Prediction of Gestational Age-Dependent Induction of In Vivo Hepatic CYP3A Activity
Based on HepaRG Cells and Human Hepatocytes

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Non-Standard Abbreviations:

GH, growth hormone; PGH, placental growth hormone; GHs, growth hormone plus placental growth hormone; PRH, pregnancy-related hormones; E₂, 17β-estradiol; P, progesterone; C, cortisol; RIF, rifampin; DEX, dexamethasone; WME, William’s medium E; DPBS, Dulbecco’s phosphate-buffered saline; SCHH, sandwich cultured human hepatocytes; PXR, pregnane X receptor; CTRL, control; AIC, Akaike information criterion; DI H₂O, deionized water.
ABSTRACT

In pregnant women, CYP3A activity increases by 100% during the third trimester (T3). Due to logistical and ethical constraints, little is known about the magnitude of CYP3A induction during the first (T1) and second (T2) trimesters. Our laboratory has shown that sandwich-cultured human hepatocytes (SCHH) and HepaRG cells have the potential to predict the magnitude of in vivo induction of CYP3A activity likely to be observed in T1 and T2. Therefore, we incubated SCHH and HepaRG cells with plasma concentrations of various pregnancy-related hormones (PRHs, individually or in combination) observed during T1, T2, or T3 in pregnant women. Then, CYP3A activity was measured by 1’-OH-midazolam formation. In all three trimesters, only cortisol (C) consistently and significantly induced CYP3A activity, while other individual hormones (progesterone, estradiol or growth hormones) failed to induce CYP3A activity. At physiologically relevant 1X plasma concentrations, the magnitude of CYP3A induction by C or the combination of all PRHs did not change significantly with gestational age. The pattern of induction of CYP3A activity in SCHH by the hormones was similar to that in HepaRG cells. Based on these data, we conclude that C remains the major inducer of CYP3A activity earlier in gestation. Moreover, we predict that the magnitude of CYP3A induction during T1 and T2 will be similar to that observed during T3 (~100% increase vs. postpartum). This prediction is consistent with the observation of similar increase in T2 and T3 oral clearance of indinavir (a CYP3A cleared drug) vs. postpartum.
INTRODUCTION

Despite the general apprehension surrounding the use of drugs during pregnancy, a pregnant woman is frequently prescribed medication to treat a variety of preexisting chronic conditions (e.g. HIV infection, epilepsy, hypertension, solid organ transplantation), acute conditions (e.g. influenza), or pregnancy-related conditions (e.g. nausea, gestational diabetes or hypertension). If left untreated, these conditions could have deleterious consequences to the mother and/or her fetus. Therefore, it is not surprising that between 2006-2008, about 82% of pregnant women took medication sometime during pregnancy and about 50% took medication during the first trimester (Mitchell et al., 2011).

Pregnancy is associated with a myriad of physiological changes including hepatic metabolism (Abduljalil et al., 2012). Amongst these is approximately a 2-fold induction of CYP3A activity during the third trimester (T3), as measured by 1'-OH-midazolam formation clearance (Hebert et al., 2008). Based on genotyping data and modeling and simulation, we have shown that this induction of CYP3A activity is primarily due to induction of hepatic CYP3A4 activity (Hebert et al., 2008; Ke et al., 2012). Many drugs routinely prescribed to pregnant women are cleared by CYP3A enzymes, such as protease inhibitors (e.g. ritonavir), antihypertensive drugs (e.g. nifedipine), and hypoglycemics (e.g. glyburide). The magnitude of induction in CYP3A activity observed during T3 can result in subtherapeutic plasma concentrations of drugs such as the HIV drug, indinavir (Unadkat et al., 2007). Logistical and ethical constraints make it difficult to conduct prospective mechanistic studies in pregnant women using validated CYP3A substrates, especially in 1st (T1) or 2nd trimester (T2). As a result, the magnitude of induction of CYP3A activity during T1 or T2 is not known. This lack of information makes it difficult for clinicians to design dosing regimens of CYP3A cleared drugs during T1 or T2.
The plasma concentrations of several pregnancy-related hormones (PRHs) rise substantially as gestation proceeds (Table 1). Our laboratory has previously demonstrated that a cocktail of PRHs, (the hormone combination containing cortisol (C), progesterone (P), 17β-estradiol (E2), and placental growth hormone (PGH)/growth hormone (GH); hereafter referred to as the PRH cocktail), at their T3 plasma concentrations observed in pregnant women, induced CYP3A activity in sandwich cultured human hepatocytes (SCHH) to an extent comparable to that observed in vivo during T3. Of all these hormones, cortisol was the most potent inducer of CYP3A activity in SCHH. Additionally, the induction pattern observed in SCHH was replicated in HepaRG cells (Papageorgiou et al., 2013). Both these in vitro models demonstrated their potential to predict the magnitude of CYP3A induction in early pregnancy. Therefore, we hypothesized that the magnitude of in vivo hepatic CYP3A induction during T1 and T2 can be quantitatively predicted from studies in SCHH or HepaRG cells exposed to PRHs at plasma concentrations observed in pregnant women (for brevity, referred to as “concentrations” hereafter) during T1 and T2. The studies presented here were conducted in HepaRG and SCHH to examine the temporal relationships between concentrations of PRHs and induction of CYP3A activity over the course of pregnancy, and, based on these data, to predict the magnitude of induction in CYP3A activity during T1 and T2.

MATERIALS AND METHODS

Chemicals and Reagents

Growth hormone, 17β-estradiol, cortisol, progesterone, diazepam were purchased from Sigma-Aldrich (St. Louis, MO); placenta growth hormone was obtained from GenWay Biotech, Inc. (San Diego, CA). Midazolam, 1-OH-midazolam, 1-OH-midazolam-d₄, 17β-estradiol-d₅ were purchased from Cerilliant Corporation (Round Rock, TX). Acetic acid (ACS grade), acetonitrile
(MS grade) were obtained from Fisher Scientific (Pittsburgh, PA). William Medium E (WME), Dulbecco's Phosphate-Buffered Saline (DPBS), GlutaMax-I, Penicillin-Streptomycin (10 000U/mL), and Insulin-Transferrin-Selenium (ITS) were purchased from Life Technologies (Carlsbad, CA). Matrigel® (growth factor reduced, phenol red free) was obtained from BD Biosciences (San Jose, CA).

**Hormone Depletion in HepaRG cells or SCHH**

In-house differentiated HepaRG cells or SCHH in 96 well plates were incubated (in triplicate or duplicate, respectively) with varying concentrations of PRHs (Table 3). Media was sampled at various time points up to 12 hours. To quantify cortisol or progesterone, media samples (15 µL) were protein precipitated with 85 µL acetonitrile containing diazepam (internal standard, 10ng/mL) and centrifuged at 3000g for 15 min prior to LC/MS-MS analysis. To quantify estradiol, media samples (15 µL) were spiked with 15 µL estradiol-d₅ (internal standard, 100ng/mL in 10% acetonitrile), extracted with 800µL methyl tertiary butyl ether, and then centrifuged at 3000g for 15 min. The organic phase was evaporated under nitrogen. The dried residue was reconstituted and derivatized using dansyl chloride to enhance electrospray ionization following the procedure described previously (Kushnir *et al.*, 2008).

The above samples were analyzed by LS-MS/MS using an Agilent 1290 Infinity UPLC system coupled to an Agilent 6400 Triple Quad mass spectrometer. An Acquity UPLC BEH C₁₈ Column (1.7 µm, 2.1 mm X 50 mm; Waters Corporation, Milford, MA) was used with the mobile phase consisting of 0.1% acetic acid (v/v) in water (A) or 0.1% acetic acid (v/v) in acetonitrile (B) (total flow rate 0.25 mL/min⁻¹). For cortisol and progesterone assay, the following linear gradient was used: 80% mobile phase A, 0-0.3min; 80-20%, 0.3-1.5min; 20%, 1.5-2.5min; 20%-80%, 2.5-2.6min; 20%, 2.6-4min. Multiple Reaction Monitoring (MRM) pairs selected for
cortisol, progesterone, and diazepam were 363.2/121.1, 315.2/97.1, and 285/193 respectively. For estradiol assay, the following gradient was chosen: 50% mobile phase A, 0-0.1min; 50-5%, 0.1-0.7min; 95%, 0.7-2.5min; 95%-50%, 2.5-2.6min; 50%, 2.6-4min. MRM pairs selected for dansyl-estradiol and dansyl-estradiol-d5 were 506.1/171.1 and 511/171.1, respectively.

**Hormonal Treatment of HepaRG Cells or SCHH**

Proliferative state HepaRG cells were kindly provided by Biopredic International (Overland Park, KS). The cells were in-house expanded and differentiated according to the provider’s protocols. Differentiated HepaRG cells were cultured in 96 well plates at a density of ~ 0.8 million cells/mL. Since the commercially available HepaRG maintenance and induction medium supplements contain hydrocortisone succinate, a CYP3A inducer, we conducted induction studies in the absence of hydrocortisone succinate to avoid confounding interpretation of our data (Papageorgiou et al., 2013). In brief, the differentiated HepaRG cells were maintained for 72 h in WME supplemented with GlutaMAX-I, ITS, and Penicillin-Streptomycin. At the end of maintenance period, the medium was removed and cells were treated with control (a cocktail of C, GH, E2 and P at their unbound concentrations observed in non-pregnant women; hereafter referred to as “CTRL”) or various PRHs individually or in combination at unbound, 1X, or 10X T1, T2, or T3 concentrations (Table 1). Because some of the hormones were depleted, the actual incubation concentrations were adjusted, based on their half-life of depletion (Table 3), such that the AUC/τ of the hormone media concentration approximated the values listed in Table 1. In addition, to compensate for this depletion, during the 72h induction period with the PRHs, induction media were renewed every 12 h (see Results). When comparing CYP3A activity by T1, T2 or T3 concentrations, these experiments were conducted on the same day with the same batch of HepaRG cells or SCHH. In all the experiments, a positive control (10µM rifampin, RIF)
and a vehicle control were included. All incubations contained 0.1% pH 9 DI H2O and 0.9% methanol. CYP3A activity in these HepaRG cells was determined as described below. Except for CTRL (n=6), experiments were performed in triplicate.

Cryopreserved human hepatocytes from three premenopausal donors (Table 2) were either commercially procured (Hu1587 and Hu1595; Life Technologies, Carlsbad, CA) or provided gratis (Hu4059; TRL, Research triangle, NC). Hepatocytes were plated in collagen-coated 96 well plates at a density of ~ 0.7 million viable cells/mL and overlaid with Matrigel® according to the manufacturer’s instructions. Since the hepatocyte medium supplements normally contain dexamethasone (DEX), a well-documented glucocorticoid CYP3A inducer, we omitted the addition of DEX to the maintenance and induction media. At the end of 72 h maintenance period, the SCHH were treated (including change of media) as described above for HepaRG cells. CYP3A activity in these SCHH was determined as described below. All experiments were performed in triplicate.

**Cortisol Concentration-CYP3A activity induction Relationship in SCHH**

SCHH (batch Hu1587) in collagen-coated 96 well plates were maintained in DEX free medium for 72 h and then treated with CTRL treatment or various concentrations of cortisol, ranging from T1 unbound concentration to 50X T3 concentrations as described above (concentrations were adjusted, where appropriate, to compensate for depletion). CYP3A activity in these SCHH was determined as described below. CYP3A activity in these SCHH was determined as described below. All treatments were performed in duplicate except for the unbound cortisol concentrations and CTRL treatments (n=3).

**CYP3A Activity Assay**
At the end of the induction period, induction media were aspirated, HepaRG cells or SCHH were rinsed twice with pre-warmed DPBS (~150 µL) and incubated for 60 min (HepaRG cells) or 20 min (SCHH) with 2µM midazolam dissolved in serum-free WME. Then, an equal volume of ice-cold acetonitrile containing 10 nM 1’-OH-midazolam-d4 (internal standard) was immediately added to the supernatant and assayed by LC-MS/MS as described previously (Shirasaka et al., 2013).

Statistical and Data Analysis

Data are expressed as mean ± SD values unless otherwise stated. One-way analysis of variance (ANOVA) followed by post hoc tests (Dunnett’s test when the PRH treatment groups were compared to the CTRL treatment or Tukey’s test when PRH treatment groups were compared with each other) were performed. The E_{max} and EC_{50} of induction of CYP3A activity by cortisol was estimated by fitting the simple E_{max} or the sigmoid E_{max} model to the concentration-response data using nonlinear regression (Graphpad Prism 5.0, La Jolla, CA). The baseline value of induction (E_0) was fixed to 1. The model with the smaller AIC value was selected (Ludden et al., 1994).

RESULTS

Hormone depletion when incubated with HepaRG or SCHH

In HepaRG cells, cortisol at the unbound concentration observed in non-pregnant women was depleted with a half-life of ~13 h, while at T3 1X and 10X concentrations, cortisol was not depleted (Table 3). In contrast, estradiol and progesterone were more rapidly depleted. To compensate for this depletion, the induction media in all future experiments (including SCHH) were renewed every 12h and the concentrations of the hormones in the media were adjusted so
that the average concentration (AUC/τ) approximated the corresponding average concentrations during pregnancy. GHs depletion was not measured as an assay to measure the depletion of PGH is currently not available.

**Induction of CYP3A Activity in HepaRG cells by Cortisol or Cortisol plus other PRHs**

A pilot study using T3 unbound, 1X, or 10X concentrations of individual PRHs was conducted. Except for cortisol, none of the PRHs induced CYP3A activity to a significant extent (Figure 1). Therefore, only cortisol and cortisol containing PRH combinations were included in all subsequent experiments described below (including SCHH).

As observed above, cortisol or cortisol plus other hormones consistently, significantly, but variably induced CYP3A activity at 1X or 10X (but not at the unbound) concentrations, compared with CTRL treatment (Figure 2). At 10X concentrations, treatments containing C+E₂ or C+P induced CYP3A activity greater than C alone (Figure 2B, D, E). But, this greater induction was not consistently present in treatments containing GHs. Our positive control, 10 μM RIF, significantly induced CYP3A activity (range: 5~19-fold), whereas CYP3A activity in the presence of the vehicle control was not significantly different from CTRL (data now shown).

**Gestational-Age Dependent Induction of CYP3A Activity in HepaRG Cells by Cortisol or PRH Cocktail**

The magnitude of induction of CYP3A activity was not significantly different across the three trimesters at 1X concentrations of the hormones. At 10X concentrations, significant differences emerged (Figure 3D). PRH cocktail resulted in varying temporal patterns in different batches at 10X concentrations. No consistent cortisol or PRH cocktail concentration-dependent induction in CYP3A activity was observed.
Induction of CYP3A Activity in SCHH by Cortisol or Cortisol plus other PRHs

Similar to HepaRG cells, cortisol and PRH cocktail consistently, variably and significantly induced CYP3A activity in SCHH at 1X and 10X concentrations (and in 1 out of 3 donors at the unbound concentration, data not shown). In batch Hu1587 alone, treatments containing C+GHs or C+E₂ induced CYP3A activity greater than C alone (Figure 4, A-E). In all three batches, 10 µM RIF significantly induced CYP3A activity (range: 9~16-fold). CYP3A activity in vehicle control was not significantly different from that observed in CTRL.

Gestational-Age Dependent Induction of CYP3A Activity in SCHH by Cortisol or PRH Cocktail

As in HepaRG cells, at 1X concentrations, induction of CYP3A in SCHH was independent of gestational age. At 10X concentrations, CYP3A induction was similar throughout all three trimesters except, in one batch (Hu1587), CYP3A activity was modestly increased (~30%) by PRH cocktail in T3 vs. earlier trimesters.

Concentration-Dependent Induction of CYP3A activity in SCHH by Cortisol

Cortisol induced CYP3A activity in SCHH (batch Hu1587) in a concentration-dependent manner. The simple E_max model rather than the sigmoid E_max model was considered to be the best model for the data based on the AIC value (AIC values: -43.624 and -35.716, respectively). At cortisol plasma concentrations spanning from T1 to T3, the induction of CYP3A activity reached a plateau and remained constant.
DISCUSSION

Previously, we have shown that of all the PRHs studied, cortisol was the major inducer of CYP3A activity in HepaRG cells and SCHH at T3 concentrations (Papageorgiou et al., 2013). Consistent with these findings, cortisol remained the major inducer in HepaRG cells at T1 or T2 concentrations (Figures 2, 3). Therefore, all subsequent studies examined the induction of CYP3A activity by cortisol alone or in combination with other PRHs. We chose to use the combination of GH and PGH in our incubations as both are present during pregnancy. The plasma concentration of GH decreases while that of PGH increases as pregnancy proceeds (Fuglsang and Ovesen, 2006). In addition, these two hormones interact with the same receptor with similar affinity (Baumann et al., 1991) and trigger the same intracellular signaling pathways (Silva et al., 2002). Since some of the hormones were rapidly depleted when incubated with HepaRG cells or SCHH, we adjusted the incubation concentrations of the hormones and frequency of change of media to account for this depletion.

Induction of CYP3A activity by cortisol or PRHs in HepaRG cells or SCHH was quite variable from batch to batch. The source of this variability is not clear but may be due to varying percent of hepatocyte-like cells vs. biliary cells between the different batches and passages in our in-house differentiated HepaRG cells (Schulze et al., 2012). Interestingly, in general, the batch of cells demonstrating the highest CYP3A induction by RIF also exhibited the highest CYP3A induction by cortisol alone or in combination. But, this variability in induction of CYP3A activity by cortisol (or PRHs) cannot be quantitatively explained by induction in CYP3A activity by RIF. For example, in SCHH, at T3 1X concentration, cortisol induced CYP3A activity in batch Hu4059 ~4-fold greater than in batch Hu1595. In contrast, RIF induced CYP3A activity in batch Hu4059 only ~50% greater than that in batch Hu1595.
Incubation of HepaRG cells or SCHH with the unbound concentrations of PRHs observed during T1, T2 or T3 did not result in induction of CYP3A activity. Therefore, only the 1X and 10X concentration data are shown in Figures 2-5. Although there was some batch to batch variability; in general, the induction of CYP3A activity in HepaRG cells at 1X concentrations of cortisol alone was comparable to that when cortisol was combined with other PRH(s) (Figure 2A, C, E). Of note, at 10X concentrations, induction of CYP3A activity with C+E2 or C+P was greater than C alone. However, this was not always the case when induction in CYP3A activity by the triple combinations of hormones (e.g. C+GHs+P) or the PRH cocktail was compared with C alone.

Based on these observations and due to our previous data in SCHH on C+GHs demonstrating greater induction of CYP3A activity than C alone (Papageorgiou et al., 2013), the number of hormonal combination treatments examined in SCHH was reduced. Similar to HepaRG cells, despite interbatch variability, the magnitude of induction of CYP3A activity by dual hormone treatments or PRH cocktail was in general comparable to that by C alone at both 1X and 10X concentrations. One batch of SCHH (Hu1587) exhibited a modestly greater induction of CYP3A activity by the dual hormone treatments (C+E2 or C+GHs) compared with C alone at 1X and 10X concentrations. At T1, T2 or T3 10X concentrations, the PRH cocktail induced CYP3A activity greater than C alone.

The pattern of induction observed above is consistent with our previous observations where cortisol alone was the major inducer of CYP3A activity in HepaRG cells or SCHH (Papageorgiou et al., 2013). This work, together with our previous report, provides strong evidence that cortisol is a major inducer of CYP3A activity throughout pregnancy. Indeed, unequivocal data exist that glucocorticoids induce CYP3A activity in human hepatocytes and in vivo (Watkins et al., 1989; Lu and Li, 2001). However, the mechanism(s) by which this
induction occurs is not clear. Evidence suggests that at low plasma concentrations of glucocorticoids, these hormones induce CYP3A activity via the glucocorticoid receptor (GR), while at supraphysiological concentrations, PXR is involved (Lehmann et al., 1998; Pascussi et al., 2000). Aside from glucocorticoid receptor and PXR, multiple lines of evidence suggest that a PXR-independent mechanism(s) may be involved in induction of CYP3A activity (Schuetz et al., 2000; Xie et al., 2000; Zimmermann et al., 2009). Others have also reported enhanced induction of CYP3A activity by cortisol in the presence of GH (Thangavel et al., 2011). They attributed this to greater activation and nuclear translocation of transcriptional factors (i.e. HNF-4α and PXR) and enhanced binding of these factors to CYP3A4 regulatory region (Thangavel et al., 2011). Previous studies have demonstrated that at supraphysiological concentrations estradiol or progesterone activates PXR (Handschin and Meyer, 2003; Mnif et al., 2007). However, this does not signify that estradiol, progesterone, or both, induces CYP3A activity at plasma concentrations observed in pregnant women. Indeed, consistent with our data, at these lower plasma concentrations of estradiol or progesterone, these hormones failed to consistently induce CYP3A activity in human hepatocytes (Choi et al., 2013).

Based upon the above data, we examined the induction of CYP3A activity by cortisol alone or by PRH cocktail at T1, T2, or T3 1X or 10X concentrations in both HepaRG cells and SCHH. Although the absolute magnitude of CYP3A induction varied between these two in vitro models, the induction of CYP3A activity by 1X concentrations of cortisol or PRH cocktail was independent of gestational age (Figures 3 and 5). In contrast, at 10X concentrations, some significant differences manifested, but these differences were modest.

The plasma concentration of cortisol rises considerably as pregnancy proceeds. Cortisol plasma concentration increases from T1 to T2 by ~2-fold and then remain constant during T3. But this
increase did not translate into a significant difference in induction of CYP3A activity (Figure 3A, 3B; Figure 5A, 5B). To gain insight into this observation, we determined the relationship between cortisol concentration and induction of CYP3A activity in one batch of SCHH (Figure 6). The results show that at the total cortisol plasma concentration (181.5 nM) observed in non-pregnant women (Lindholm and Schultz-Moller, 1973; Maroulis et al., 1976; Kalleinen et al., 2008; Matsuzaka et al., 2013) or in pregnant women during T1, T2, or T3, the CYP3A activity would be predicted to be induced by 45%, 120%, 170%, and 170% respectively. While the predicted induction in CYP3A activity during T1 appears numerically lower than that during T2 or T3, our experimental data show that this difference is not statistically significant (Figures 3 and 5). This is because at 1X cortisol concentrations spanning T1-T3, the induction of CYP3A activity appears to be approaching a plateau. Based on these data, we predict that the magnitude of CYP3A induction during T1 or T2 will be similar to that during T3 (~2-fold). Evidence in the literature supports our predictions. Although not an optimum CYP3A probe, urinary dextromethorphan/3-OH-morphinan ratio remains constant throughout pregnancy (Tracy et al., 2005). Also, the clearance of CYP3A substrates, indinavir and nifedipine, during T2, is comparable to that observed during T3 (Marin et al., 2007; Cressey et al., 2013).

In summary, this work, together with our previous report, provides strong evidence that cortisol is the major inducer of CYP3A activity throughout pregnancy in HepaRG cells and SCHH. Moreover, we predict that the magnitude of induction in CYP3A activity during earlier trimesters will be about 2-fold, similar to that in the third trimester. Although these predictions should ideally be validated with CYP3A probe studies, our predictions are consistent with the limited clinical data on the disposition of CYP3A cleared drugs (dextromethorphan, nifedipine and indinavir) during T2 and T3. The mechanism(s) by which cortisol induces CYP3A activity
remains unclear. Given the consistency between HepaRG cells and SCHH in the pattern of induction of CYP3A activity by PRHs, we propose that HepaRG cells can serve as a model to elucidate the molecular mechanism(s) by which cortisol induces CYP3A activity during pregnancy.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Zhang, Farooq, Prasad, Grepper, and Unadkat.

Conducted experiments: Zhang and Farooq.

Contributed to the development of analytical assays: Prasad.

Performed data analysis: Zhang.

Wrote or contributed to the manuscript: Zhang, Farooq, Prasad, Grepper, and Unadkat.
REFERENCES


FOOTNOTES

a. This work was supported by National Institute on Drug Abuse [Grant P01DA032507].

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FIGURE LEGENDS

Figure 1 CYP3A activity in HepaRG cells incubated with T3 plasma concentrations of individual PRHs observed in pregnant women or 10µM Rifampicin (RIF). Fold induction (mean ± SD; n=3) is expressed relative to control treatment (CTRL, n=6; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women). Data were analyzed by one-way ANOVA followed by Dunnett’s test. *, p<0.05; **, p<0.01; ***, p<0.001 compared with CTRL.

Figure 2 CYP3A activity in HepaRG cells incubated with T1 (A, B), T2 (C, D), or T3 (E, F) at 1X (A, C, E) or 10X (B, D, F) plasma concentrations of PRHs observed in pregnant women or 10µM Rifampicin (RIF). Fold induction (mean ± SD; n=3) is expressed relative to control treatment (CTRL, n=6; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women). Data were analyzed by one-way ANOVA followed by Dunnett’s test. *, p<0.05; **, p<0.01; ***, p<0.001 compared with CTRL. §, p<0.05; §§, p<0.01; §§§, p<0.001 compared with cells exposed to C alone.

Figure 3 CYP3A activity in HepaRG cells incubated with 1X or 10X plasma concentrations of cortisol (A and C respectively) or PRH cocktail (B and D respectively) observed in pregnant women. Fold induction (mean ± SD; n=3) is expressed relative to control treatment (CTRL, n=6; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women). Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison to detect any difference in the magnitude of CYP3A induction amongst T1, T2, and T3. §, p<0.05; §§, p<0.01; §§§, p<0.001 compared with indicated trimester.

Figure 4 CYP3A activity in SCHH incubated with T1 (A, B), T2 (C, D), or T3 (E, F) at 1X (A, C, E) or 10X (B, D, F) plasma concentrations of PRHs observed in pregnant women or 10µM Rifampicin (RIF). Fold induction (mean ± SD; n=3) is expressed relative to control treatment...
(CTRL; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women).
Data were analyzed by one-way ANOVA followed by Dunnett’s test. *, p<0.05; **, p<0.01; ***, p<0.001 compared with CTRL. §, p<0.05; §§, p<0.01; §§§, p<0.001 compared with SCHH exposed to C alone.

**Figure 5** CYP3A activity in SCHH incubated with 1X or 10X plasma concentrations of cortisol (A and C respectively) or PRH cocktail (B and D respectively) observed in pregnant women. Fold induction (mean ± SD; n=3) is expressed relative to control treatment (CTRL, n=3; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women). Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison to detect any difference in the magnitude of CYP3A induction amongst T1, T2, and T3. §, p<0.05; §§, p<0.01; §§§, p<0.001 compared with the indicated trimester.

**Figure 6** CYP3A activity in SCHH (Hu1587) incubated with various plasma concentrations of cortisol. Fold induction is expressed relative to control treatment (CTRL, n=3; unbound plasma concentrations of C+GH+E2+P observed in non-pregnant women). Data are expressed as individual data points. $E_{\text{max}}$ and EC$_{50}$ (estimate and 95% confidence interval) were estimated by fitting the simple $E_{\text{max}}$ model to the data.
### TABLES

Table 1 Plasma Concentrations of Pregnancy-related Hormones Observed in Pregnant Women during 1st (T1), 2nd (T2) or 3rd (T3) Trimester and Targeted to Use in This Study after Correcting for Depletion

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1. Huang et al., 2007; 2. Carr et al., 1981; 3. Demey-Ponsart et al., 1982;

Data represent weighted arithmetic means from the above cited studies.
Table 2 Demographic Information of the Hepatocyte Donors

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<tr>
<td>Hu4059</td>
<td>17</td>
<td>Caucasian</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Hu1587</td>
<td>43</td>
<td>Caucasian</td>
<td>F</td>
<td>Vitamin D, multivitamin, caltrate plus</td>
<td>N/A</td>
</tr>
<tr>
<td>Hu1595</td>
<td>31</td>
<td>Caucasian</td>
<td>F</td>
<td>Diazepam, fluticasone furoate, hydrocodone-acetaminophen, methocarbamol</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A – not available
Table 3 Depletion Half-lives of Steroid Hormones when incubated with HepaRG cells or SCHH

<table>
<thead>
<tr>
<th>Hormone</th>
<th>HepaRG Cells</th>
<th>SCHH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc (nM)</td>
<td>Half life (h)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1X 51 NQ</td>
<td>319 2.0±0.5*</td>
</tr>
<tr>
<td></td>
<td>10X 510 3.3±0.7</td>
<td>3187 1.6±0.4*</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1X 395 7.5±1.0</td>
<td>705 1.4±0.4*</td>
</tr>
<tr>
<td></td>
<td>10X 3950 9.2±0.7</td>
<td>19100 1.6±1.0*</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Unbound 16.3 13.0 ±2.4*</td>
<td>48.8 NQ</td>
</tr>
<tr>
<td></td>
<td>1X 798 ND</td>
<td>798 ND</td>
</tr>
<tr>
<td></td>
<td>10X 7980 ND</td>
<td>7980 ND</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD; ND, no depletion; NQ, not quantified; *, n= 3
Figure 2

Fold Induction (relative to CTRL)
Fold Induction (relative to CTRL)

Figure 4

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Figure 6

E_{max}: 3.8 (3.2 - 4.4)
EC_{50}: 293.5 (143.3-601.0) nM