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Poster Award Finalist Abstracts

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Poster Award Finalist Abstracts

A1. Inherited genetic variations at the UGT1A locus are associated with prostate cancer recurrence after radical prostatectomy

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Background. Inherited variations in hormone-metabolizing genes such as UDP-glucuronosyltransferases (UGTs) have been studied for a possible association with prostate cancer (PCa) risk but their prognostic value in the context of recurrence after radical prostatectomy (RP) remains unexplored. *Hypothesis and objective*. Inherited genetic variations in UGTs, by modifying function/expression of the glucuronidation pathway, might significantly affect inactivation of sex-steroid hormones in a wide range of tissues including in residual prostate cancer cells following the surgical procedure, and alter subsequent risk of cancer recurrence. Our objective was to investigate *UGT1A* genetic variations as candidate prognostic markers and their relationships with circulating steroids. *Study design*. The study included 526 Caucasian men who underwent RP for clinically localized and locally advanced PCa with a median follow-up of 7.4 years. We genotyped 17 variants across the *UGT1A* locus previously reported to mark common variants in regulatory domains, exons and intron-exon boundaries. [1] Biochemical recurrence (BCR) risks were estimated using adjusted Cox proportional hazards regression and Kaplan-Meier analysis. We additionally investigated whether variants are predictive of plasma hormone levels using linear regression. Plasmas (n = 500) collected the morning of the surgical procedure were available for endogenous steroid measures. Specific and sensitive mass spectrometry–based methods were used to quantify unconjugated (n = 7) and conjugated (n = 8) steroids. Analysis of covariance (ANCOVA) was used to compare means of log10-transformed hormonal variables between groups, adjusted for age. *Results*. Overall, 130 patients (24.7%) experienced PSA recurrence. As expected, preoperative PSA values and pathological biopsy Gleason scores were associated with BCR. After adjusting for these clinico-pathologic risk factors, a significant increased risk of BCR was revealed for 3 *UGT1A* tag SNPs. Hazard ratio (HR) values were of 1.59 (p = 0.016) 1.77 (p = 0.005) and 1.84 (p = 0.003) for tag SNPs located in *UGT1A8*/*UGT1A9*. Furthermore, a reduced BCR risk was also observed in relation to an intronic *UGT1A9* tag SNP (HR = 0.56; p = 0.003). No significant association was observed in the analysis of the functional *UGT1A1*28* variant (rs34815109; c.–54/–53insTA) and other tag SNPs located in *UGT1A3*, *UGT1A4*, *UGT1A6* or in the common *3′UTR* region. Alterations in circulating steroids associated with some of these *UGT1A* genetic variants further support the notion that germline variations have the potential to modify hormonal exposure and risk of recurrence. *Conclusion*. This study provides the first evidence of *UGT1A* germline variations as potential biomarkers for prostate cancer recurrence. Predicting biochemical recurrence after radical prostatectomy in patients with clinically localized prostate cancer may thus be improved by taking into consideration patients' genetic information related to the UGT genes. *This work was supported by Prostate Cancer Canada.* [1] Menard, V et al. *Analysis of inherited genetic variations at the UGT1 locus in the French Canadian population*. Hum Mutat. 2009. **30** (4): p. 677–87

A2. The neonatal development of UGT1A9: Implications for pediatric pharmacokinetics

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The isoform UDP-glucuronosyltransferase 1A9 (UGT1A9) is found in many tissues including the liver, intestines, and kidneys and is the major metabolic pathway for several drugs, pesticides, and hormones. Despite the importance of the UGTs, the developmental dynamics of most isoforms, including 1A9, have not been elucidated. Herein, using 4-methylumbelliferone (4MU) with specific inhibition by niflumic acid and specific antibodies, UGT1A9 activities and protein expression were examined in the neonatal and pediatric liver. Subsequently, *in silico* pharmacokinetic (PK) and physiologically-based pharmacokinetic (PBPK) modeling was used to determine maturation of UGT1A9 and hepatic clearance. One-phase exponential association models were most appropriate and returned maximal enzyme activity of 27.94 nmol/min/mg protein at 4 months of age, with high concordance with the average V_{max} in 45 individual adult livers of 28.98 nmol/min/mg protein, with a 7.5-fold range (4.1– 54.5 nmol/min/mg protein). The expression of 1A9 protein increased with postnatal age and activity, and positively correlated to protein expression in neonates (Spearman r = 0.84), but over 1 year of age the positive correlation was lost. When the hepatic clearance of 4MU by UGT1A9 was scaled with an allometric PK model, a high clearance at birth falling to adult levels (1.12 l/hr/kg at 18.1 years for well-stirred or 1.23 l/hr/kg at 18.7 years for parallel tube) was described. In contrast, using a PBPK model (Simcyp, Sheffield, UK), low clearance at birth increasing to adult levels of 0.97 l/hr/kg at 1.3 months (well-stirred) and 1.11 l/hr/kg at 1.0 months (parallel tube) were returned. Neither activities nor hepatic clearances mediated by UGT1A9 differed with gender or ethnicity. Seemingly, UGT1A9 has high capacity for xenobiotics leading to high extraction. This may explain why UGT1A9 substrates, such as propofol, SN-38, and mycophenolic acid require greater or equal doses in children compared to adults.

A3. Can P-glycoprotein at the human blood-brain barrier be induced by rifampin? A PET imaging study

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While inhibition of P-glycoprotein (P-gp) at the human blood-brain barrier (BBB) has been clearly demonstrated by our laboratory, no such data are available on the induction of P-gp at the human BBB. This question needs to be addressed to better predict P-gp based drug-drug interactions at the human BBB, and to develop therapeutic strategies for the treatment of CNS diseases that might benefit from increased BBB P-gp activity (e.g. Alzheimer's disease). Therefore, the goal of our investigation was to determine whether P-gp at the human BBB can be induced by a FDA-approved potent P-gp inducer (rifampin) using Positron Emission Tomography (PET) imaging. With regulatory approval and informed consent, ^{11}C-verapamil (~0.1 mCi/kg, ~0.07 μg/kg) was administered intravenously to 7 healthy volunteers (3 women and 4 men) before and after 10-21 days of rifampin treatment (600 mg nightly). Prior to each ^{11}C-verapamil administration, ^{15}O-H_2O (~0.5 mCi/kg) was administered intravenously to measure cerebral blood flow (Q). During each PET-imaging session, brain images and frequent arterial blood samples were obtained for 5 (^{15}O-H_2O) or 20 (^{11}C-verapamil) mins to measure radioactivity content in these tissues. Plasma ^{11}C-verapamil and metabolites radioactivity were quantified by rapid solid-phase extraction[1]. The tissue uptake ($AUC_{tissue}/AUC_{plasma}$ ratio), distribution clearance (CL_{12}) and extraction ratio, ER (CL_{12}/Q), of ^{11}C-radioactivity for the whole brain, gray and white matter during the first 10 mins post-injection were determined for both control and post-rifampin treatment, and compared using the paired Student's t-test. As expected, rifampin treatment induced ^{11}C-verapamil metabolism via the CYP3A-mediated pathway (^{11}C -polar metabolites, ^{11}C-PM). Compared to baseline, the plasma radioactivity content of ^{11}C-PM increased from 15 ± 9% to 28 ± 8% at 10 mins post-^{11}C-verapamil injection. A 2-tissue compartment model using a single input function of total plasma radioactivity was determined to be the best model for estimating CL_{12} and ER. When compared with baseline, rifampin treatment did not significantly ($p>0.05$) change the $AUC_{tissue}/AUC_{plasma}$ ratio for the brain (11 ± 29%), gray matter (9 ± 26%), or white matter (16 ± 42%). Likewise, verapamil CL_{12} and ER were unaffected by rifampin pre-treatment. These data show that while therapeutic doses of rifampin induced systemic CYP3A-metabolism of ^{11}C-verapamil, they did not induce P-gp activity at the human BBB. These results are consistent with a study that reported PXR expression in the human

brain microvessel endothelial cells (hBMECs) is low[2], although these results await confirmation. In addition, the expression at the human BBB of other nuclear receptors that regulate P-gp expression needs to be evaluated.

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A4. Comparison of cytochrome b5 interactions with some human cytochrome P450 isoforms

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[Objective] Cytochrome P450 (CYP) enzymes are important contributors to xenobiotic metabolism, among which the CYP3A4 and CYP2 family account for ~80% of the total liver P450s. Cytochrome b5 (cyt b5 or holo b5) interacts with many CYP isoforms and can markedly modulate their activities. The effect is both substrate- and CYP-dependent, either increasing, decreasing or having no effect on CYP activities. In addition, cyt b5 devoid of heme (apo b5) can stimulate the activities of some CYP isoforms (3A4, 2A6, 2C9) to a similar extent as holo b5, but not the activities of other CYP isoforms (2E1, 2D6). The underlying molecular mechanism for this differential modulation has not yet been determined. The current investigation was focused on the surface interactions of cyt b5 with various CYP isoforms as possible contributing factors in the differential modulation. [Methods] Recombinant human CYPs (3A4, 2A6, 2C9, 2D6 and 2E1) were each reconstituted with cyt b5. Interacting sites between CYP isoforms and cyt b5 were identified using the zero-linker cross-linking reagent 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), coupled with mass spectrometric analysis. Computer models of their interactions were built using the cross-linked sites as constraints in addition to the crystal structures of individual CYPs and a homology model of human cyt b5. Interactions between apo b5 and CYPs were also investigated using the same methods. In order to confirm the biological significance of the amino acid residues on CYP3A4 involved in cross-linking with cyt b5, site-directed mutagenesis was carried out followed by reassessment of CYP3A4 activities using Vivid Green and testosterone as probe substrates. [Results] Cross-linking sites between CYPs and holo b5/apo b5 were identified, and computer models of their interactions were developed. The heme in holo cyt b5 and in CYP2A6/2C9/3A4 are not as closely associated as in the published interaction model of holo b5-CYP2E1, indicating holo b5 stimulates the activities of these three CYPs differently than CYP2E1. The single or triple mutations of cross-linked residues (K96A, K127A, K421A) on CYP3A4 decreased its activity to various degrees. The conserved amino acid R446 was predicted to be critical in the interaction between cyt b5 and CYP3A4 based on a holo b5-CYP3A4 interaction model. Mutation at this position R446A abolished the testosterone 6-beta hydroxylation and Vivid Green metabolism activity of CYP3A4. Overall, the data indicates that sites on CYPs that interact with cyt b5, as identified by chemical cross-linking, likely play important roles in the activities of some CYP isoforms. [Conclusion] The present study indicates that cyt b5 modulates CYP2A6/2C9/3A4 activities without direct electron transfer. This is a different mechanism than that proposed for the interaction of cyt b5 with CYP2E1, which likely involves electron transfer between the heme groups of these two cytochromes. Acknowledgment: Supported by NIH/NIGMS Program Project Grant No. GM32165 and the Univerisity of Washington Proteomics Resource center Grant No. UWPR95794. We thank Dr. David R. Goodlett and Dr. Priska von Haller for their assistance with the proteomics analysis, and the Collaboratory for MS3D Portal.

A5. Potent inhibition of human SULT1A1 by 17α-Ethinylestradiol

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Potent Inhibition Of Human SULT1A1 By 17α-Ethinylestradiol (EE2). Sulfotransferases (SULTs) have a major role in metabolizing endogenous and exogenous compounds in the human body. SULTs are Phase II conjugating enzymes that activate or inactivate compounds by transferring a sulfonate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the hydroxyl or amine group. Human SULT1A1 is highly expressed in liver and is also widely expressed in other tissues including brain, breast and intestine where it is associated with the sulfation of a wide variety of

small phenols, dietary polyphenols, thyroid hormones and N-hydroxyaromatic amines. SULT1A1 also conjugates estrogens including 17α-ethinylestradiol (EE2), the active ingredient in many oral contraceptives. EE2 is a lipophilic molecule that passes the outer membrane of the cell and targets estrogen receptors to mediate hormone-regulated gene expression. SULT1A1 sulfates EE2 with a Km of approximately 600 nM. This study focused on the ability of EE2 to potently inhibit the SULT1A1 catalyzed sulfation of 17β-estradiol (E2), p-nitrophenol, and β-naphthol. The Ki values ranged from 8.9 to 19 nM for the inhibition of the SULT1A1 catalyzed sulfation of these compounds. The Ki for EE2 inhibition of E2 sulfation by intact human MCF-7 breast cancer cells that express high levels of SULT1A1 activity was 20 nM. We were interested in the mechanism by which EE2 could inhibit SULT1A1 at low nanomolar concentrations while being a substrate at greater than 30-fold higher concentrations. PAP(S) binding results in a structural rearrangement that decreases the volume of the PAPS and substrate binding sites. Models of SULT1A1 were generated with the Molecular Operating Environment programs using the crystal structures of human SULT2A1 resolved with bound PAPS (closed) and of the enzyme without bound PAPS (open) as a template. The Kd for EE2 binding to pure SULT1A1 was determined to be 150 nM to the enzyme without bound PAP and 4.3 nM to the enzyme previously incubated with PAP. EE2 docked into the active site of the SULT1A1 open model in a catalytically competent orientation with low affinity, whereas, if PAPS was bound to SULT1A1 to generate a closed conformation, EE2 bound with a greater affinity in a non-catalytically competent orientation. This demonstrates that when EE2 is bound to SULT1A1 before PAPS is bound, EE2 is in a catalytically orientation for sulfation; however, when PAPS is bound to SULT1A1 first EE2 acts as a non-sulfated potent competitive inhibitor. The potent inhibition of SULT1A1 by low nanomolar EE2 concentrations may be a source of drug interactions in women using EE2 containing contraceptives. (Supported by NIH GM38953)

A6. Do cryopreserved sandwich cultured human hepatocytes better predict *in-vivo* CYP3A inactivation DDIs than human liver microsomes?

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Predictions of mechanism-based drug interactions (MBDDIs) have historically been based on in-vitro parameters derived in human liver microsomes (HLMs). Typically, these parameters are k_{inact}, the maximum inactivation rate constant and K_I, the concentration that results in the half maximum inactivation rate constant describing the mechanism based inactivation (MBI) of the enzyme. When predicting these MBDDIs, a critical assumption is made. This assumption states that the intrahepatocyte unbound concentration of the perpetrator drug is approximated by the extracellular unbound plasma concentration of the drug, i.e. minimal net transport of the perpetrator drug into or out of the hepatocytes. Since drug transporters are expressed at the sinusoidal and canalicular membrane of the hepatocytes, this assumption is unlikely to be true. When cultured appropriately, sandwich-cultured human hepatocytes (cSCHHs) are able to reestablish bile canalicular networks and to express many of the transporters (e.g. P-glycoprotein; P-gp) expressed in the liver. Therefore, we selected 8 CYP3A-based MBIs which have resulted in either over or underprediction of in vivo DDIs, and asked the question "Do cSCHHs better predict these in vivo CYP3A MBDDIs than do HLMs? In addition we asked if the differences in MBI parameters determined in HLMs and cSCHHs is a result of efflux transporters (e.g P-gp). CYP3A inactivation parameters (K_I and k_{inact}) were determined in HLMs (n = 3) or in cSCHHs, (n = 3; in the presences or absence of elacridar) for the following MBIs: ritonavir, nelfinavir, amprenavir, lopinavir, verapamil, troleandomycin, diltiazem or erythromycin using midazolam 1′-hydroxylation as the CYP3A measure. k_{inact} was significantly lower in cSCHHs compared to HLMs for all 8 inactivators, ranging from ~2-fold (diltiazem) to ~130-fold (amprenavir). K_I, was ~8–10-fold lower in HLMs compared to cSCHHs for lopinavir and nelfinavir, whereas the K_I for verapamil was ~8-fold higher in HLMs compared to cSCHHs. P-gp inhibition by elacridar resulted in more potent inactivation of CYP3A by ritonavir and troleandomycin, but had no effect on amprenavir, lopinavir, verapamil or erythromycin. Previously published MBDDIs between the MBIs listed above and midazolam were predicted using a comprehensive mechanistic static prediction model with k_{inact} and K_I parameters generated in HLMs or cSCHHs. Midazolam DDIs were more accurately predicted for ritonavir (high dose only) and nelfinavir using cSCHHs. Diltiazem, verapamil and erythromycin mediated DDIs were in general slightly overpredicted by HLMs but slightly underpredicted by cSCHHs. Troleandomycin mediated DDIs were drastically underpredicted by both HLMs and cSCHHs. CYP3A inactivation was different in HLMs and cSCHHs, and P-gp played a significant role in this discrepancy for ritonavir and troleandomycin. However, for the remaining MBIs, other mechanisms must play a role in differences in CYP3A inactivation between HLMs and cSCHHs. Nevertheless, cSCHHs provide comparable, if not better, prediction accuracy of in vivo DDIs by the MBIs evaluated.

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A7. Decipher the transcriptome during mouse liver development by RNA-Seq

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During development, fetal liver mainly functions as a hematopoietic organ. After birth, profound changes occur in hepatic gene expression when liver rapidly transitions into the major organ for xenobiotic metabolism and nutrient homeostasis. Although much is known in adults regarding the regulation of drug-processing genes in liver, very little is known during the pediatric period, placing newborns and children at a higher risk of adverse drug reactions than adults. More importantly, recent studies demonstrate that many genes have multiple mRNA isoforms that may lead to different translation potential and protein activity. However, there is limited knowledge regarding age-specific mRNA isoforms of drug-processing genes during liver development that may influence the capacity of drug biotransformation. RNA-Seq allows unbiased detection of novel isoforms on a transcriptome-wide scale. The purpose of this study was to determine the ontogeny of hepatic transcriptome by RNA-Seq, and unveil novel mRNA isoforms of essential drug-processing genes during liver development. Male C57BL/6 mouse livers were collected at 12 ages (prenatal: GD17.5; neonatal: Day1, 3, 5, and 10; adolescent: Day15, 20, 25, and 30; adult: Day45 and Day60), and mRNA transcriptome was determined by RNA-Seq on Illumina HiSeq2000 with 200 cycles paired-end (n = 3 per age). RNA-Seq generated an average of 160-180 million reads per sample which were aligned to the mouse reference genome NCBI37/mm9. More than 60% of reads were mappable by TopHat. The abundance of genes and transcripts was estimated by Cufflinks. 18,622 genes had a positive FPKM (Fragments per kilobase of exon per million fragments mapped) value within at least one age during development. Among all 12 ages, a histogram showed that the expression of genes centered on a FPKM value of 5. A heatmap of the whole transcriptome showed that more mRNAs appear to be produced at perinatal ages (between GD17.5 and Day5) than at later developmental ages (Day10 and after). Correlation analysis showed three distinct age clusters, with high correlation of gene expression within each cluster: cluster 1 (Day-2 and Day0), cluster 2 (Day1, 3 and 5), as well as cluster 3 (Day10 to 60). The mRNAs from a total of 3852 genes were significantly altered during development ($p<0.05$). Ingenuity Pathway Analysis showed that compared to Day60 adult age, genes that were significantly higher at perinatal and neonatal ages are mainly important in pathways such as cell cycle, amino acid and carbohydrate metabolism, protein synthesis, and RNA post-transcriptional modifications; whereas at adult age, genes that were significantly increased are involved in lipid metabolism and xenobiotic metabolism. Cufflinks identified 9331 novel isoforms within the 3 ages examined, corresponding to 5217 genes in liver. Among 238 critical drug-processing genes, novel mRNA isoforms were observed for 42 phase-I enzymes, 15 phase-II enzymes, and 22 transporters. In conclusion, the present study decoded age-specific transcriptome signatures with broader coverage across multiple ages during liver development. This work has also unveiled distinct expression patterns of novel isoforms for critical drug-processing genes, paving the path for further understanding mechanisms of adverse drug reactions in developing liver (Supported by NIH ES-019487 and RR-021940.)

A8. 17β-Estradiol (E2) enhances cytochrome P450 (CYP) 2B6 expression via cooperative actions of estrogen receptor (ER) and constitutive androstane receptor (CAR)

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Pregnancy alters hepatic drug metabolism in a cytochrome P450 (CYP) pathway-specific manner [1]. Pregnancy-related physiological changes, such as rising concentrations of estrogen, are potentially responsible. However, it remains unknown whether estrogen, at concentrations attainable during pregnancy, affects hepatic drug metabolism. The objective of this study is to characterize the effects of major estrogen, E2, on hepatic expression of major CYP enzymes and elucidate the underlying molecular mechanisms. To this end, human hepatocytes were treated with vehicle (ethanol) or E2 (1 μM), and mRNA expression levels of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were determined by quantitative RT-PCR. E2 increased expression of CYP2A6, CYP2B6 and CYP3A4 but not those of other CYP

enzymes. Metabolism of probe drugs for CYP2A6, 2B6 and 3A4 was also increased in E2-treated hepatocytes. In HepG2 cells, luciferase reporter assays of CYP2B6 upstream regulatory region revealed that E2 enhances promoter activity of CYP2B6 via activation of CAR. E2 triggered nuclear translocation of EGFP-tagged human CAR in rat hepatocytes, further confirming that E2 activates CAR. Luciferase reporter assays in HepG2 cells revealed that E2 enhances CYP2B6 promoter activity also via ER transactivation. DNA-binding domain of ER was found dispensable in this induction, suggesting involvement of non-classical mechanism of ER action in upregulation of CYP2B6. Results from luciferase reporter assay using 5′-deletion reporter constructs for CYP2B6 upstream region showed that -1839/-1461 of CYP2B6 mediated the ER action. The region harbors 2 putative binding sites for activator protein-1 (AP-1): -1782/-1776 and -1664/-1658. Results from mutation assays indicated that both AP-1 motifs were critical in ER-mediated activation of CYP2B6 promoter by E2. Electrophorectic mobility shift and supershift assays demonstrated that in addition to AP-1 proteins, ER bound to the distal AP-1 motif and the binding was enhanced in HepG2 cells treated with E2. Concurrent activation of ER and CAR by E2 activated the promoter activity of CYP2B6 in a synergistic manner. Our data indicate that at concentrations attainable during pregnancy, E2 upregulates CYP2B6 expression by cooperative activation of CAR and ER, and the ER action is mediated through AP-1. These results suggest potential increases in metabolism of CYP2B6 substrates during pregnancy.

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A9. Mechanisms by which hepatic CYP3A enzymes are induced during pregnancy

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Cytochrome P450 3A4/5 enzymes (CYP3A4/5) collectively metabolize more drugs than any other cytochrome P450 enzymes. Our laboratory has found that the oral clearance of the HIV protease inhibitor, indinavir (a CYP3A substrate), is much greater during pregnancy than post-partum [1] and we have shown that CYP3A activity (as measured by midazolam oral clearance) is induced in pregnant women [2]. The increased clearance of the protease inhibitors observed in pregnant women was reproduced in mice and was found to be due to increased Cyp3a mRNA/protein expression in the liver [3] and positively correlated with higher hepatic mRNA levels of the transcription factors *HNF6* and *ERα* (estrogen receptor alpha), which are respectively major players in the growth hormone (GH) and estrogen signaling pathways. Based on the above data, we have hypothesized that, during human pregnancy, the increase in plasma concentration of placental growth hormone (PGH), which is secreted by the placenta and gradually replaces the pituitary GH, progesterone, estrogens (primarily 17β-estradiol), and corticosteroids activates a network of nuclear receptors and transcriptional factors to induce hepatic CYP3A activity. To test our hypothesis we incubated these hormones with sandwich-cultured human hepatocytes from healthy women (post- or premenopausal), using the total or 10X the maximum total plasma concentration of each hormone observed during human pregnancy. Primary cultures of human hepatocytes were incubated for 72 hrs with or without the pregnancy hormone(s). At the end of the incubation, CYP3A activity was measured using midazolam hydroxylation. Additionally we quantified using quantitative Real-Time PCR (qPCR), the mRNA expression of CYP3A4 and 3A5 and the respective hormone receptors: glucocorticoid receptor, *GR*; estrogen receptor alpha, *ERα*, and growth hormone receptor, *GHR*. Our results show that 17β-estradiol or cortisol significantly induced CYP3A activity (~4-6 fold in premenopausal donors at the total concentration of each hormone) and *CYP3A4* mRNA (~12-50 fold in postmenopausal and ~8-9 fold in premenopausal donors) expression in human hepatocytes. However, when the hormones were incubated in combination, they remarkably induced CYP3A activity (~12-fold in premenopausal donors at the total concentration of each hormone) and *CYP3A4* mRNA expression (~70-fold). In addition, qPCR analysis of the mRNA levels of the hormone receptors, revealed only a modest increase in *ERα* transcript levels (~3-6 fold in pre- and postmenopausal donors respectively) in the presence of estradiol, while there was no biologically significant increase in the transcript levels of the GHR or GR. These data implicate cortisol and 17β-estradiol as mediators of hepatic CYP3A induction during pregnancy and provide important leads regarding the molecular mechanisms of this induction.

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A10. p97 and 19S Proteasomal cap AAA ATPases in CYP3A endoplasmic reticulum (ER)-associated degradation (ERAD)

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Cytochromes P450 3A are ER membrane-anchored hemoproteins that are predominantly responsible for hepatic metabolism of endo- and xenobiotics. Hence mechanisms regulating hepatic CYP3A content can significantly influence drug metabolism, therapeutics and clinical drug-drug interactions. Our goal therefore is to characterize the molecular mechanisms involved in the stabilization and turnover of CYP3A. In the rat/human liver, native and suicidally inactivated CYPs 3A are degraded via ubiquitin-dependent 26S proteasomal degradation. The proteasomal targeting of CYP3A4 requires phosphorylation at residues S478, S420 and T264 (based on in-vitro data and S^{35}-pulse-chase analyses of wild type and mutant CYPs 3A expressed in HepG2 cells), followed by ubiquitination by the mammalian ubiquitin ligase complexes, and its subsequent p97 AAA ATPase-dependent ER-extraction into the cytosol. However, given that the CYP3A bulk is in the cytosol and a significant 26S proteasomal fraction is ER-associated, it was plausible that the 26S proteasomal AAA ATPases (particularly the Rpt4 19S cap subunit which has been shown to extract ER-membrane proteins into the cytosol) were also involved in CYP3A extraction. To determine the relative roles of p97 and 26S proteasome, we used lentiviral shRNA-mediated knockdown of rat hepatic p97 and proteasomal Rpt4 subunit in combination with MG132 (a proteasomal inhibitor) or hemin (a reported proteasomal AAA ATPase inhibitor) as probes. Knockdown of p97 or Rpt4 did not impair 20S proteasomal proteolytic core function. However, p97 knockdown (90%) nearly completely abolished the membrane extraction of CYP3A, resulting in a marked accumulation of functionally active parent CYP3A and its ubiquitinated species that remained firmly ER-tethered. We document that CYP3A ubiquitination precedes its membrane extraction. Upon p97 knockdown, little CYP3A was extracted into the cytosol, even after MG262-proteasomal inhibition. Hemin blocked p97 AAA ATPase activity and consistent with this inhibition both parent and ubiquitinated CYP3A species accumulated in the ER. Thus, p97 plays a major and obligatory role in the ER extraction of CYP3A. By contrast following 92% of Rpt4 knockdown, S^{35}-CYP3A pulse chase analyses revealed that CYP3A extraction into the cytosol was unhindered. However, once CYP3A was extracted into the cytosol it was modestly stabilized. These findings are consistent with mutually exclusive albeit sequential roles for p97 and Rpt4 AAA ATPases in CYP3A ERAD, wherein Rpt4 functions at the proteasomal degradation step following ER extraction of CYP3A by p97. These studies elucidate key steps in the mechanism of regulation of liver CYP3A turnover via ERAD.

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A11. The inductive expression of citrate transporter SLC13A5 gene is regulated by the xenobiotic receptor CAR and PXR in human liver

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The solute carrier family 13 member 5 (SLC13A5) is a newly identified sodium-coupled transporter that mediates the cellular uptake of the tricarboxylic acid cycle (TCA) intermediate citrate, which plays an important role in the synthesis of fatty acid and energy homeostasis. Recently, the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR), initially characterized as xenobiotic sensors, have been functionally linked to the regulation of various physiological processes that associated with lipid metabolism and energy homeostasis. Here, we show that SLC13A5 is robustly up-regulated by the prototypical CAR activator phenobarbital (PB) and PXR activator rifampicin (RIF) in human primary hepatocytes (HPHs). Two distal enhancer modules located upstream of the *SLC13A5* gene transcription start site are identified and postulated as the key elements governing the induction of SLC13A5. In electrophoretic mobility shift assays, these elements demonstrated strong binding to PXR/CAR proteins in the presence of their heterodimer partner, retinoid X receptor. Cell-based reporter assays in HepG2 cells and HPHs further showed that SLC13A5 reporter construct, containing both elements could be transactivated by CAR and PXR upon exposure to PB and RIF, respectively. Moreover, our chromatin immunoprecipitation (CHIP) assays in HPHs showed that after PB and RIF exposure, significantly increased amounts CAR and PXR proteins were recruited to these distal enhancers. Together, our results clearly indicate that CAR and PXR are crucial for chemical-mediated induction of the *SLC13A5* gene, and

the regulation network of CAR/PXR-SLC13A5 may contribute to the overall energy homeostasis under metabolic and chemical stresses.

A12. Intestinal CYP3A4 and midazolam disposition *in vivo* show seasonal variation and associate with VDR polymorphisms

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Vitamin D, whose levels vary seasonally with UV sunlight, binds the vitamin D receptor (VDR) and transcriptionally regulates intestinal CYP3A4 expression. We tested the hypothesis that intestinal CYP3A4 expression and midazolam disposition in vivo varies seasonally and is associated with VDR genotype. We genotyped six polymorphisms in the VDR gene and determined their associations with CYP3A4 intestinal expression and activity. Significant associations were observed between the VDR polymorphism in intron 8 rs1544410 (BsmIG>A, g.63980G>A) and (a) CYP3A4 jejunal protein expression and activity, and (b) CYP3A4 duodenal mRNA expression, and (c) midazolam dose adjusted area under the curve (AUC). Intestinal CYP3A4 expression/activity was significantly higher in biopsies with the VDR promoter polymorphisms rs11568820 (Cdx2-3731G>A) and rs4516035 (GATA-1012A>G) that are known to increase binding of Cdx2 and GATA and increase VDR transcriptional activation of target genes. Duodenum CYP3A4 mRNA levels was significantly higher between March and July than between October and February. Dose-adjusted midazolam p.o. AUC and oral bioavailability were each significantly higher (and weight adjusted midazolam oral clearance was lower) in September-January compared to March thru July. We conclude that these data provide support for VDR polymorphisms as predictors of intestinal CYP3A4 expression, and that intestinal CYP3A4 expression and midazolam dose-adjusted AUC and bioavailability show seasonal variation that is likely related to annual changes in UV sunlight and vitamin D levels. This is the first example of a gene (VDR)/environment (seasonal variation in UV sunlight and presumably Vitamin D) interaction regulating intestinal CYP3A4.