

Land O'Lakes Conference on Drug Metabolism/Applied Pharmacokinetics
Posters 101-112 presented in 2006
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Programmed Blood and Bile Collections from Freely-Moving Rats: A Study Tracking Nicotine and its Cotinine Metabolite

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Objectives: Bile is essential to mammalian health. Animal models that continuously divert bile (for collection) can't be maintained for long due to the adverse consequences to animal health which ensue. A new model for bile collection from freely-moving rats was evaluated by tracking nicotine, and its cotinine metabolite. **Methods:** The common bile duct and duodenum were catheterized and exteriorized to the dorsal scapular area in male SD rats (450 g). Jugular and femoral vein catheters were placed in the same subjects for IV dosing and blood sampling, respectively. All animals were installed on an automated pharmacology system providing food, water, freedom of movement and catheter maintenance until preprogrammed collections of blood or bile were initiated. Subjects were evaluated daily for clinical signs, food and water intake, urinary output, and overall motor activity. Analysis was performed using LCMSMS. Urine was collected but not analyzed, although it was available for that purpose. The nicotine dose (0.5 mg/Kg) was infused over 10 minutes while the serial bile and blood samples were collected for 24 hours postdose. **Results:** Nicotine peaked 15 minutes postdose in plasma and 120 minutes in bile. Cotinine T_{max} was 120 minutes postdose for plasma, while in bile, concentrations peaked 240-420 min post dose. **Implications:** Subjects could be maintained for periods up to 10 days, permitting redosing of the same compounds and comparisons within the same subject. This approach was valuable in providing comparisons of parent and metabolites among multiple matrixes in the same subject.

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Investigation of the Pharmacokinetic Properties of Certolizumab Pegol, an Anti-TNF Agent

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Purpose: Certolizumab pegol is a PEGylated Fab' fragment of a humanised anti-tumor necrosis factor (TNF) monoclonal antibody. PEGylation (site-specific addition of polyethylene glycol [PEG]) improves the pharmacokinetic (PK) properties of the Fab' fragment. This discussion characterised the disposition of certolizumab pegol after iv and sc administration over a range of doses in non-human primates and human subjects. **Methods:** Certolizumab pegol was administered either iv or sc across a dose range to human volunteers and cynomolgus monkeys. PK evaluation and modelling, which also comprised allometry investigations, were performed on primate and human plasma levels of certolizumab pegol. **Results:** PK properties of certolizumab pegol were consistent across primate and human studies and had a linear PK profile. The PK profile of certolizumab pegol was in line with those of other large biological molecules. The apparent volumes of distribution of certolizumab pegol in primates (46 mL/kg) and humans (45 mL/kg) were equivalent to the species-relevant plasma volumes. The clearance rate was 0.29 mL/h/kg in primates and 0.72 mL/h/kg in humans. Clearance and volume of distribution were amenable to allometric scaling (upon addition of preclinical data from other species). The

elimination $t_{1/2}$ was 192 hours in primates and 311 hours in humans. Modelling of certolizumab pegol indicated at least 100% bioavailability in humans and primates. **Conclusion:** Certolizumab pegol demonstrates predictable linear PK in both humans and primates. Its PK profile shows sustained subcutaneous absorption, high bioavailability, low clearance and prolonged elimination half-life, reflecting the advantages of conjugating a PEG moiety with a Fab' fragment.

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Biliary Excretion and Metabolite Profiles of ERB-041, an Estrogen Receptor-beta Selective Agonist, in the Rat

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Purpose: To determine the biliary excretion and metabolite profiles of ERB-041, an orally active and highly selective estrogen receptor- β agonist, in the rat. ERB-041 is currently in clinical development as a treatment for rheumatoid arthritis and endometriosis. **Methods:** Excretion of [^{14}C]ERB-041 (10 mg/kg) was investigated in intact and bile-duct cannulated male rats, and the metabolite profiles in urine, feces, and bile were determined by HPLC-radio flow detection. The identities of the metabolites were established by LC-MS and NMR analysis following oral administration of ERB-041 at 100 mg/kg to bile-duct cannulated rats. **Results:** In intact rats, 54% of the dose was excreted in feces and 26% in urine within 24 hrs. Little phase I transformation was observed. The major metabolic pathways were phenolic glucuronidation and sulfation. Unchanged ERB-041 was the major component (~90%) in feces. [^{14}C]ERB-041 represented 13% of the radioactivity in urine. Urinary metabolites included mono-glucuronides, mono-sulfates, a di-glucuronide and traces of glucuronide-sulfate di-conjugates. In bile-duct cannulated rats, ERB-041 was well absorbed and rapidly excreted with bile representing the major route of elimination (50%, 0-24 hr). Urine and feces contained 21 and 16% of the dose, respectively. The biliary metabolites (0-4 hr) included: 4'-glucuronide (9%), 5-glucuronide (6%), 4'-sulfate (4%), 5-sulfate (7%), two glucuronide-sulfate di-conjugates (32 and 30%), 4',5-diglucuronide (6%), with lesser amounts of 4',5-di-sulfate and ERB-041. **Conclusions:** The biliary route was the major route of excretion of [^{14}C]ERB-041 in rats. Glucuronidation and sulfation were the major metabolic pathways. The sites of ERB-041 conjugation were determined by LC-MS and NMR analysis.

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Pharmacokinetics and Interspecies Scaling of A Novel Anti-tumor Agent, Zalypsis[®] (PM00104)

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Objectives: Zalypsis[®] (PM00104) is a new synthetic alkaloid that demonstrated a significant *in vitro* and *in vivo* activity against solid and non-solid tumors. The objective of this study is to determine pharmacokinetics of Zalypsis[®] in preclinical species. **Methods:** The studies were conducted in CD-1 mice, guinea pigs, rabbits, and dogs. A dose of Zalypsis[®] was given i.v. bolus to each of male and female animals. Blood was collected and plasma was harvested for analysis. LC-MS/MS assay was used to determine PM00104 plasma concentration. **Results:** In general, PM00104's plasma concentration-time profile displayed multi-compartmental kinetics without notable sex differences. The plasma levels dropped rapidly after i.v dose, followed by a much more gradual decrease. The plasma PM00104 concentrations declined with terminal half-lives ($t_{1/2}$) of 7, 8, 10 – 16, and 11 – 12 hours in mice, guinea pigs, rabbits and dogs, respectively. The plasma clearance (CL_p) values were 49 – 88, 73 – 84, 51 – 57, and 14 – 19 mL/min/kg for mice, guinea pigs, rabbits and dogs, respectively. The volume of distribution at steady state ($V_{d_{ss}}$) was much higher than plasma volume for all tested species, indicating extensive extravascular distribution of PM00104. Allometric scaling of PM00104 produced the following relationship:

$CL_p = 60.915 (\text{weight}^{0.6219})$, which predicted CL_p of 12.22 mL/min/kg for man. The observed mean CL_p value is 10.33 ± 2.38 mL/min/kg. **Conclusions:** Pharmacokinetic properties were established for Zalypsis[®] in preclinical species. Interspecies scaling projects a CL_p of 12.22 mL/min/kg in humans.

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Metabolism of Naveglitazar, a Peroxisome Proliferators-activated Receptor (PPAR) α - γ dual, γ -dominant Agonist in Mice, Rats, Monkey and Healthy Human Subjects

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Objectives: Naveglitazar (LY519818) is a non-thiozolidinedione PPAR α - γ dual, γ -dominant agonist in clinical development for the treatment of type 2 diabetes. The objectives of this study are to characterize the metabolism of naveglitazar in mice, rats, monkeys and humans and to determine if the human metabolism of naveglitazar is represented by the selected animal species. **Methods:** [14 C]naveglitazar was administered orally and/or intravenously, and whole blood, urine, feces and bile (bile cannulated rats only) were collected. Total radioactivity in various matrices was determined by LSC. Parent and metabolites were quantified by HPLC radioprofiling and identified by LC/MS/MS and/or NMR. **Results:** Naveglitazar-related radioactivity was well absorbed, extensively metabolized and predominantly excreted via feces across all species evaluated. Naveglitazar was the most abundant circulating entity in the plasma of all species evaluated. LY591026 (*R*-enantiomer) was a major circulating metabolite observed in the plasma of all species, which is formed through enzymatic chiral conversion of naveglitazar (*S*-enantiomer). In addition, *para*-hydroxylation of the biphenyl ether moiety (M2) followed by sulfate and/or glucuronide conjugation (M1, M3 and M12A-C) was another major metabolic pathway in all species. In humans, oxidative dehydrogenation (M7) and oxidative dehydrogenation plus taurine conjugation (M19) was observed as another major metabolic pathway. M7 was detected in all animal species, but M19 was only detected in humans. However, a taurine conjugate of parent (M18) was observed as a major metabolite in mouse. **Conclusion:** All metabolic pathways in humans have been observed in at least one of the selected animal species.

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Evaluation of a Higher Throughput Fluorescence Polarization Assay Method for Glucuronidation Assessment in Drug Discovery Profiling

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Glucuronidation is an important metabolic route for the inactivation and elimination of many drugs, environmental chemicals, and endogenous compounds such as bilirubin, bile acids and hydroxysteroids. The glucuronidation reaction is catalyzed by a family of UDP-glucuronosyl transferase (UGT) enzymes, which facilitate the conjugation of glucuronic acid from its co-substrate UDP-glucuronic acid to an acceptor substrate, generating a glucuronide and uridine diphosphate (UDP). Rapid UGT assays are now needed in the drug discovery process to identify Phase II metabolic liabilities, define the UGT specificity and reaction kinetics, and assist in the prediction of human pharmacokinetics of new chemical entities. The TransreenerTM UGT Assay (BellBrook Labs, Madison, WI) is a competitive, fluorescence polarization immunoassay, which directly detects the displacement by UDP of a UDP-AlexaFluor®-488 tracer from an anti-UDP antibody. This interaction reduces the effective molecular volume of the tracer and its rotational relaxation time, which results in a decrease in polarization value. UGT activity thus may be indirectly measured through a change in the fluorescence polarization of the displaced tracer. The assay platform was evaluated with several membrane preparations of the human recombinant isozyme UGT2B7 and two representative substrates, hyodeoxycholic acid and octyl gallate. Preliminary experiments have demonstrated that the TransreenerTM UGT Assay is

straightforward to perform and quite robust, with a Z' factor value greater than 0.5. With some additional methodological optimization, this high throughput assay may serve as a useful tool for early ADMET profiling in drug discovery.

CYP1A2 Polymorphism in the Beagle Dog

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Objectives: Cytochrome P450 plays a significant role in the oxidative metabolism of xenobiotics and endogenous substances. CYP450 activity can be affected by inhibition, induction and genetic polymorphism. Dogs are commonly used for pharmacological and ADME/toxicology studies of new drug candidates. A single nucleotide polymorphism, CYP1A2 1117 C>T that yields an early stop codon (truncated inactive protein) has been reported in dogs by Tenmizu. Past studies, conducted by Tenmizu and Mise suggest that 10-15% of dogs could be CYP1A2 deficient. Inter-individual pharmacokinetic variability may be observed in dogs after administration of drug candidates that are predominately eliminated by CYP1A2. Several drugs, such as, AC-3933 and YM-64227 have been reported to exhibit polymorphic metabolism in beagle dogs. In these studies, mutant dogs (TT) exhibited 5-17 fold higher mean plasma concentrations of unchanged drug than wild type or heterozygous dogs (CC/CT). **Methods:** Our internal dog colony was genotyped to identify animals for this CYP1A2 polymorphism which may affect the ADME/toxicology outcome of studies. Blood was taken from male beagle dogs, DNA was isolated, and genotyped. **Results:** Seventy nine dogs were genotyped, of these 61 (77%) were CC (wild type), 15 (19%) were CT (heterozygous), and 3 (4%) were TT (homozygous mutants). A possible explanation for our lower frequency of TT mutations may be the source of the dogs used in these various studies. **Implications:** Having knowledge of this polymorphism and identifying animals with this CYP1A2 deficiency can be useful in understanding and interpreting the metabolism and elimination of drugs from this common ADME/toxicology model.

Drug-Drug Interaction: Effect of Thiazolidinedione Analogues, Rosiglitazone and Pioglitazone, on Pharmacokinetics of Cilostazol in Rats

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Objective: To study the drug interaction potential of Rosiglitazone and Pioglitazone, potent PPAR γ agonists, with Cilostazol (anti-platelet drug) in Wistar rats by comparing the pharmacokinetics of Cilostazol and one of its active metabolite 3-4-dehydro Cilostazol. **Methods:** The *in vivo* pharmacokinetic interactions after oral administration of Cilostazol with and without oral administration of Rosiglitazone or Pioglitazone in rats were carried out. Either single dose or multiple doses (seven once daily doses pretreatment) of Rosiglitazone 3 mg/kg.b.wt or Pioglitazone 10 mg/kg.b.wt were administered simultaneously with single dose 10 mg/kg.b.wt Cilostazol. The plasma samples were analyzed for Cilostazol and its metabolite. **Results:** Single dose of Rosiglitazone/Pioglitazone have not significantly altered the pharmacokinetic parameters of Cilostazol and its metabolite where as the multiple dose pretreatment of Rosiglitazone significantly increased C_{max} (p<0.01) & AUC (p<0.01) of Cilostazol and significantly decreased C_{max} (p<0.01) & AUC (p<0.001) of the active metabolite. Multiple dose pretreatment of Pioglitazone has not changed the C_{max} but increased the AUC (p<0.05) of Cilostazol and significantly increased C_{max} (p<0.001) & AUC (p<0.001) of the active metabolite. The *in vitro* plasma un-bound fractions of Cilostazol were not altered in presence of Rosiglitazone. Significant reduction in levels of cilostazol metabolite is due to the moderate inhibitory potency of

Rosiglitazone on CYP3A4. And the increase in levels of cilostazol metabolite by Pioglitazone pretreatment needs to be studied further. **Conclusion:** These results suggest the potential for pharmacokinetic changes with Cilostazol in concomitant use of Rosiglitazone and Pioglitazone in diabetic patients.

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Efficacious Exposure Levels of ABT-869, an Orally Active Multi-targeted Kinase Inhibitor, in Human Xenograft Models

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Objectives: ABT-869 is a multi-targeted kinase inhibitor potent (IC₅₀ values < 100 nM) against all members of the VEGF and PDGF receptor families that is active in a range of tumor models. The goal of the present study was to determine the relationship between tumor growth inhibition (TGI) and drug exposure (plasma and tumor) in solid tumor xenograft models. **Methods:** TGI was assessed by measuring tumor size with calipers. Drug levels in plasma and tumor samples were measured by HPLC. **Results:** Oral dosing ABT-869 to an exposure (AUC_{24h}) as low as 0.7 µg•hr /mL resulted in 50% TGI in the HT1080 human fibrosarcoma xenograft model. At a higher exposure (2.7 µg•hr /mL) 75% TGI was achieved. This relationship between efficacy and exposure was representative of a group of xenograft models (HT1080, DLD1, MX1, H1299 and SW620). Other models (MiaPaCa, HCT116 and PC3M) were somewhat less sensitive (25-45% TGI) to ABT-869 whereas the MV4-11 and A431 models were more sensitive (tumor regression) at the same level of plasma exposure. Tumor exposure ranged from 2-fold to 7-fold the corresponding plasma exposure, indicating that ABT-869 was readily accessible to tumors in all models. In terms of duration of exposure an efficacious dose (70% TGI, 10 mg/kg/day bid) resulted in plasma concentrations that exceed the IC₅₀ for inhibition of cellular KDR in the presence of plasma (0.08 µg/mL) for only 3-4 of the 12 hours in the bid dosing cycle.

Implications: Based on these results, clinical pharmacokinetic targets were identified as an AUC_{24h} value of 2.7 µg•hr/mL and plasma concentrations above 0.08 µg/mL for ≥7 hours.

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Kinetic Equations for Modeling Drug-Drug Interactions (DDI) in a Multi-Drug System

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Objective: To establish a kinetic link between *in vitro* drug metabolism parameters and *in vivo* hepatic clearance for a system including multiple inhibitors and inducers. **Method:** Under the assumptions of steady-state and Michaelis-Menten kinetics, equations have been derived to depict the relationship between *in vitro* inhibition and induction parameters and the hepatic clearance of a drug. The proposed model provides a kinetic approach to transform the inhibitory effect of inhibitors into an enzyme reduction effect so that the net impact of DDI on intrinsic clearance may be evaluated. Based on this model, a drug interaction index (χ) has been defined. χ relates to the change in hepatic clearance due to DDI. **Implications:** This model attempts to provide a kinetic approach to normalize the effect of inhibitors and inducers in a multi-drug system. The net cumulative DDI effect may be projected using χ and drug candidates may be ranked for DDI potential using the parameters defined in this model. The intention of this model is to predict quantitatively *in vivo* hepatic clearance from *in vitro* kinetic parameters of both inducers and inhibitors.

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Analytical Validation and Performance Qualification of a new Accelerator Mass Spectrometry (AMS) Core Facility.

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Accelerator Mass Spectrometry (AMS) is an ultra-sensitive method able to quantify the amount of a radiocarbon-labeled compound in biological samples with attomole (10^{-18} M) sensitivity. AMS is an isotopic analysis method in which ions extracted from a sample are accelerated by high voltages, separated according to their momentum, charge and energy, and finally counted by an ion detector after identification by nuclear mass and charge. Increasingly, AMS is being applied to challenging problems in pharmacokinetics and metabolism. In January 2006, a new state-of-the-art AMS service facility dedicated to biomedical research was commissioned in Seattle. The facility features a National Electrostatics Corporation (NEC) Model 1.5SDH-1 Pelletron Accelerator. **Objective:** The objective of this study was to validate the analytical performance of Accium's AMS instrument and to demonstrate the exquisite sensitivity of this detection platform. **Methods:** The analytical performance of the AMS system was thoroughly assessed by means of extensive tests carried out under a strict Good Laboratory Practice (GLP) quality system using a suite of internationally accepted standards. **Results:** The measurements demonstrated that precision and accuracy of better than 0.4% are routinely achieved. A machine background corresponding to an isotopic ratio lower than 10^{-15} level was measured. **Implications:** AMS-based pharmacokinetics, drug metabolism and mass balance studies are increasingly providing critical supporting data or allowing earlier decision-making for drugs at various stages of development. FDA's recent release of the Guidance for Exploratory IND Studies (microdosing) further extends the need for increasingly sensitive bioanalytical methods.

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Accelerator Mass Spectrometry (AMS) Quantification of the Biodistribution and Pharmacokinetics of 157377, an Early Stage Highly Potent Inhibitor of Protein Tyrosine Phosphatase 1B (PTP1B) in Mice.

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Although several therapies are available for treatment of diabetes, many have significant side effects or later become ineffective. One potential new approach is to target the insulin signaling pathway directly through Protein Tyrosine Phosphatase 1B (PTP1B). An early stage highly potent lead molecule (157377) from a chemical series designed to specifically inhibit PTP1B, was found to be efficacious in a mouse diabetes and obesity model (C57Bl/6J ob/ob). Traditional pharmacokinetic analysis revealed oral bioavailability to be <6% with serum $T_{1/2}$ of 1.5 hours. **Objectives:** Based on the reported role of PTP1B in peripheral tissues, accelerator mass spectrometry (AMS) was employed to determine whether the observed efficacy of 157377 was due to residence time in specific tissues. **Methods:** 157377 was lightly labeled with ^{14}C and administered to mice by oral (20 mg/kg) or IV (4 mg/kg) routes. $^{14}\text{C}/^{12}\text{C}$ ratios in plasma, various tissues and residual carcass were quantified by AMS. **Results:** Plasma ^{14}C in both groups was cleared rapidly with a C_{max} of 1.5-2 hours in the oral group. No appreciable ^{14}C was detected in brain tissue. In heart, liver, kidneys, testes, muscle, fat and bone homogenates, both IV and oral groups showed rapid distribution and elimination of ^{14}C within 25 minutes post dose. Further PK and distribution parameters will be presented. **Implications:** This information helped to establish the potential correlation between tissue exposure and effect. AMS sensitivity extended the

detection range in plasma and tissue by several orders of magnitude using a lightly labeled ^{14}C compound.

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***In vitro* Metabolism of β -Lapachone (ARQ 501) in Mammalian Hepatocytes**

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Objectives: ARQ 501 (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione, β -lapachone) isolated from the lapacho tree (*Tabebuia avellanedae*) has been shown to be cytostatic or cytotoxic in different human tumor cells, and a fully synthetic version is currently in Phase II clinical trials. In order to elucidate the metabolic pathways of ARQ 501 in both animals and humans, *in vitro* cross-species metabolism of [^{14}C] ARQ 501 was conducted in mouse, rat, dog and human hepatocytes. **Methods:** Metabolites were characterized using liquid chromatography-mass spectrometry-accurate radioactivity counter (LC-MS-ARC). **Results:** ARQ 501 was found to be more stable in mouse, rat and dog hepatocytes than in human hepatocytes. Monoglucuronide conjugates were the major metabolites in mouse and human hepatocytes, whereas monoglucuronide and monosulfate conjugates were the major metabolites in rat hepatocytes, and monosulfate conjugates were the major metabolites in dog hepatocytes. Diglucuronide and oxidative glucuronide conjugates were detected in all of the studied species, while oxidative sulfates were only detected in rat and dog hepatocytes. **Conclusions:** No unique metabolites were identified in human hepatocytes that were not already identified in mouse, rat, or dog hepatocytes. The metabolic pathway of ARQ 501 was identified in human hepatocytes.

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Synthesis and Characterization of the Metabolites of ARQ 501 in Whole Blood

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Objective: ARQ 501 (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione, β -lapachone) is a potent and selective anti-cancer agent that has demonstrated anti-tumor activity that is mediated via checkpoint pathway activation in Phase 1 and 1b clinical trials. The goal of the present study was to characterize the *in vitro* metabolites of ARQ501 in human, dog, and mouse whole blood and confirm their identity by comparison to synthetic standards. **Methods:** Cross-species metabolite profiles from whole blood incubated with [^{14}C] ARQ 501 were obtained using liquid chromatography-mass spectrometry-accurate radioactivity counter. The metabolites were characterized using ultra-high performance liquid chromatography time-of-flight mass spectrometry. The synthetic standards were prepared using wet-chemistry techniques and their structural identity was confirmed using ^1H and ^{13}C NMR (DEPT, gHMQC, gHMBC, and NOESY). **Results:** ARQ 501 is metabolized more rapidly in mouse and rat blood than in dog, monkey and human blood. The primary metabolic pathways of ARQ 501 in blood involved the oxidation of the two adjacent carbonyl groups to produce dicarboxylic and monocarboxylic metabolites, the elimination of a carbonyl group to form a ring-contracted metabolite, and the lactonization to produce two metabolites with a pyrone ring. The synthetic standards yielded the same mass spectral pattern and co-eluted with the whole blood samples on HPLC. **Conclusion:** The metabolic profile of ARQ501 was established in human, dog, and mouse whole blood and the structural identity of the metabolites was confirmed using chemical synthesis.

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Simultaneous Assessment of Compound-Mediated Inhibition of Six Human Cytochrome P450 Enzymes in Human Liver Microsomes Using High-Throughput LC-MS

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Objective: A high-throughput liquid chromatography-mass spectrometry (LC-MS) approach for detection of potential cytochrome P450 (CYP) inhibition, called the “cocktail assay”, is becoming the industry standard. A number of scientists have reported new assays that include five cytochrome P450s, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 utilizing a LC-MS method with a six minute or longer gradient. **Methods:** Herein, we describe a cocktail assay that simultaneously characterizes compound-mediated inhibition of six major cytochrome P450 enzymes in pooled human liver microsomes through the use of specific CYP probe substrates, and a rapid, three minute LC-MS analytical method. The specific CYP substrates used in this cocktail assay included phenacetin, amodiaquine, tolbutamide, *S*-mephenytoin, dextromethorphan, and midazolam for CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5, respectively. The LC-MS method incorporates baseline separation of the six CYP substrates along with their respective metabolites, and an internal standard, labetalol. **Results:** In a cross-validation analysis, the concentrations of each CYP probe substrate in the assay had no effect (*i.e.*, inhibition or activation) on the other CYP activities. Furthermore, the assay conditions for the cocktail were validated against the single isoform assays using 22 compounds with known CYP inhibition liabilities. The inhibitory constant (K_i) determined with this cocktail assay was highly correlated ($r^2 \geq 0.94$ for each isoform) with that of the single isoform substrate assay for the 22 CYP inhibitors. **Implication:** This CYP inhibition cocktail assay has increased the efficiency of our efforts to assess compounds for inhibition of the six major CYP isoforms in a drug discovery setting.

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Evaluation of the use of U-HPLC and Accurate Mass LC-MSⁿ for the Determination of Metabolites in Species Comparison Studies

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Objectives: The objectives of this work were to investigate the use of U-HPLC and accurate mass spectrometry for the early evaluation of metabolism data and selection of appropriate toxicology species to reflect drug behaviour in man. **Methods:** Parallel incubations of radiolabelled and non-radiolabelled diazepam (10 μ M) were undertaken with liver microsomes from various species. Analysis was carried out using a LTQ Orbitrap hybrid mass spectrometer and on-line radiodetection. Metabolites of diazepam were located through accurate mass or radioactivity, with coverage compared from the two detection methods. Potential metabolites formed using radiodetection and accurate mass LC/MSⁿ were compared and productivity gains observed when utilising U-HPLC were investigated. **Results:** Accurate mass U-HPLC-MS data using predicted m/z values for metabolites confirmed it as capable of identifying major metabolites when compared to radio-HPLC. Time savings of 70% and improvements in sensitivity were also achieved using U-HPLC-MS. Product ion spectra containing peaks with mass accuracies < 3 ppm enabled comprehensive assignment of the product ion mass spectra for

metabolite identification. **Implications:** Accurate mass LC-MSⁿ provides a pragmatic alternative to the use of costly radiolabelled compounds for utilisation in early metabolite profiling and screening studies. Species comparison results obtained during this investigation correlated with those in the literature.

Rapid Identification of Compounds that are Unstable to CYP Mediated Metabolism

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Objective: The purpose of the study was to develop a computational method to identify metabolically unstable compounds at the early drug discovery stage. **Methods:** The intrinsic clearance (CL_{int}) of each compound was evaluated by measuring compound disappearance from human liver microsome incubates (0.5mg/ml @ 37 °C). The apparent CL_{int} values of 6309 compounds were used to develop and validate a binary classification model using physicochemical properties plus functional class fingerprints and support vector machine (SVM) algorithms. The model was trained and validated using 3141 and 3168 compounds categorized as metabolically stable or unstable compounds with a CL_{int} of 100 µl/min/mg as cutoff criteria. **Results:** The CL_{int} SVM classification model achieved overall 80% prediction accuracy with 80% selectivity and 80% specificity when applied to the validation compound set (n=2193). It was demonstrated that the distance of each compound to the separating surface in the feature space ($|d|$) of the SVM model could be used as confidence index for the prediction. The predictivity of the model increased to 88% for the compounds with $|d| > 0.5$ in the validation set. This model accurately predicted 87% of the compounds with $|d| > 0.5$ in an external validation set (n=1498), where CL_{int} values were evaluated experimentally after the model was developed. **Conclusions:** The high classification accuracy indicates that this SVM binary classification model can be used as a rapid computational filter for identifying metabolically unstable compounds and for prioritizing the synthesis of new compounds at early stages of drug discovery.

CYP-mediated Bioactivation of a Thiazole and *p*-Cresol-containing Compound

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Thiazole and *p*-cresol moieties are present in a variety of medicinal compounds and natural products. In the current study, biotransformation of Compound I containing both thiazole and *p*-cresol was investigated in human and rat liver microsomal incubations with NADPH. Metabolic profiling using HPLC/UV/MSⁿ demonstrated a P450-catalyzed metabolic activation on the thiazole moiety, producing a substantial amount of a dihydrodiol metabolite (Met1) and a ring-opened metabolite (Met2, thiourea). Subsequent glutathione (GSH) trapping experiments indicated the presence of a reactive intermediate through the detection of two thiazole-GSH adducts (Met4 and Met5). Based on the structure of identified metabolites and adducts, it is proposed that the thiazole moiety was oxidized by CYPs to form a reactive epoxide intermediate, followed by subsequent nucleophilic attack by water or GSH to give Met1, Met4 or Met5, respectively. It was documented the toxic properties of thiourea and thioamid resulted from thiazole bioactivation in laboratory animals. The *p*-cresol portion of Compound I was also found to be metabolized in microsomal incubations. A *p*-quinol metabolite, Met3, was characterized by NMR spectroscopy after isolation. Its formation is hypothesized via P450-mediated *ipso*-oxidation of the *p*-cresol. The toxicity of *p*-toluquinol has been reported in literatures and was

attributed to its property to covalently bind to DNA, RNA and other cellular macromolecules. The metabolic findings from the present study, along with other literature reports, provided additional evidence of CYP-catalyzed bioactivation of thiazole and *p*-cresol, which should either be avoided or be placed in perspective during lead optimization.

In Silico, In Vitro, and In Vivo Data Generation to Select and Optimize Lead Series in Early Drug Discovery.

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Objectives: Selecting the best possible series is usually driven by potency and chemical do ability. Ignoring ADME properties during series selection could result in significant delays, as incorporating ADME properties later in a series might be challenging or not possible at all. Hence, beginning at the hit triage stage, we employ *in silico*, *in vitro* and *in vivo* data to select and guide the optimization of lead series. **Methods:** First, multi-parametric analysis (MPA), which combines physicochemical properties, *in silico* ADME and potency, is used to rank order series. Next, for the most attractive series, selected ADME data is generated on a few compounds representing the chemical space of that particular series. The relationship between ADME and potency space can then be established through re-trained *in silico* ADME models. Additionally, for example assessing brain penetration, the physicochemical and *in silico* understanding is calibrated with a few *in vivo* data points and subsequently used for series evaluation as well as follow on library designs. **Results/Implications:** Consequently, the series with over lapping ADME and potency space is selected for hit-to-lead optimization. In this presentation, this concept will be described using MPA, *in vitro* microsome stability and Pgp liability, and *in vivo* brain penetration as examples.

In Vivo Microdialysis Study of Cortisol Metabolism in Monkey Adipose Tissue

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Purpose: Cortisol metabolism is related to the pathogenesis of metabolic diseases such as obesity and type 2 diabetes. 11 β -hydroxysteroid dehydrogenase type 1 enzyme (11 β -HSD1) regenerates cortisol from inactive cortisone in tissues. To better understand the tissue-specific 11 β -HSD1 regulated cortisol metabolism, a microdialysis sampling coupled with an LC/MS/MS assay was developed to detect 11 β -HSD1 activity *in vivo*. **Methods:** Stable isotope labeled (SIL) cortisone was used as substrate and delivered to Rhesus monkey adipose tissue via a local infusion through the microdialysis probe. The rate of conversion from SIL-cortisone to SIL-cortisol was used as a biomarker of 11 β -HSD1 activity. The extraction efficiencies (E_d) of microdialysis probe for SIL-cortisone and cortisol were tested *in vitro* in Ringer's solution and *in vivo* by retro-dialysis. Different concentrations of SIL-cortisone were infused to monkey adipose tissue by microdialysis and SIL-cortisol production profiles were obtained. **Results:** At flow rates 1.0 and 0.5 μ L/min, average E_d for SIL-cortisone and SIL-cortisol were between 60 and 70%. SIL-cortisol production was observed when 100, 500 and 1000 ng/mL SIL-cortisone was infused at flow rates of 0.5 and 1.0 μ L/min to monkey adipose tissue. **Conclusions:** Results show that it is feasible to sample cortisone and cortisol in monkey adipose tissue by *in vivo* microdialysis. The probe was well tolerated throughout the study. The optimal SIL-cortisol response was observed when 500 ng/mL SIL-cortisone was infused at 0.5 μ L/min, considering sufficient microdialysis E_d and sample

collection volume. This methodology could potentially be utilized to study *in vivo* tissue cortisol metabolism.

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Use of the IC50-Shift Assay for Identification of Time-Dependent CYP3A inhibition in Drug Discovery

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In vitro screening for drug-drug interactions is well established in drug discovery, particularly for detection of reversible inhibition of cytochrome P450 enzymes. Compared to reversible inhibition, irreversible or time-dependent inhibition is of greater concern, because enzymes are irreversibly inactivated and enzyme activity can only be restored by *de novo* synthesis. Testing for time-dependent CYP inhibition is a time-consuming and labor-intensive study because it requires multiple parameters such as time-, NADPH- and concentration-dependency to be quantified. A miniaturized version of the time-dependent CYP inhibition (TDI) assay, the IC50-shift assay, was used to allow for a more rapid evaluation of discovery compounds. The IC50-shift assay is based on the observation that time-dependent inhibitors cause the IC50 to decrease when pre-incubated with NADPH. Similar to the TDI assay, the method uses pooled human liver microsomes and midazolam-1'hydroxylation as marker substrate reaction. Based on the evaluation of a number of marketed drugs and Novartis compounds, the IC50-shift assay was assessed in terms of its predictability and reproducibility. The results of this evaluation, and the advantages as well as limitations of the IC50-shift assay will be discussed.

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Expression Profiling of Drug Transporter, Cytochrome P450 and Nuclear Receptor Genes in Human Hepatocytes and Caco-2 Cells using DTExtm Microarrays: The Effect of Drug Treatment on Cytochrome P450 Induction

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Objectives: To investigate the modulation of drug transporter (efflux and uptake), cytochrome P450 and nuclear receptor gene expression levels in both freshly isolated human hepatocytes and Caco-2 cells treated with either beta-naphthoflavone [BNF] or rifampin [RIF] as a function of treatment duration. **Methods:** We have developed a cDNA microarray which permits the examination of changes in drug transporter (efflux and uptake), cytochrome P450 and nuclear receptor gene expression levels (DTExtm). Caco-2 cells were cultured in 100mm dishes for 21 days and then treated with either beta-naphthoflavone or rifampin. Fresh plated human hepatocytes were incubated overnight in medium and then treated with either beta-naphthoflavone or rifampin. Total RNA was isolated at 2, 4, 6, 8 and 24 hours from both cell types using TriZol reagent. Total RNA [20ug] was reverse-transcribed with SuperScript II and converted to labelled cDNA using Cy5-random pentadecamers. Microarrays were hybridised for 18 hours at 45°C. Microarray images were acquired with ScanArray and image analysis was performed with QuantArray. Matrix plots and clustering dendrograms were generated using GeneLinker Gold. **Results:** The experiments indicate that gene expression profiles are distinct for different drugs and involve both the induction and suppression of drug transporter (efflux and uptake), cytochrome P450 and nuclear receptor gene expression as a function of the duration of drug exposure. **Implications:** Induction of cytochrome P450 and nuclear receptor gene expression by drugs can be demonstrated using DTExtm microarrays. These gene expression profiles could be employed as surrogate indicators of potential drug:drug interaction and/or adverse drug effects.

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Phenacetin as a CYP1A2 Probe Substrate in the Dog

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Objectives: Phenacetin is a substrate of cytochrome P450 1A2 (CYP1A2) and is widely used as an *in vitro* probe. Phenacetin undergoes O-deethylation by CYP1A2 to form acetaminophen. This study investigates the use of phenacetin as a probe substrate to phenotype CYP1A2 activity in dogs. **Methods:** Beagle dogs were previously genotyped for a single nucleotide polymorphism, CYP1A2 1117 C>T that yields a truncated inactive protein. Based on genotype, dogs were selected and placed into three groups: CC (wild type), CT (heterozygous), and TT (homozygous mutants). The dogs (n=3/group) were dosed orally (PO) and intravenously (IV) with 15 mg/kg of phenacetin. Blood samples were taken over 72 hours. Samples were analyzed by LC/MS/MS for phenacetin and acetaminophen. **Results:** The exposures of phenacetin and acetaminophen were compared between the three groups of dogs after IV and PO administration. After IV dosing, all groups showed similar exposures to phenacetin. After PO dosing, the exposure of phenacetin in CC & CT dogs was similar, but was 2-fold greater in TT dogs. The mean acetaminophen:phenacetin ratio in TT dogs was about half that observed in the CC. **Implications:** Although oral exposure of phenacetin was higher in CYP1A2 deficient dogs, the results were considered modest. Previous studies using other CYP1A2 specific substrates in TT dogs displayed 5-17 fold higher oral exposures of unchanged drug than CC/CT dogs, which are greater than those observed in this study. Therefore, although phenacetin is regarded as an ideal CYP1A2 substrate *in vitro*, it may not be a specific *in vivo* probe substrate in the dog.

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In silico Prediction of Biliary Excretion of Drugs in Rats Based on Physicochemical Properties

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OBJECTIVE: Evaluating biliary excretion (BE), a major elimination pathway for many compounds, is important in drug discovery. The bile duct-cannulated (BDC) rat model is commonly employed to determine the percent of dose excreted as intact parent into bile. However, a study using BDC rats is time-consuming and cost-ineffective. The present study aims to establish a computational model to predict BE of intact parent in rats as a percent of dose. **METHODS:** The model was based on BE data (ranging from 0.1 to 100%) of 50 compounds from various chemotypes. The compounds were intravenously dosed to BDC rats and bile was collected for at least 8 hours after dosing, and the recoveries of intact drug in bile were determined by LC/MS. **RESULTS:** Individual correlation analysis found a high correlation coefficient (r) between BE and polar surface area (PSA) (r = 0.76), and delta G solvation in water (ΔG) (r = -0.67). In addition, the presence of a carboxylic acid (CA) moiety in the test compounds resulted in a significant correlation with high BE. An equation to predict BE in rats was established from fitting the BE data via multiple regression. For the training set, a high correlation (r = 0.94) was seen between the predicted and observed BE results. This model also successfully (r = 0.88) predicted the extent of biliary excretion of twenty four published compounds. **CONCLUSIONS:** A computational model has been established to predict biliary excretion of intact parent in rats.

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The Pharmacokinetics properties of BL-3020, a Melanocortin Receptor-4 Agonist for the Treatment of Obesity

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Purpose: The melanocortin signaling pathway is a key endocrine system involved in body weight homeostasis. Binding of the MC4R to its ligand α MSH induced satiety and increases energy expenditure, thus leading to weight loss. BL-3020 is a cyclic peptide based on the minimal sequence of α MSH, and capable of specifically activating the MC4 receptor. Backbone cyclization has been employed in order to improve in-vivo stability of the peptide while maintaining binding affinity. In this study we have evaluated in-vitro stability and pharmacokinetic parameters of BL-3020. **Methods:** The metabolic stability and intestinal permeability of BL-3020 were evaluated using Brush Border Vesicles and Caco-2 monolayers, respectively. The pharmacokinetic profile following IV administration was assessed in cannulated rats. **Results:** BL-3020 was found to be stable to enzymatic degradation with less than 5% reduction in concentration following 90 minutes incubation. The linear equivalent peptide was unstable with a 40% reduction of the initial concentration. Permeability coefficient of the BL-3020 cyclic peptide was high suggesting that the mechanism of intestinal absorption is transcellular. PK studies revealed that the elimination $t_{1/2}$ was 3h and the volume of distribution at steady state is 3.9L. These high values indicate that the cyclic BL-3020 is stable and that distribution is not confined to blood or intercellular fluid. Moreover, examination of the rats' brain revealed that BL-3020 crosses the blood brain barrier and is detectable in the brain for at least 8h. **Conclusion:** Using backbone cyclization we improved stability and the pharmacokinetic profile of BL-3020, a potential anti-obesity drug.

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Metabolism and Pharmacokinetics of a potent kappa-selective opioid agonist (PNU-50,488) in male Sprague-Dawley rats.

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In the past two decades, the quest for strong analgesics with a minimal abuse potential has been the focus of active pharmacological research. Discovery of the benzacetamide amines¹, was an important milestone in this direction, the prototype of this series being the kappa selective agonist PNU-50,488. **Objectives:** The objective of the present study was to determine the disposition of PNU-50,488 after a single intravenous (*i.v.*) bolus (1 mg/kg) and oral (10 mg/kg) dose, in Sprague-Dawley rats. **Results:** Upon *i.v.* dosing, plasma concentrations of PNU-50,488 declined rapidly with a terminal half-life of 1.25 hr; the corresponding total plasma clearance (CL_p) of 141 ± 0.23 mL/min/kg exceeded hepatic blood flow in the rat. PNU-50,488 demonstrated a moderate volume of distribution at steady state ($V_{d,ss}$) at 9.1 ± 0.22 L/kg. Following oral administration, mean peak plasma concentration (C_{max}) of 56.3 ± 0.21 ng/mL was attained at a T_{max} of 1 hr. The corresponding $AUC_{0-\infty}$ was 164 ± 0.18 ng-hr/mL. The absolute oral bioavailability of PNU-50488 was very low (13%). Studies in portal- and jugular-vein-cannulated rats revealed that the fraction absorbed across the gut lumen was moderate at 51 %. **Conclusions:** These observations in conjunction with the observed total CL_p indicate an extra-hepatic component

contributing to the elimination of PNU-50,488 *in vivo*. Identification of the metabolic liabilities of PNU-50,488 could aid in further deducing this extra-hepatic component. In summary, the present study represents the first report on preclinical disposition of PNU-50,488 in male Sprague-Dawley rats.

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The Development of an Early and Rapid Screening Assay to Assess the Unbound Intrinsic Clearance in Microsomes

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Objectives: Unbound intrinsic clearance is an *in vitro* pharmacokinetic parameter which if determined early in the drug screening funnel, can add more value than a standard metabolic stability assay. In the proposed screening paradigm, unbound intrinsic clearance would be determined in a single species and extrapolated to multiple species.

Methods: The unbound intrinsic clearance was determined for several marketed compounds in rat, dog, and human liver microsomes. To determine unbound intrinsic clearance, the intrinsic clearance at 4 protein concentrations (0.1, 0.25, 0.5, and 1 mg/mL) was measured. For use as a primary screen the time points used were 0, 15, and 30 minutes. Unbound intrinsic clearance for human, rat, and dog was calculated by back extrapolating the four intrinsic clearance values to 0 mg/mL protein. Additionally, the free microsomal fraction was calculated by dividing the extrapolated unbound microsomal clearance by the intrinsic clearance at 0.5 mg/mL. Microsomal unbound fraction was determined experimentally using equilibrium dialysis as a comparison with the microsomal unbound fraction calculated from the unbound intrinsic clearance for each species. **Implications:** Use of assay methods to determine the unbound intrinsic clearance at an early stage in the drug discovery process will allow more meaningful data through realization of SAR of a pure pharmacokinetic parameter that is not confounded by differences in non-specific binding. Additionally, utilization of the unbound intrinsic clearance may prevent misinterpretation and prioritization or de-prioritization of compounds which are highly bound to microsomal protein.

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Survey of Reactive Intermediate Formation: Conjugate Detection Across a Variety of Species

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Adverse drug reactions (ADR) are a serious concern to the medical community and have been the focus of considerable research. Idiosyncratic drug reactions (IDR) are a subset of ADRs that occur in a small percentage of patients, are not dose dependent, and are not associated with known metabolism of a drug. IDRs for a new drug carry significant risk, since no a priori warning is detected prior to widespread use. A number of factors contribute to the development of an IDR, including an individual's balance between metabolic bioactivation and detoxification pathways. Generally, it has become accepted that bioactivation of some drugs can lead to reactive intermediates capable of covalent attachment to proteins, possibly culminating in a toxic response. **Objective:** In preparation to address this issue, studies were designed to evaluate the formation and detection of GSH conjugates for a series of compounds with a known propensity to form reactive intermediates. **Methods:** A series of compounds was studied using various hepatic systems, including microsomes and S9 fractions from various species (rat, dog, monkey and human). Samples were analyzed by multiple LC/MS methods. **Results:** Mass spectral detection of glutathione adducts can be method and compound dependent. Data analyses for several compounds are presented. **Implications:** Species differences and mass spectral analysis type can be important parameters in designing broad screens for reactive metabolite detection.

Is CYP2C9 an Important Determinant of Diclofenac Disposition in Humans?

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Objectives: Our previous studies suggested that the clearance of diclofenac in humans may be largely determined by acyl glucuronide (AG) formation, with smaller contribution from CYP2C9-catalyzed metabolism to 4'-hydroxydiclofenac. This contrasts with the belief that 4'-hydroxylation accounts for a major portion of diclofenac elimination, as suggested by excretion of ~50% of the dose as 4'-hydroxydiclofenac and its glucuronic acid conjugate. We proposed earlier that a portion of the 4'-hydroxydiclofenac glucuronide could arise via a unique CYP2C8-catalyzed 4'-hydroxylation of diclofenac AG. Here, we present data in support of this emerging view of diclofenac metabolism in humans. **Methods:** The intrinsic clearances of 4'-hydroxydiclofenac and diclofenac AG formation were determined in 14 individual human liver microsome preparations to determine their relative efficiency for diclofenac elimination. Role of CYP2C8 and 2C9 in the formation diclofenac metabolites in human hepatocytes was evaluated using selective inhibitors of these enzymes. **Results:** Formation of diclofenac AG contributed ~65% of total diclofenac metabolism, while metabolism to 4'-hydroxydiclofenac accounted for only ~35%. Sulfaphenazole, a potent CYP2C9 inhibitor, had no effect on diclofenac AG formation but reduced the formation of unconjugated 4'-hydroxydiclofenac by 80-90%. In contrast, formation of 4'-hydroxydiclofenac glucuronide was inhibited by only ~50%, indicating that up to 50% of the 4'-hydroxydiclofenac glucuronide in human hepatocytes may arise from CYP2C9-independent pathways. **Implications:** These data suggest that acyl glucuronidation may be a major determinant of diclofenac clearance and question the validity of diclofenac pharmacokinetics and/or the net (free+conjugated) formation of 4'-hydroxydiclofenac as a selective phenotypic probe for CYP2C9 activity in humans.

Imidazole Ring Scission: An Uncommon Metabolic Pathway

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Objectives: Imidazole ring scission resulting in loss of C-4,5 carbons, potentially in the form of glyoxal, has rarely been reported in the literature. The pesticide imazalil is an imidazole containing compound in which this N-dealkylated metabolite was reported in rat (Meuldermans et al., 1977b). In the current study, NVP-XYZ123, is proposed to undergo oxidative metabolism to yield this ring cleaved N-dealkylated metabolite (M1). **Methods:** LCMS and LCMS/MS characterization of M1 were used to produce the fragmentation pattern and accurate mass within 5ppm suggesting loss of 2 carbons precisely from the imidazole ring. To understand enzymes and species capable of mediating this metabolic pathway, several incubation conditions were tested. **Results:** Incubations with and without the cofactor NADPH or the partial CYP450 inactivator Triton-X indicate the involvement of CYP450s. M1 was observed in rat, dog, monkey and human liver microsomes indicating this is not a species specific mechanism. M1 was also detected in dog plasma after iv administration. Tests with human and monkey liver microsomes demonstrate that the ring scission pathway is more extensive with NADPH as compared to dog where it is more extensively formed without NADPH. Rat microsomal incubations showed no significant difference with and without NADPH. **Conclusions:** The varying relative amounts formed across species (+/- NADPH) suggest the involvement of multiple enzyme systems.

Studies with specific inhibitors and/or recombinant enzymes are ongoing to help elucidate the enzymes involved in this metabolic process.

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Identification of Novel Diclofenac Metabolites in Rats.

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Objectives: Two novel metabolites of diclofenac were identified in rat bile and rat and human hepatocyte incubations: a benzyl-S-glutathione conjugate and 2-(2,6-dichlorophenylamino) benzoic acid. A mechanism for bioactivation of diclofenac involving decarboxylation is proposed. Diclofenac is a NSAID that is widely prescribed for the treatment of osteoarthritis, rheumatoid arthritis, and acute muscle pain conditions. Treatment with diclofenac can cause a rare, but severe, incidence of hepatic injury often described as idiosyncratic toxicity. Although the exact mechanism of diclofenac hepatotoxicity is not understood, it has been proposed that metabolic activation of the drug and subsequent covalent modifications of proteins by reactive metabolites may directly impair cellular signal transduction cascades or indirectly eliciting an immune response. **Methods/Results:** Here we describe the use of high resolution accurate mass spectrometry to identify two unique metabolites of diclofenac. The glutathione adduct was detected in rat bile following intravenous administration of diclofenac and following incubation of diclofenac with rat or human hepatocytes. **Implications:** A possible bioactivation pathway leading to a reactive intermediate is rationalized. It is our hypothesis that, in addition to the mechanisms proposed by others, bioactivation of diclofenac also occurs at the carbon alpha to the carboxylic acid. The presence of the glutathione conjugate in human hepatocyte incubations indicates that the bioactivation process is not rat specific and can occur in humans.

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A Bioluminescent Assay System for Measuring UDP Glucuronosyltransferase (UGT) Activity

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Objectives: The UDP glucuronosyltransferase (UGT) family of enzymes is involved in the metabolism of various compounds in the body. The UGTs act on various endogenous substrates as well as xenobiotics and drugs. The function of these enzymes is essential for the clearance of drugs and other toxins from the body and alteration of UGT activity could potentially cause drug-drug interactions *in vivo*. The current methods for assessing UGT enzyme activity are laborious and involve protein precipitation and/or chromatographic separation steps, which are not amenable to higher throughput screening applications for UGT inhibitors or activators.

Methods: Here, we present a new bioluminescent assay system for measuring UGT enzyme activity *in vitro*. Our assay does not involve any protein precipitation or chromatographic steps and is easily performed in a multi-well plate format. **Results:** We have shown the ability of our assay to measure activity of many recombinant UGT enzymes as well as assessing endogenous UGT activities from animal tissue microsomes. We were able to detect inhibition by compounds known to inhibit numerous isozymes and we verified published data showing that some isozymes are inhibited by the HIV protease inhibitors liponavir and ritonavir while other isozymes are relatively unaffected. Assay variability, as measured by Z' values, have been calculated for UGT 1A1 [Z' = 0.83] and UGT 2B7 [Z' = 0.67]. **Implications:** Our new assay format could greatly

increase the throughput for assessing UGT activity and enable efficient screening of UGT isozymes against compound libraries.

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Assessment of CYP1A2, CYP2B6, and CYP3A4 Enzyme Induction in Primary Cultures of Human Hepatocytes Using a High Throughput Cocktail Assay

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Objectives: The current study investigates the feasibility and reliability of utilizing a cocktail substrate method to simultaneously assess CYP1A2, CYP2B6, and CYP3A4 enzyme induction in primary cultures of human hepatocytes. The simultaneous assessment of multiple cytochrome P450 enzyme activities increases study productivity and efficiency by reducing sample numbers, laboratory time, and raw materials (e.g. primary human hepatocytes). **Methods:** Primary human hepatocytes were cultured in the presence of vehicle (0.1% DMSO), 3-MC (2 μ M), phenobarbital (1000 μ M), or rifampicin (10 μ M) for 48 and 72 hours prior to enzyme activity analysis. A substrate cocktail consisting of 100 μ M phenacetin, 500 μ M bupropion, and 200 μ M testosterone was incubated with hepatocyte monolayers (*in situ*) or with microsomes prepared from cultured hepatocytes to determine cytochrome P450 enzyme activity. Incubation samples were extracted and analyzed using LC-MS/MS. Total RNA was isolated for each time point and qRT-PCR was utilized to quantify CYP1A2, CYP2B6, and CYP3A4 mRNA content. **Results:** Preliminary data from incubations with human liver microsomes indicated that production of isoform-specific metabolites from all three probe substrates were linear with respect to time and protein concentration. Enzymatic activity data derived from substrate cocktail incubations were also demonstrated to be consistent with traditional single-probe approaches. **Implications:** The substrate cocktail method offers a robust and efficient method to determine the cytochrome P450 induction profile of many new chemical entities in primary human hepatocytes. Due to their limited supply, this method will also greatly facilitate the use of this model system in cytochrome P450 induction profiling.

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A Comparison of CYP3A4 Induction as Measured by Activity Versus mRNA Transcript in Cryopreserved Human Hepatocytes

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The induction of cytochrome CYP3A4 can have important clinical significance. CYP3A4 gene expression is inducible by numerous xenobiotics, resulting in altered drug metabolism and drug-drug interactions. Induction of CYP3A activity in the clinic can result in therapeutic failure such as tissue rejection in transplant patients or unwanted pregnancy, among others. The objective of this investigation was to evaluate several known inducers of CYP3A4 in humans, using cryopreserved human hepatocytes, and to compare activity versus mRNA data. Cryopreserved human hepatocytes were treated with several known CYP3A4 inducers at a range of concentrations for 72 hours. CYP3A4 induction was assessed by enzymatic activity (testosterone 6 β -hydroxylase) as well as by mRNA analysis via two-step real-time PCR. Consistently, the mRNA fold-induction data results were generally equivalent or higher when compared to the activity data. Even though the enzymatic activity has been the preferred route for assessing induction, mRNA levels may be more informative in cases where the drug is also an enzyme inhibitor. The data generated from this study demonstrates that measurement of mRNA, while

providing a useful supplement to activity data, may also serve as a more sensitive and convenient tool for assessing CYP3A4 induction *in vitro*.

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¹⁴C-Acetaminophen Metabolism And Excretion In Chronic Bile Duct Cannulated Dogs

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Objectives: This study aimed at development and evaluation of a chronic bile duct cannulation model allowing bile collections from a conscious dog. **Methods:** Female Beagle dogs were implanted with a “T” type catheter with central occlusion bulb, anterior catheter for bile collection and posterior catheter for infusion of bile replacement fluid. Approximately 5 months after the surgery, 3 dogs received a single oral (capsule) dose of 250 mg ¹⁴C-acetaminophen (80 µCi). Blood, bile and urine samples were collected periodically over 24 hours post-dosing. All samples were analyzed for total radioactivity and profiled using radio-HPLC. The identity of the drug metabolites was determined by LC-MS/MS. **Results:** C_{max} in plasma was reached at 2 or 3 hours post-dosing and ranged from 16.7 to 29.0 µg/g, AUC ranged from 141 to 226 µg×hr/g; and half-life was between 7 and 11 hours. Similar amounts (expressed as % of dose) were recovered over the 24-hour post-dose period in the urine (70%) and bile (9 to 13%) of all 3 animals. Metabolite profiling of the plasma metabolites demonstrated the presence of the parent drug and up to 2 metabolites; the later were present in the bile and urine and were identified as the glucuronide and sulphate conjugates of acetaminophen. **Conclusion:** This chronic bile duct cannulation model proved suitable for drug metabolism studies in dogs.

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MIST: Changing Strategies for Early Metabolite Identification

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Introduction: With recent publication of the FDA Guidance for Industry; Safety Testing of Drug Metabolites, there is increasing focus on strategies for ADME study design, and how metabolite data can be obtained earlier to influence decision making. We discuss a strategy to accelerate early attainment of metabolite data, using samples from a variety of pre-clinical studies. **Methods:** An LTQ Orbitrap mass spectrometer coupled with UPLC provides a powerful high resolution tool for data generation. The strategy uses samples generated during *in vitro*, toxicology, radiolabelled and early clinical studies. Data dependant analysis generates information on the number, quantity and character of metabolites present. **Results:** We will present data demonstrating information that can be obtained from a variety of sample matrices, highlighting reduction of analysis time, increased analytical resolution, and increased data confidence, supporting our interpretation of the data required by the MIST guidelines. In samples from repeat dose toxicology studies, over 30 metabolites were observed. The results were compared with those from subsequent radiolabelled *in vivo* metabolism studies and data from *in vitro* studies. We demonstrated that analysis of non-radiolabelled samples was successful in identifying all the major metabolites and many minor metabolites. **Conclusions:** We compared metabolite data from *in vitro* incubations, and after single dose or repeat dosing to toxicology species. Accurate mass UPLC-MS/MS identified 30 major metabolites from toxicology study samples, and detected minor metabolites not observed by radio-HPLC. Information on species specific metabolites were obtained without the need for additional sampling or studies.

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Automation of Surface Ionization Technology for Determination of Drug Candidates from Blood and Serum without Sample Preparation.

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Extensive sample preparation is normally required for analysis of blood and serum prior to high throughput DMPK analysis by using LC/MS/MS. Ambient surface desorption ionization technology, in this case direct analysis in real time (DART®), has been shown to provide a means to ionize biological samples such as blood, plasma, and urine seconds. We have interfaced the DART ionization source to an ABI / MDS SCIEX API-4000 LC/MS while incorporating a LEAP Technologies CTC CTS PAL laboratory robot to permit high throughput sampling. DART technology ionizes samples from surfaces, therefore we have developed sample holders for introduction of the biological fluid into the ionization region of the system without sample preparation. Protocols that enable efficient ionization and subsequent detection of drug candidates present in the biological fluids with MS/MS are shown to facilitate high throughput quantitative analysis of those candidates. The poster will describe the improvement in sensitivity and quantitative capability associated with development of novel vacuum assisted sampling technology that was not available with the original DART. Experimental results obtained with this instrument configuration show an improvement in response for a broad range of analytes and improved CV's of >25% to <15% with implementation of the more reproducible positioning of samples. An examination of the relative response of compounds as a function of sampler shape, composition and location was completed.

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CAR and PXR – Mediated Regulation of CYP2C9 in Primary Human Hepatocytes

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Cytochrome P450 2C9 (CYP2C9) expression is regulated predominantly by the nuclear receptors CAR and PXR. The relative importance of these receptor pathways to drug-induced expression of CYP2C9 remains unclear. CYP2B6 and CYP3A4 are currently thought to be the most sensitive genes to CAR and PXR respectively. Comparing the co-regulation of CYP2C9 with these genes using selective inducers for each pathway can provide a useful approach to characterize the profile of CYP2C9 induction. We used the PXR activators clotrimazole, rifampin and ritonavir, the mixed-function nuclear receptor activator (CAR/PXR) phenobarbital, and the CAR agonist CITCO and examined their effects on CYP2C9. Concentration-response profiles were determined for CYP2C9 in primary human hepatocytes treated with phenobarbital [CAR/PXR], phenytoin [CAR] and rifampin [PXR]. The EC₅₀ values were 519 μM, 11 μM, and 0.75 μM, respectively, similar to those for CYP3A4 induction. Rifampin increased mean basal CYP2C9 activity from 59 ± 43 to 143 ± 68 pmol/(min*mg) and the fold induction ranged from 1.4- to 6.4-fold. Additional studies with rifampin, CITCO and PB produced potency and efficacy trends consistent with the concept that regulation of CYP2C9 by common inducers is analogous to that of CYP3A, rather than CYP2B. Overall, our studies show that hepatic CYP2C9 activity is differentially induced by a number of xenobiotics identified as agonists of CAR and PXR. CYP3A4 and CYP2C9 share common regulatory mechanisms in response to inducers, providing

further evidence that PXR plays a major role in the induction of CYP2C9 by drugs and other xenobiotics.

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Effect of BDE-47 on Hepatic Drug Metabolizing Enzymes in the Rainbow Trout

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Polybrominated diphenyl ethers such as BDE-47 are used extensively as flame retardants in commercial products. BDE-47 exposure has neurobehavioral, endocrinological and developmental effects and there are increasing concerns about the effects of bioaccumulation in fish and fish-eating animals, including humans. BDE-47 alters gene expression in *Oncorhynchus mykiss* (rainbow trout) and is a concentration-dependant inhibitor and inducer of CYP1A. Sexually mature male rainbow trout were orally dosed BDE-47 (55 µg/Kg/day) for 17 days and used to fertilize eggs from a single, untreated female. The resulting offspring (F1) treated with vehicle or BDE-47 at 6 months of age for 19 days. Livers of F1 progeny were perfused, harvested, S9 fractions prepared and activities of cytochrome P450 1A (CYP1A), 3A (CYP3A), UDP glucuronosyltransferases (UGT) and sulfotransferase (SULT) analyzed. In addition, inhibition potential on CYP enzymes was analyzed in fish hepatic microsomes. Our results demonstrate that BDE-47 up-regulates CYP1A activity, with a greater effect when parent is treated, likely due to concomitant CYP1A inhibition in F1 livers. This induction suggests increased clearance and potentially decreased bioaccumulation of CYP1A substrates. There was a moderate increase in CYP3A activity when parent but not F1 was treated. BDE-47 had no effect on hepatic UGT activity. There was a significant decrease in hepatic SULT activity in all BDE-47 treated groups, indicating decreased clearance and potential for increased bioaccumulation of SULT substrates. Our data demonstrates that BDE-47 exposure causes transgenerational changes in the major xenobiotic metabolizing enzymes and on the bioaccumulation potential in rainbow trout.

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Evaluation of the In Vitro Induction Potential of Xenobiotics Known to be Transactivators of Human Pregnane X Receptor

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The pregnane X receptor (PXR) is a key regulator of enzyme expression of cytochrome P450 3A4 and 2B6 (CYP3A4 and CYP2B6). We recently demonstrated that terbinafine, diclofenac, sildenafil, glimepiride, montelukast and ticlopidine significantly transactivate PXR, even though there are no reports of CYP3A4 induction or drug-drug interactions. To assess the potential of these therapeutics to contribute to drug interactions mediated by CYP3A4 or CYP2B6 induction, we used primary cultures of human hepatocytes treated these for 3 days with each test compound and rifampicin (as a positive control). Terbinafine did not induce CYP3A4 activity but was a moderate inducer of CYP3A4 RNA; CYP2B6 was unchanged. Diclofenac showed a small, non concentration-related increase in CYP3A4 activity and RNA and more robust CYP2B6 induction. Sildenafil decreased CYP3A4 activity, suggesting concomitant inhibition and induction, with a moderate increase in CYP2B6. Glimepiride increased expression and activity of both enzymes while montelukast was not an inducer. Ticlopidine increased CYP3A4 activity at the median concentrations and decreased activity at higher concentrations; CYP2B6 activity was decreased at

all concentrations, while there was no change in RNA expression. Although all six of the compounds used for these studies are PXR activators, this translated to increased CYP3A4 activity with only glimepiride and ticlopidine under our experimental conditions. Since none of these compounds are known to cause drug interactions due to CYP3A4 induction, our studies further show that multiple factors need to be considered to predict potential clinical induction of drug candidates e.g. plasma C_{max} , hepatic exposure and inhibition potential.

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Influence of Cell Concentration on Intrinsic Clearance Values in Cryopreserved Human Hepatocytes.

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Objectives: Cryopreserved human hepatocytes are utilized routinely to evaluate the metabolic stability of compounds *in vitro*. However, experimental conditions employed in these studies vary between laboratories. The goal of the current study was to assess the influence of cell concentration on intrinsic clearance (Cl_{int}) in cryopreserved human hepatocytes. **Methods:** Cryopreserved human hepatocytes were re-suspended in Williams' Medium E (WEM). Cytochrome P450 substrates (midazolam, phenacetin, dextromethorphan, testosterone) prepared in WEM were placed in duplicate wells of 12-well non-coated plates and allowed to pre-incubate. Final cell and substrate concentrations were 0.25, 0.5, 1.0 and 2.0×10^6 cells/mL and 0.5 μ M and 1 μ M, respectively. Plates were incubated on a shaker for 15, 30, 60, 90 and 120 minutes. Negative controls consisted of samples void of cells. Cl_{int} was determined by monitoring the disappearance of parent by LC-MS/MS analysis. **Results:** Severe depletion (<1% parent remaining by 60 min) was frequently observed and independent of substrate at a concentration of 2.0×10^6 cells/mL. The 0.25×10^6 cells/mL concentration yielded the lowest R^2 value in most cases. Actual Cl_{int} values obtained for all four substrates did not differ significantly between the 0.5 and 1.0×10^6 cells/mL concentrations. **Implications:** In general, cell concentrations of 0.5 and 1.0×10^6 cells/mL produced the most robust results for all four prototypical substrates. It is recommended to avoid the use of either too high or too low of a cell concentration in order to utilize the hepatocytes in the most efficient manner.

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Differential time- and NADPH-dependent inhibition of CYP2C19 by isomers of fluoxetine in vitro.

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Objectives: Fluoxetine [\pm -N-methyl-3-phenyl-3-[(α , α , α -trifluoro-p-tolyl)oxy]propylamine)], a widely used selective serotonin reuptake inhibitor, is prescribed as the racemate. Fluoxetine and its isomers are potent reversible inhibitors of CYP2D6 and the racemate has been shown to be a mechanism-based inhibitor of CYP3A4. Racemic fluoxetine also demonstrates time-dependent inhibition of CYP2C19 catalytic activity in vitro. In the course of enhancing our laboratory's time-dependent inhibition assay for CYP2C19, we tested model compounds that might serve as a positive control test inhibitor that is more reliable than ticlopidine, which is only weakly inhibitory. **Methods:** In this study, we compared fluoxetine, its (R)- and (S)-isomers and also ticlopidine and the reversible CYP2C19 inhibitor S-benzyl nirvanol. **Results:** In a reversible

inhibition protocol (30 min preincubation with liver microsomes without NADPH), we found (R)-, (S)- and the racemate to be moderate inhibitors with IC_{50} values of 17, 67 and 27 μM . However, when the preincubation was supplemented with NADPH, IC_{50} -values shifted to 4.1, 3.4 and 1.8 μM . Thus, (S)-fluoxetine demonstrated a 20-fold shift in the IC_{50} value. Follow up K_I and k_{inact} determinations were found to be consistent with these data, showing a good negative correlation between the shifted IC_{50} and the k_{inact}/K_I ratio [e.g. $K_I = 46 \mu\text{M}$ and $k_{inact} = 0.064 \text{ min}^{-1}$ for (S)-fluoxetine]; $K_I = 5.3 \mu\text{M}$, 0.018 min^{-1} for (R)-fluoxetine]. By contrast, ticlopidine showed an approximate 2-fold shift in IC_{50} -value and S-benzylrivanol exhibited no shift, as expected. These data demonstrate that relative to (R)-fluoxetine, the (S)-isomer is less potent as a reversible CYP2C19 inhibitor, but is more rapidly converted to a CYP2C19-inactivating metabolite. **Implications:** These data may have implications for drug interactions following fluoxetine administration. From a practical viewpoint, (S)-fluoxetine appears to be a much improved positive control inhibitor for time-dependent inhibition of liver microsomal CYP2C19 compared to ticlopidine.

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Validation Of Cytochrome P450 Time Dependent Inhibition Assays Featuring a Two Time Point IC_{50} Shift Approach To Facilitate k_{inact} Assay Design

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Objective: Assessment of time-dependent inhibition (TDI) of cytochrome P450 (CYP) often includes determination of K_I/k_{inact} . A challenge in k_{inact} assay design is selecting pre-incubation times to define slopes of inactivation rate curves. A unique IC_{50} shift assay was developed to facilitate k_{inact} studies. **Methods:** IC_{50} shifts, K_I and k_{inact} values were determined in human liver microsomes (HLM) using LC/MS/MS for CYP1A2/ phenacetin/ alpha-naphthoflavone/ furafylline; CYP2B6/ bupropion/ ketoconazole/ ticlopidine; CYP2C9/ diclofenac/ sulfaphenazole/ tienilic acid; CYP2C19/ S-mephenytoin/ S-benzylrivanol/ S-fluoxetine; CYP2D6/ dextromethorphan/ quinidine/ paroxetine; CYP3A4/ testosterone, midazolam/ ketoconazole/ azamulin, verapamil, diltiazem. For IC_{50} shifts, inhibitors were incubated with HLM with/without NADPH for 10 or 30 min prior to 10x dilution into secondary incubations containing probe substrate at K_m . For K_I/k_{inact} s, inhibitors were incubated with HLM/NADPH for multiple time points prior to 10x dilution into incubations containing probe substrate at $\sim 5X K_m$. IC_{50} "shift" was calculated as the ratio of the IC_{50} s in absence and presence of NADPH. K_I/k_{inact} values were determined by non-linear regression. **Results:** A two-time point IC_{50} shift assay design optimized selection of pre-incubation times for the K_I/k_{inact} assay. Rapid acting inhibitors showed no change in IC_{50} shift between the 10 min and 30 min pre-incubation times suggesting short and narrow spacing of time points for the follow-up K_I/k_{inact} study, whereas slow acting inhibitors showed substantial change between the 10 min and 30 min pre-incubation times indicating longer and wider spacing of time points. **Conclusions:** In vitro assays to characterize TDI of CYP isoforms have been validated using a two pre-incubation time point IC_{50} shift experiment to optimize the selection of appropriate K_I/k_{inact} assay parameters.

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Inter-individual Variability of CYP1A2, 2B6 and 3A4 Basal and Induced Activity in Primary Cultures of Human Hepatocytes

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Objectives: The cytochrome P450 (CYP) induction potential of drug candidates is commonly assessed during drug discovery and development. Human hepatocytes are the gold-standard test system. In this investigation, we have evaluated the induction of CYP1A2 (N=53), CYP2B6 (N=30) and CYP3A4 (N=64) by prototypical inducers in primary cultured human hepatocytes.

Methods: Induction was measured by enzyme catalytic activity in situ using probe substrates and normalized to cell protein to control for potential differences in the number of adhered cells at the time of the assay. Hepatocytes were cultured in 24-well collagen I-coated plates and treated with 20 μ M rifampicin (RIF), 20 μ M β -naphthoflavone (BNF) or 2 mM phenobarbital (PB) with a daily media change and replenishment of inducers for 3 days. After treatment, the catalytic activities of CYP1A2, CYP2B6 and CYP3A4 were determined in situ by measuring acetamidophenol, nirvanol and 6 β -hydroxytestosterone formation from corresponding probe substrates.

Results: The results showed that significant fold induction variability was found for all enzymes. The fold induction ranged from 2.1-58 (median = 17) for CYP1A2, 3.1-70 (median = 18) for CYP2B6 and 1.4-162 (median = 20) for CYP3A4. Unlike CYP1A2 and CYP2B6, the induced activity of CYP3A4 appeared to vary less and to reach an upper limit in the induction response. However, this finding was not seen for CYP1A2 and CYP2B6 induction.

Implications: In conclusion, the inducibility of CYP1A2, 2B6 and 3A4 is highly donor-dependent and this supports the need to test multiple donors to assess induction potential of drug candidates.