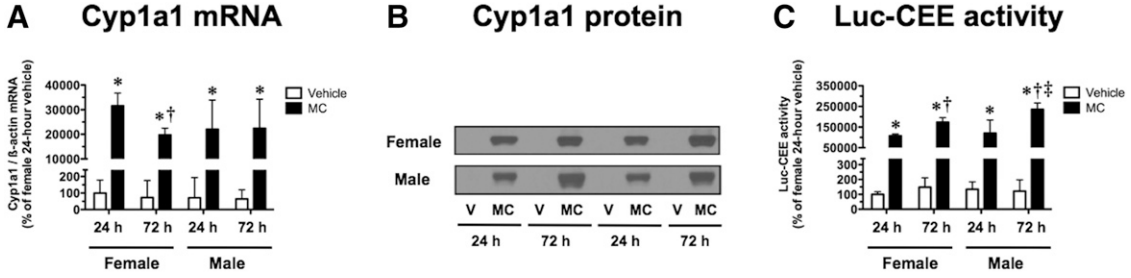

Fig. 1. Effects of MC treatment, time, and sex on hepatic Cyp1a1 mRNA levels (A), Cyp1a1 protein levels (B), and luciferin 6′-chloroethyl ether (Luc-CEE) activity (C). (B) Immunoblot of microsomal protein (5 μg) using MAb 1-31-2 against rat Cyp1a1, showing results for two vehicle-treated (V) or MC-treated mice per time point. (A and C) Data represent the mean ± S.D. of determinations from four (male 24-hour vehicle group) or five (all others) mice per group, expressed as a percentage of the mean for the female 24-hour vehicle group. Data were analyzed initially by three-way analysis of variance (ANOVA), and the *P* values for the ANOVA main effects are shown in Supplemental Table 1. Outcomes from Bonferroni-corrected post tests were as follows: * denotes significantly different ($P < 0.05$) from sex- and time-matched vehicle control; † denotes significantly different ($P < 0.05$) from sex- and treatment-matched 24-hour group; and ‡ denotes significantly different ($P < 0.05$) from time- and treatment-matched female group. The 24- and 72-hour designations indicate the time postdosing with vehicle or MC.

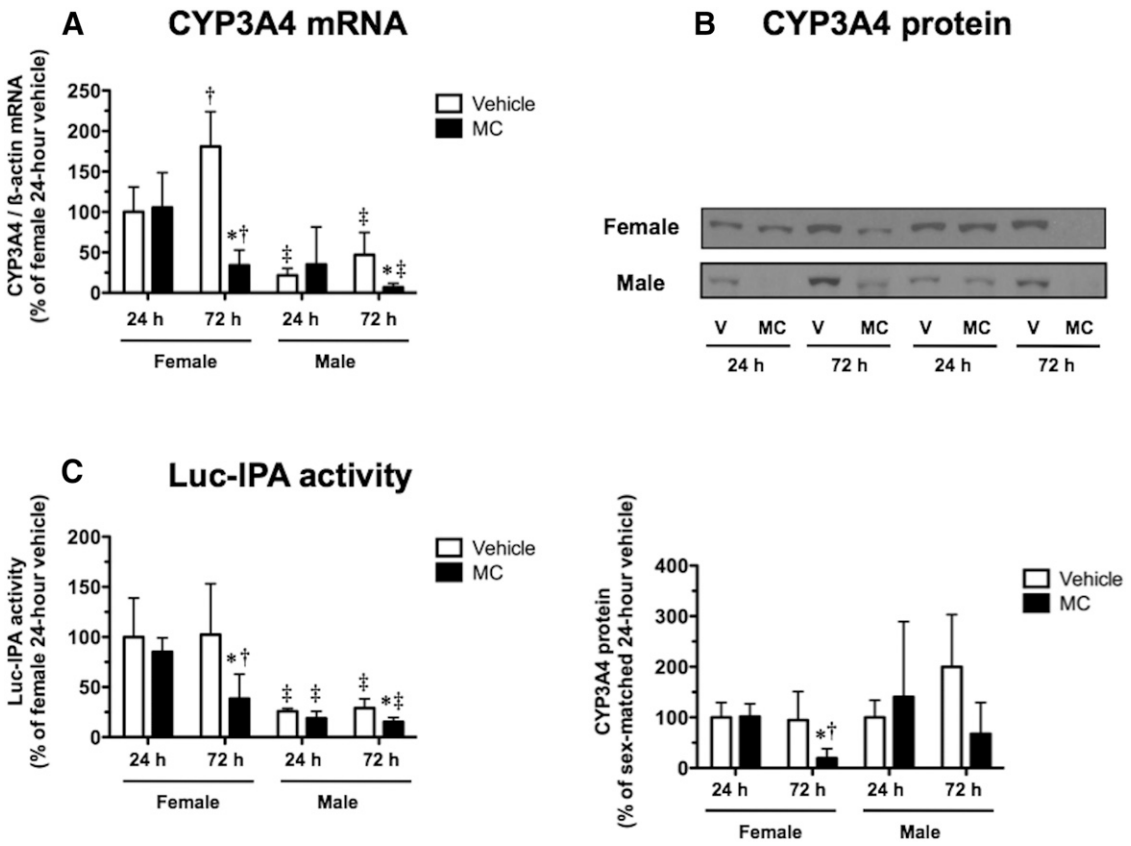

Fig. 2. Effects of MC treatment, time, and sex on hepatic CYP3A4 mRNA levels (A), CYP3A4 protein levels (B), and luciferin isopropyl acetal (Luc-IPA) activity (C). (B) Immunoblot of microsomal protein (5 μg for female and 25 μg for male) using MAb 275-1-2 against human CYP3A4, showing results for two vehicle-treated (V) or MC-treated mice per time point. (A–C) Data represent the mean ± S.D. of determinations from four (male 24-hour vehicle group) or five (all others) mice per group, expressed as a percentage of the mean for the female 24-hour vehicle group (A and C) or the sex-matched 24-hour vehicle group (B). Data were analyzed initially by three-way analysis of variance (ANOVA) (A and C) or two-way ANOVA (B), and the *P* values for the ANOVA main effects are shown in Supplemental Table 1 (A and C) and Supplemental Table 2 (B). Outcomes from Bonferroni-corrected post tests or nonparametric Mann-Whitney tests were as follows: * denotes significantly different ($P < 0.05$) from sex- and time-matched vehicle control; † denotes significantly different ($P < 0.05$) from sex- and treatment-matched 24-hour group; and ‡ denotes significantly different ($P < 0.05$) from time- and treatment-matched female group. The 24- and 72-hour designations indicate the time postdosing with vehicle or MC.

MC treatment suppressed hepatic *CYP3A4* in female and male mice at 72 hours postdosing at the mRNA (Fig. 2A) and catalytic activity (Fig. 2C) levels. The downregulation of CYP3A4 protein levels by MC (Fig. 2B) achieved statistical significance in females but not males, likely because of the very low and variable levels of CYP3A4 protein seen in the liver of male mice. At the protein level, similar results were obtained using an independent primary rabbit polyclonal raised against a peptide specific for human CYP3A4 (product no. 458234; Corning-Gentest, Woburn, MA) at 1:1000 dilution (data not shown). The influence of sex on the developmental profile of hepatic *CYP3A4* expression in humanized PXR-CAR-CYP3A4/3A7 mice has not been reported. In other *CYP3A4*-transgenic mouse models (Yu et al., 2005; Cheung et al., 2006) prepubertal males and females display similar hepatic *CYP3A4* expression. It would be interesting to study the regulation of hepatic *CYP3A4* expression by PAHs in humanized PXR-CAR-CYP3A4/3A7 mice at earlier prepubertal developmental stages, keeping in mind that the sexually dimorphic pituitary GH secretion profiles are not yet established (Gabriel et al., 1992) and hepatic factors needed to impart GH-controlled sex differences in liver gene expression patterns are absent in prepubertal rodents (Choi and Waxman, 2000).

To explore potential mechanistic links between AHR activation and *CYP3A4* suppression, we measured hepatic mRNA levels for the metabolic hormone FGF21. Depending on dose, AHR agonists can either induce (Cheng et al., 2014; Lu et al., 2015) or suppress (Girer et al., 2016) mouse hepatic *Fgf21* expression. Elevated hepatic FGF21 levels can trigger phosphorylation-dependent trapping of PXR in the cytoplasm, leading to decreased PXR-stimulated *CYP3A4* transcription (Woolsey et al., 2016). We found that MC treatment induced hepatic Fgf21 mRNA levels at 72 hours postdosing, but only in male mice (Fig. 3). Although interesting, this result does not support a role for Fgf21 in the suppression

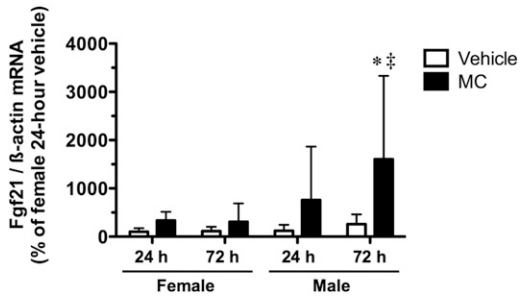

Fig. 3. Effects of MC treatment, time, and sex on hepatic Fgf21 mRNA levels. Data represent the mean ± S.D. of determinations from four (male 24-hour vehicle group) or five (all others) mice per group, expressed as a percentage of the mean for the female 24-hour vehicle group. Data were analyzed initially by three-way analysis of variance (ANOVA), and the *P* values for the ANOVA main effects are shown in Supplemental Table 1. Outcomes from Bonferroni-corrected post tests were as follows: * denotes significantly different ($P < 0.05$) from sex- and time-matched vehicle control; and ‡ denotes significantly different ($P < 0.05$) from time- and treatment-matched female group. The 24- and 72-hour designations indicate the time postdosing with vehicle or MC.

of *CYP3A4* expression by MC, which was observed in both sexes. Levels of two FGF21 target transcripts (c-Fos and early growth response protein 1) were not impacted by MC treatment (data not shown). We also explored other pathways commonly implicated in P450 downregulation responses and MC was found to have no significant effect on the following marker transcripts: endoplasmic reticulum stress (78-kDa glucose-regulated protein; activating transcription factor-4; X-box binding protein-1 spliced); inflammation mediators (interleukin-6 and tumor necrosis factor-α) and markers (suppressor of cytokine signaling-3 and serum amyloid protein P); and peroxisome proliferator-activated receptor-α target (pyruvate dehydrogenase kinase-4) (data not shown).

This study shows that PAH exposure in an in vivo context results in suppression of hepatic *CYP3A4* expression and activity. Importantly, this contrasts with a PXR-mediated induction response seen in human continuous cell lines (e.g., HepG2), likely related to altered basal and inducible expression of P450s, transcription factors, and coregulators in cell lines. The humanized PXR-CAR-CYP3A4/3A7 mouse model will facilitate further studies of the mechanisms and consequences of *CYP3A4* suppression by PAHs.

Acknowledgments

We thank Chunja Lee for technical assistance as well as Kristopher Krausz and the late Dr. Harry Gelboin (National Cancer Institute, Bethesda, MD) for providing antibodies.

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Authorship Contributions

Participated in research design: Crosby, Riddick.
Conducted experiments: Crosby, Riddick.
Performed data analysis: Crosby, Riddick.
Wrote or contributed to the writing of the manuscript: Crosby, Riddick.

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SUPPLEMENTAL DATA

Suppression of hepatic CYP3A4 expression and activity by 3-methylcholanthrene in humanized PXR-CAR-CYP3A4/3A7 mice

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SUPPLEMENTAL TABLE 1

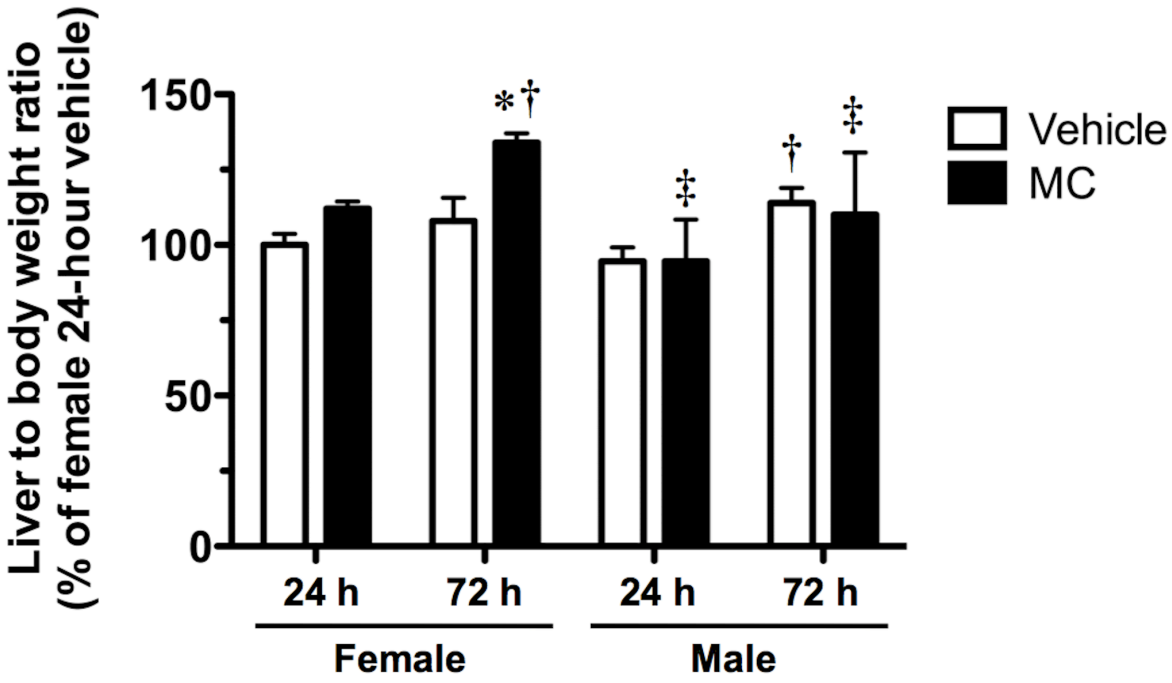
P value outputs from the three-way ANOVA used as the initial layer of data analysis

	Dependent variable					
Independent variable	Cyp1a1 mRNA	Luc-CEE activity	CYP3A4 mRNA	Luc-IPA activity	Fgf21 mRNA	Liver:Body weight
MC Treatment	< 0.0001	< 0.0001	0.0003	0.0047	0.0186	0.0094
Time	0.1708	< 0.0001	0.8693	0.1837	0.3243	< 0.0001
Sex	0.4139	0.0376	< 0.0001	< 0.0001	0.0615	0.0035
Treatment x Time	0.1732	< 0.0001	< 0.0001	0.0987	0.4966	0.4681
Treatment x Sex	0.4190	0.0377	0.0090	0.0869	0.1190	0.0019
Time x Sex	0.1428	0.1637	0.7702	0.1910	0.3151	0.7368
Treatment x Time x Sex	0.1441	0.1627	0.0230	0.2073	0.4474	0.1848

SUPPLEMENTAL TABLE 2

P value outputs from the two-way ANOVA used as the initial layer of data analysis

	Dependent variable	
Independent variable	CYP3A4 protein (female)	CYP3A4 protein (male)
MC treatment	0.0336	0.3357
Time	0.0139	0.7739
Treatment x Time	0.0270	0.0805



Supplemental Fig. 1. Effects of MC treatment, time, and sex on the liver to body weight ratio. Data represent the mean ± S.D. of determinations from four (male 24-hour vehicle group) or five (all others) mice per group, expressed as a percentage of the mean for the female 24-hour vehicle group. Data were analyzed initially by three-way ANOVA and the *P* values for the ANOVA main effects are shown in Supplemental Table 1. Outcomes from Bonferroni-corrected post tests were as follows: *significantly different ($P < 0.05$) from sex- and time-matched vehicle control; †significantly different ($P < 0.05$) from sex- and treatment-matched 24-hour time point; ‡significantly different ($P < 0.05$) from time- and treatment-matched female group. The 24- and 72-hour designations indicate the time post-dosing with vehicle or MC.