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Evaluation of the Assumptions of an Ontogeny Model of Rat Hepatic Cytochrome P450 Activity

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List of Abbreviations: 4NC, ρ-nitrocatechol; 6-OH-CZX, 6 hydroxychlorzoxazone; BW, body weight; CYP, cytochrome P450; CZX, chlorzoxazone; HPLC, high performance liquid chromatography; HSF, hepatic scaling factor; ISF, infant scaling factor; LW, liver weight; MP, microsomal protein; MR, methoxyresorufin; MROD, methoxyresorufin O-dealkylation; OSF, ontogeny scaling factor; PD, postnatal day; PNP, ρ-nitrophenol; POD, phenacetin O-dealkylation; UMB, umbelliferone

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Abstract

We previously reported an ontogeny model of hepatic cytochrome P450 (CYP) activity that predicts *in vivo* CYP elimination from *in vitro* intrinsic clearance. The purpose of this study was to conduct investigations into key assumptions of the CYP ontogeny model using the developing rat model system. We used two developmentally dissimilar enzymes, CYP2E1 and CYP1A2, and male rats (n=4) at age groups representing critical developmental stages. Total body and liver weights and hepatic microsomal protein contents were measured. Following HPLC analysis, apparent K_M and V_{Max} estimates were calculated using nonlinear regression analysis for CYP2E1- and CYP1A2-mediated chlorzoxazone 6-hydroxylation and methoxyresorufin O-dealkylation, and V_{Max} estimates for ρ-nitrophenol and phenacetin hydroxylations, respectively. Hepatic scaling factors and V_{Max} values provided estimates for infant scaling factors (ISF). The data show microsomal protein contents increased with postnatal age and reached adult values after postnatal day (PD) 7. Apparent K_M values were similar at all developmental stages except at ≤ PD7. Developmental increases in probe substrate V_{Max} values did not correlate with the biphasic increase in immunoquantifiable CYP. The activity of two different probe substrates for each CYP covaried as a function of age. A plot of observed ISF values as a function of age reflected the developmental pattern of rat hepatic CYP. In summation, these observations diverge from several of the model's assumptions. Further investigations are required to explain these inconsistencies and to investigate whether the developing rat may provide a predictive paradigm for paediatric risk assessment for CYP-mediated elimination processes.

Intentional and inadvertent exposures (i.e. environmental contamination, breast milk) of the developing neonate to xenobiotics raises significant concerns over the potential risks posed to the exposed neonate. However, ethical impediments and limited toxicity data in human paediatric populations hinders the assessment of such risk. Furthermore, evidence for age-dependent differences in susceptibility to toxicity precludes a simple adult-to-neonate extrapolation of toxicity risk based upon toxicology studies in adult populations (Clark et al., 2004; Ginsberg et al., 2004b). A need exists, then, for predictive paradigms for risk assessment in human paediatric populations.

Pharmacokinetic processes, particularly elimination mechanisms, often underlie these age-related differences in susceptibility to toxicity. Most elimination processes undergo significant ontogeny (Alcorn and McNamara, 2002a) and their maturation status in the developing neonate may determine toxicological outcomes following a xenobiotic exposure (Ginsberg et al., 2004a; Barton, 2005). Consequently, knowledge of a neonate's elimination capacity is critical for toxicological risk assessments. To address this issue, recent efforts to use available data on the ontogeny of xenobiotic elimination mechanisms in humans and animals have lead to models that allow predictions of xenobiotic elimination in the developing neonate (Alcorn and McNamara, 2002b; Brent, 2004; Barton, 2005; Jarabek et al., 2005; Bjorkman, 2006; Edginton et al., 2006; Johnson et al., 2006; Nong et al., 2006) These models are either based upon physiological data from paediatric patients and adults or upon developmental toxicological assessments in suitable animal systems. In the only validated model approach (Nong et al., 2006), the authors reported that a physiologically based pharmacokinetic model premised upon knowledge of age- and subject-specific human CYP2E1 protein content and known physiological variables can be used to evaluate interindividual variation in internal dosimetry estimates for toluene (CYP2E1

substrate) exposures in children and represents a feasible approach for risk assessment purposes. Predictions of xenobiotic elimination capacity alone, though, offer little understanding on mechanisms of toxicity and mitigating genetic and environmental factors contributing to interindividual variation in toxicity. To facilitate our understanding of age-dependent differences in susceptibility to toxicity an appropriate developmental animal model system remains mandatory (Graeter and Mortensen, 1996; Koren et al., 2003; Brent, 2004).

Our labs previously developed an ontogeny model of hepatic CYP-mediated elimination based on an *in vitro-in vivo* extrapolation of published CYP activity data from age-specific human fetal and infant hepatic microsomes. Our review outlines the theoretical framework for the model (Alcorn and McNamara, 2002b). Briefly, the model involves scaling *in vitro* hepatic intrinsic clearance data to *in vivo* intrinsic clearance and the extrapolation of known adult intrinsic clearance values to any age during postnatal development. This extrapolation occurs via a scaling factor (the Infant Scaling Factor), which incorporates age-dependent changes in hepatic scaling factors and CYP activity. Critical assumptions of the model include: 1) microsomal protein content is constant and independent of developmental age; 2) K_M values are constant and not a function of developmental age; 3) V_{Max} is proportional to functional enzyme level and activity from one substrate will accurately predict activity for all substrates of that enzyme; 4) uptake and efflux transporters do not contribute significantly to intrinsic clearance; 5) the drug is a low extraction ratio drug; and 6) the elimination pathways in the adult are known and the infant shares the same pathways.

Since, interspecies extrapolation of developmental toxicity data remains a practical option to elucidate the impact of development on susceptibility to toxicity, we propose a similar model approach for rat hepatic CYP ontogeny as a means of providing a predictive paradigm for risk

assessment. Comparative analyses of human and rat CYP enzyme maturation (Cresteil, 1987; Rich and Boobis, 1997) and CYP enzyme substrate specificity and regulation highlight important species differences between the rat and human. Furthermore, differences in the rates and patterns of CYP maturation (Alcorn and McNamara, 2002a) may result in species differences in the parameterization of the model. Nevertheless, CYP enzyme mechanisms are similar across species, which suggests model assumptions remain the same regardless of species. However, before any toxicokinetic model of CYP ontogeny in rat can be developed and used to provide robust predictions of hepatic CYP-mediated elimination, we must first evaluate the appropriateness of the model's underlying assumptions.

The purpose of this study is to conduct investigations into the key assumptions of the model of hepatic CYP ontogeny. We use two developmentally dissimilar CYP enzymes, CYP2E1 and CYP1A2 and male Sprague-Dawley rats at various fetal, neonatal, juvenile, and adult age groups to provide critical information on the model's assumptions. The implications of the findings on the validity of the model are discussed.

Methods

Chemicals and Reagents. Chlorzoxazone (CZX), 6 hydroxychlorzoxazone (6-OH-CZX), umbelliferone (7-hydroxycoumarin) (UMB), ρ-nitrophenol (PNP), ρ-nitrocatechol (4NC), salicylamide, phenacetin, acetaminophen, caffeine, resorufin, methoxyresorufin (MR) and all chemicals used for microsomal preparation, determination of microsomal protein content and enzyme assays were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Rat CYP1A2 and CYP2E1 supersomes were acquired from BD Biosciences (Woburn, MA, USA). Peroxidase-conjugated antibody (Goat anti-rabbit IgG HRP conjugate), prestained SDS-PAGE standards, Immun-Star HRP kit, Bio-Rad Trans-Blot cell and polyvinylidene difluoride (PVDF) membranes (0.2 μm) were obtained from Bio-Rad Laboratories (Hercules, CA). Rabbit anti-Human/Rat CYP2E1 and sheep anti-rat CYP1A2 polyclonal antibodies and rabbit anti-sheep IgG HRP conjugate antibody were purchased from Chemicon International (Temecula, CA). All other chemicals used were analytical grade.

Animals. Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, PQ). Eight pregnant rats were received at 14 day pregnancy and allowed to acclimatize for one week. At day 21 of pregnancy, four pregnant rats were anesthetized under isoflurane and the fetuses were collected. Given the difficulty with sex-typing fetal rats, pups from both genders were used. The remaining pregnant rats were allowed to give birth and one male pup from each dam was collected at postnatal ages 1, 3, 5, 7 and 10 days. Other age groups (2, 3, 4, 6, 9, 12 and 16 weeks) (n = 4) were obtained from Charles River Canada one week before the required age during which time rats were allowed to acclimatize. All rats received standard laboratory rat chow and water *ad libitum* throughout the acclimatization period. At the appropriate age, body weights were recorded. Rats were then killed by guillotine under light isoflurane anaesthesia, the

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livers rapidly excised and rinsed in cold 0.9% NaCl, weighed and flash frozen in liquid nitrogen, and stored at –80°C until microsomal preparation. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

Preparation of Hepatic Microsomes. Hepatic microsomes were prepared as previously described (Elbarbry et al., 2007). Protein concentrations of microsomal preparations were determined in triplicate by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as a calibration standard.

Microsomal Incubations with CYP2E1 and CYP1A2 Probe Substrates. Preliminary experiments in 50-day old male rat hepatic microsomes were conducted to determine the optimum incubation conditions (incubation time and microsomal protein content) to give linear metabolite formation kinetics for CYP2E1-mediated hydroxylation of chlorzoxazone (CZX) and ρ-nitrophenol (PNP) and CYP1A2-mediated phenacetin O-dealkylation (POD) and methoxyresorufin O-dealkylation (MROD) activities. Michaelis-Menten parameters, K_M and V_{Max}, for CYP2E1 and CYP1A2 were determined by measuring metabolite formation velocities of CZX and MROD at substrate concentrations of 0 to 1000 μM and 0 to 1000 nM, respectively. With PNP and phenacetin, metabolite formation velocities were measured at saturating probe substrate concentrations (500 μM and 150 μM, respectively) in age-specific rat hepatic microsomes to provide estimates of V_{max}.

HPLC Analysis. For CYP2E1, 6-hydroxylation of chlorzoxazone in rat hepatic microsomal preparations was quantified using an HPLC analysis as described (Elbarbry et al., 2007). For the second probe substrate, PNP hydroxylation (CYP2E1 probe) was assayed as reported previously

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(Elbarbry et al., 2006). Calibration curves were constructed from known concentrations of 6-OH CZX and 50 µL of the internal standard (0.078 mM umbelliferone) or known concentrations of 4-nitrocatechol and 50 µL of internal standard (salicylamide, (6 µg/mL) added to heat-inactivated (55°C for 5 min) rat hepatic microsomes. The calibration standards were diluted with 50 mM phosphate buffer (pH 7.4) to achieve calibration standards of 0.31-40 µM for 6-OH CZX or 0.1-40 µM for 4NC in a total volume of 0.5 mL. Three quality control (QC) samples at 0.625 µM (low), 1.25 µM (medium), and 5 µM (high) for 6-OH CZX or 0.5 µM (low), 5 µM (medium), and 20 µM (high) for 4NC were prepared independent of those used for the calibration curves. Coefficients of determination were greater than 0.999 for all calibration curves. Intra-day and inter-day precisions were less than 14%. The accuracy of estimated metabolite concentrations ranged from 91-113%.

For CYP1A2, Phenacetin-O-dealkylation (CYP1A2 probe) was assayed by a published HPLC method (von Moltke et al., 1996) using caffeine as an internal standard. UV absorbance was monitored at 254 nm (λ_{max} for acetaminophen). The mobile phase consisted of 50 mM potassium dihydrogen phosphate and acetonitrile at a ratio of 85:15 (v/v (%)) and delivered at 1.0 mL/min. Calibration curves were constructed from known concentrations of resorufin or acetaminophen and the internal standard (caffeine 5 µg/mL) added to heat-inactivated (55°C for 5 min) rat hepatic microsomes and diluted with 50 mM phosphate buffer (pH 7.4) to achieve calibration standards of 0.04-2 nM for resorufin and 0.2-20 µM for acetaminophen in a total volume of 0.5 mL. Three quality control (QC) samples at 0.1 nM (low), 0.4 nM (medium), and 2 nM (high) for resorufin and 0.5 µM (low), 2 µM (medium), and 10 µM (high) for acetaminophen were prepared independent of those used for the calibration curves. Coefficients of determination were greater than 0.999 for all calibration curves. Intra- and interday precision was <14% and

<9% for resorufin and acetaminophen, respectively, and accuracy was within 15% of the nominal value for both HPLC assays.

CYP1A2 and CYP2E1 Protein Quantification. Hepatic microsomal proteins were separated on a sodium dodecyl sulfate gel containing 10% polyacrylamide (SDS-PAGE) by standard methods. The gel was loaded with rat CYP1A2 purified protein (20, 80, 200 and 500 pmol) or heat denatured microsomes (0.4 μg of protein) for CYP1A2 and rat CYP2E1 purified protein (20, 50 and 100 pmol) or heat denatured microsomes (10.5 μg of protein) for CYP2E1, and electrophoresis conducted for 1.5-2 h at 125V. Proteins were transferred onto a PVDF membrane (0.2 μm) with a Bio-Rad Trans-Blot cell (Bio-Rad Laboratories, Hercules, CA) at 25V for 50 minutes. The membrane was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBSt) and subsequently blocked with PBSt containing 5% milk with overnight incubation at 4°C. For CYP1A2, the membrane was then incubated overnight at 4°C with sheep anti-rat CYP1A2 polyclonal antibody prepared in PBS solution (1:1000 dilution). For CYP2E1, the membrane was incubated for 3h at 20°C with rabbit anti-human/rat CYP2E1 polyclonal antibody prepared in the blocking solution (1:750). Following the appropriate incubation period, membranes were washed three times with PBSt and once with PBS and then incubated with peroxidase-conjugated antibody prepared in the blocking solution (1:3000) for 2h at 20°C. The membrane was further washed three times with PBSt and once with PBS and then immunodetection was performed using the Immun-Star HRP kit. To estimate the amount of reacting protein, the blot was scanned with an LKB Laser Densitometer (Amersham Pharmacia Biotech, Piscataway, NJ) using reference samples to calibrate determinations. The results were expressed as absorbance unit per μg protein (OD/μg protein).

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Scaling Factors. Hepatic scaling factors for a particular age ($HSF_{(t)}$) were estimated from the product of microsomal protein yield ($MP_{(t)}$) (mg microsomal protein per gram liver) and liver weight (g) ($LW_{(t)}$) normalized to body weight (g) ($BW_{(t)}$) as a function of developmental age, t, according to Equation 1.

$$HSF_{(t)} = MP_{(t)} \times \frac{LW_{(t)}}{BW_{(t)}} \quad (1)$$

Infant scaling factors at a particular age, t ($ISF_{(t)}$) were estimated from the product of $HSF_{(t)}$ and V_{Max} values at the same age, t ($V_{Max,(t)}$) normalized to the product of the respective adult values according to Equation 2.

$$ISF_{(t)} = \frac{HSF_{(t)}^{infant} V_{Max,(t)}^{infant}}{HSF^{adult} V_{Max}^{adult}} \quad (2)$$

Data Analysis. All data within the same age was reported as mean±SD. Metabolite formation velocity as a function of substrate concentration was fit to a one-enzyme site Michaelis-Menten equation. The parameters, V_{Max} and K_M, were estimated by an iterative nonlinear least squares regression analysis using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Significant differences in total microsomal protein content, CYP protein content, and V_{Max} and K_M estimates between the different age groups were assessed with one-way Analysis of Variance with Tukey's post-hoc test for pair-wise multiple comparisons using GraphPad Prism. For CZX K_M estimates a Wilcoxon signed-rank test was used for pooled data. Pearson correlation coefficients were determined to compare the V_{Max} values of two different substrates of the CYP. The level of significance was set at $P<0.05$.

Variation associated with the computed $ISF_{(t)}$ values was represented as a coefficient of variation according to Equation 3, where $\bar{X}$ is the infant ISF mean value, $\bar{Y}$ is the adult ISF mean

value, X/Y is the ratio of the mean infant ISF to the mean adult ISF values, SD is the standard deviation and n is the sample size.

$$CV_{(X/Y)} = \sqrt{\left(\frac{SD/\sqrt{n}}{\bar{X}}\right)^2_{Infant} + \left(\frac{SD/\sqrt{n}}{\bar{Y}}\right)^2_{Adult}} \qquad (3)$$

Results

Hepatic scaling factor (HSF) as a function of developmental age. To evaluate whether microsomal protein content remains constant with developmental age (the first major assumption of the ontogeny model of CYP enzyme activity) we measured total body and liver weights and liver microsomal protein content (mg per g liver) in fetal (gestation day 20), neonatal, juvenile, and adult age groups (Table 1). As expected, mean total body and liver weights increased with postnatal age, with the most prominent increases occurring during puberty (PD42-PD63). The age-dependent increase in liver and body weights was associated with marked interindividual variation, which reflects the normal variation in rates and patterns of body and organ growths typically observed in animal species. The average microsomal protein content per gram of liver (MP) was significantly lower than adult levels in fetal and early postnatal age groups. After PD5, MP levels were similar to adult values.

Closer examination of the data showed mean liver weight normalized to total body weight remained relatively constant with postnatal age. The pattern observed in the mean HSF values with age (Figure 1) mirrored the developmental pattern of microsomal protein content per gram liver (Table 1). HSF values between gestational day 20 and postnatal day 5 remained approximately at 50-70% of the adult value. HSF values steadily increased to reach ~2-fold the adult value by PD42. By PD63, mean HSF values were similar to adult values.

K_M as a function of development. To evaluate whether K_M values remain constant with developmental age (the second major assumption of the ontogeny model of CYP enzyme activity) we conducted enzyme kinetic studies in rat hepatic microsomes with probe substrates specific for CYP2E1 and CYP1A2, namely CZX and MR, respectively. For CYP2E1 limiting tissue availability necessitated the use of pooled microsomes (pooled from four animals). We

detected no activity in gestation day 20 and PD1 hepatic microsomes and CZX apparent K_M values were significantly lower than adult values (35-40% of adult values) at PD3 and PD5 (data not shown). By PD10, apparent K_M values were similar to adult values and stayed relatively constant thereafter.

For CYP1A2, the apparent K_M estimates for MROD for PD5 and PD7 were significantly higher than adult values (>2.5-fold adult values) (Figure 2). No significant differences in apparent K_M estimates were observed for PD age groups ≥ 10 days. The data did not allow estimation of K_M values in fetal, PD1, and PD3 age groups, although activity was detected at low levels at PD3.

V_{Max} as a function of development. To assess whether enzyme activity (V_{Max}) is proportional to enzyme level during ontogeny (the third major assumption of the ontogeny model of CYP enzyme activity) we measured CYP1A2 and CYP2E1activity at saturating probe substrate concentrations in rat hepatic microsomes at different developmental stages and compared this activity to immunoquantifiable CYP levels at the same developmental age. MROD activity (Figure 3A) was first detected at PD3, but below the level of quantification of the HPLC assay. PD5 and PD7 hepatic microsomes demonstrated significantly lower MROD activity at 11% and 20% adult activity, respectively. MROD activity increased significantly to reach ~1.75-fold the adult values by PD28. However, after weaning (PD28) and during puberty (PD42-63) V_{Max} values fell to adult values. Although, the marked increase in MROD activity between PD14 and PD21 corresponded to a marked increase in age-dependent hepatic scaling factors (Figure 1), activity declined after PD28 to the adult value by PD42, unlike the hepatic scaling factor.

We did not detect CZX hydroxylase activity (Figure 3B) in fetal and PD1 livers. CZX hydroxylase activity was first detected in PD3 livers (70% of the adult value), which increased

significantly to reach maximum levels by PD14 (~1.6-fold adult value) and generally declined thereafter to adult values. The marked postnatal increase in V_{Max} values for CZX hydroxylase preceded hepatic scaling factor values, where the predominant age-dependent increase occurred between PD14 and PD21 and continued to increase until PD42 (Figure 1).

Immunoquantifiable CYP1A2 and CYP2E1 exhibited a biphasic pattern of development (Figure 4). Rapid increases in immunodetectable levels of CYP1A2 and CYP2E1 occurred during the early postnatal period (≤PD7) followed by a slower, more gradual increase to adult values. The rapid rise in immunodetectable CYP1A2 and CYP2E1 in the early postnatal period seemed to parallel increases in MROD and CZX hydroxylase activity. Thereafter, a marked discordance between CYP protein and activity was observed and V_{max} values did not correlate (Pearson r = 0.35, P>0.05 for CYP1A2; Pearson r = -0.08, P>0.05 for CYP2E1) with changes in immunoquantifiable levels observed in the same age group (Figure 4).

To determine whether enzyme activity (V_{Max}) from one substrate will accurately predict the level of activity for all substrates of the enzyme we correlated the V_{Max} values at each age group from one CYP probe substrate with the second probe substrate (Figure 5). Figure 5 suggests a strong and significant correlation between the activity of one specific substrate relative to a second specific substrate for both CYP1A2 (r = 0.68, P<0.05; data not shown due to low variability in the POD data relative to the MROD data) and CYP2E1 (r = 0.69, P<0.05).

Figure 6 shows the postnatal developmental pattern of CYP1A2 and CYP2E1 when scaled to the whole body. For both CYP enzymes whole body CYP activity is low at birth and rapidly increases to maximum levels at the end of weaning (PD28). Thereafter, whole body CYP activity declines towards adult values by the end of puberty (PD 63).

Discussion

The very limited toxicological data in human paediatric populations prompted efforts to advance physiologically based pharmacokinetic models in animal developmental toxicology systems for human paediatric risk assessments (Brent, 2004; Clark et al., 2004). By relating xenobiotic elimination capacity to the time course of CYP maturation, the previously reported ontogeny model of CYP activity (Alcorn and McNamara, 2002b) contributes to such efforts. A valid and robust model can facilitate study design and dosage selection for developmental toxicology studies and identify when age-dependent differences in xenobiotic elimination account for differences in susceptibility to toxicity. To this end, we evaluated the underlying assumptions of the CYP ontogeny model (Alcorn and McNamara, 2002b) in a toxicologically relevant animal system. The assessments in the rat identified important departures from model assumptions and we discuss their possible underlying causes and overall significance below.

Hepatic Scaling Factor

In vitro-in vivo extrapolation of intrinsic clearance data requires knowledge of developmental changes in microsomal protein content, liver weight, and body weight (Carlile et al., 1997; Houston and Carlile, 1997; Obach et al., 1997). In the human CYP ontogeny model, we obtained information on the age-dependent changes in liver and body weight from the literature, but assumed hepatic microsomal protein content on a per gram liver basis remained constant throughout postnatal development and used a published literature value (Alcorn and McNamara, 2002b). Any age-related changes in hepatic scaling factors, then, became dependent solely on developmental changes in liver weight normalized to body weight.

In this study rat hepatic microsomal protein content per gram liver increased significantly during the neonatal period, reached a maximum at puberty and declined to adult levels in the

early post-pubertal stage. The data is consistent with rat hepatic microsomal content described in a limited number of postnatal, prepubertal and pubertal age groups and adults in the literature (Borlakoglu et al., 1993; Kishi et al., 2005). Postnatal changes in microsomal protein content in the rat suggests a need for similar investigations with human paediatric livers (Johnsrud et al., 2003; Johnson et al., 2006). Interestingly, the age-dependent changes in rat hepatic scaling factors became a function of changes in microsomal protein content rather than liver weight to body weight ratio, which stayed relatively constant and independent of developmental stage. Although these findings contradict a model assumption, this contradiction does not necessarily weaken its potential value. These results merely suggest the need to measure hepatic microsomal protein content in paediatric livers to incorporate its developmental change into the modeling process to provide predictive estimates of the infant scaling factors.

Clearance as a Function of Hepatic Metabolic Enzyme Activity

The ontogeny model makes the limiting assumption that systemic clearance is a function of hepatic metabolic enzyme activity (i.e. the xenobiotic is a low hepatic extraction ratio compound) and uptake and efflux transporters make no contribution to intrinsic clearance. We used probes (Kobayashi et al., 2002) that are known low hepatic extraction ratio compounds, but these probes have not been assessed as transporter substrates. Intrinsic clearance estimates are affected by substrates where membrane transporters act as a rate limiting process of metabolic clearance (Ito et al., 1998). At present the model makes no accommodation for such substrates but has the potential to incorporate transport processes in the infant scaling factor determinations. Nevertheless, the model can be applied to many xenobiotics as most are low extraction ratio compounds and passive diffusion governs their distribution into the liver.

Intrinsic Clearance

The experimental data failed to support two critical assumptions of the ontogeny model (i.e. age-dependent changes in intrinsic clearance reflect changes only in V_{Max} and K_M remains constant throughout development) (Alcorn and McNamara, 2002b). The data suggests a need to incorporate developmental changes in K_M estimates in the model building process (i.e. model the V_{max}/K_M ratio) instead of modeling only changes in V_{Max}. The incorporation of a new parameter, which undergoes interindividual variability during development, will increase the model's complexity and possibly result in greater uncertainty in its parameter estimates. However, study design limitations may explain this apparent divergence from model assumptions. Specifically, we question probe substrate specificity in paediatric hepatic microsomal preparations. At best probes are selective (not specific) for the CYP of interest (Kobayashi et al., 2002; Lu et al., 2003) and selectivity is dependent upon substrate concentration (Kato and Yamazoe, 1994). Since different CYP enzymes undergo variable rates and patterns of development (Borlakoglu et al., 1993; Rich and Boobis, 1997; Alcorn and McNamara, 2002a), CYP enzymes that contribute a small percentage to probe metabolism in adult stages may play a larger role during specific paediatric stages. In the study, differences in K_M values may reflect the contribution of other CYP enzymes and the estimated K_M values will then reflect a hybrid of contributing enzymes. Eadie-Hofstee plots (data not shown), though, gave no evidence of systematic deviation from a single site Michaelis-Menten model at higher substrate concentrations for CZX, but curvilinear plots were observed for MROD suggesting the contribution of at least a second low affinity enzyme site. Furthermore, nonspecific binding to microsomal protein (Obach, 1997; Obach et al., 1997) and differences in the membrane environment of the endoplasmic reticulum (Dallner et al., 1966) can influence apparent K_M estimates. We cannot ignore the potential for age-dependent

changes in such factors. Although the data does not rule out the possibility for developmental changes in the inherent properties of CYPs, we failed to find any information regarding changes in CYP enzyme active sites with postnatal maturation. Further investigations are needed to explain these postnatal changes in K_M values. For example, recombinant CYPs, singly expressed and mixed in proportion to CYP levels present in hepatic microsomes at different age groups, may facilitate assessment of the contribution of different CYP isoforms to the estimated K_M value at a given age (Tang et al., 2005).

The data on the age-dependent changes in CYP2E1 and CYP1A2 activity are consistent with the few reports of rat CYP ontogeny (Jahn et al., 1993; Wu and Cederbaum, 1993; Rich and Boobis, 1997; Iba et al., 2000; Anand et al., 2006). However, the observed lack of correlation between developmental increases in CYP activity and immunoreactive protein levels violates an important model assumption. Posttranslational modifications with development that markedly influence enzyme activity may explain the apparent discordancy. Alternatively, other CYP enzymes whose development precedes CYP2E1 and CYP1A2 maturation may contribute significantly to probe metabolism at particular postnatal age stages. Finally, inadequate antibody specificity (i.e. cross-reactivity with other CYP enzymes) may also contribute to this lack of congruence. For these reasons, then, CYP immunoreactive protein levels may not necessarily reflect enzyme activity (Johnsrud et al., 2003). Interestingly, immunohistochemical analysis revealed homogeneous expression of CYP1A2 and CYP2E1 in the hepatic acinus in fetal and early postnatal livers with increasing preferential localization of CYP1A2 and CYP2E1 expression in perivenous hepatocytes with postnatal development (Elbarbry et al., 2007). With intrasinusoidal gradients in oxygen tension and substrate supply, age-dependent changes in intrahepatic CYP expression patterns will likely have some influence on CYP regulation and

activity. Further investigations into antibody specificity, involvement of alternative CYP enzymes in probe substrate metabolism and the role of developmental changes in intrahepatic CYP expression patterns are necessary to explain the lack of correlation between probe substrate activity (V_{Max} estimates) and immunoquantifiable CYP.

Finally, we observed that the activity of two different probe substrates for each CYP covaried as a function of age, thus satisfying an important model assumption. However, activity was generally detected earlier with the less specific CYP substrate, which suggests the involvement of additional CYP enzymes. Although the data does not necessarily refute an implicit assumption of the ontogeny model, namely the infant shares the same metabolic pathways as the adult, it does suggest that the relative contribution of different pathways involved in the metabolism of a particular substrate may have varied importance during development. This will influence both qualitative and quantitative predictions and the model will need to accommodate the potential for CYPs to have different contributions at different stages of development.

Conclusion

The data identified several important inconsistencies with the underlying assumptions of the CYP ontogeny model. The observed age-related changes in hepatic microsomal protein content suggest a need to incorporate its developmental change into the modeling process. Inconstant K_M values during early postnatal development may reflect questionable probe substrate specificity during CYP ontogeny rather than age-dependent changes in the inherent properties of substrate-CYP interactions. Further investigations are needed to determine the reason for age-dependent changes in the apparent K_M values. Consistent with model assumptions, the apparent V_{Max} estimates increased postnatally to reach a maximum prior to or during weaning, and the activity of two separate substrates for a given CYP covaried as a function of age. The lack of correlation

between age-dependent changes in V_{Max} estimates and immunoreactive CYP requires further investigation. Future studies are planned to 1) explain the incongruence between the experimental data and several model assumptions and 2) to develop a model of rat CYP ontogeny that provides reasonable predictions of *in vivo* intrinsic clearance of CYP probe substrates.

References

Alcorn J and McNamara PJ (2002a) Ontogeny of hepatic and renal systemic clearance pathways in infants: part I. *Clin Pharmacokinet* **41**:959-998.

Alcorn J and McNamara PJ (2002b) Ontogeny of hepatic and renal systemic clearance pathways in infants: part II. *Clin Pharmacokinet* **41**:1077-1094.

Anand SS, Kim KB, Padilla S, Muralidhara S, Kim HJ, Fisher JW and Bruckner JV (2006) Ontogeny of hepatic and plasma metabolism of deltamethrin in vitro: role in age-dependent acute neurotoxicity. *Drug Metab Dispos* **34**:389-397.

Barton HA (2005) Computational pharmacokinetics during developmental windows of susceptibility. *J Toxicol Environ Health A* **68**:889-900.

Bjorkman S (2006) Prediction of cytochrome p450-mediated hepatic drug clearance in neonates, infants and children : how accurate are available scaling methods? *Clin Pharmacokinet* **45**:1-11.

Borlakoglu JT, Scott A, Henderson CJ and Wolf CR (1993) Expression of P450 isoenzymes during rat liver organogenesis. *Int J Biochem* **25**:1659-1668.

Brent RL (2004) Utilization of juvenile animal studies to determine the human effects and risks of environmental toxicants during postnatal developmental stages. *Birth Defects Res B Dev Reprod Toxicol* **71**:303-320.

Carlile DJ, Zomorodi K and Houston JB (1997) Scaling factors to relate drug metabolic clearance in hepatic microsomes, isolated hepatocytes, and the intact liver: studies with induced livers involving diazepam. *Drug Metab Dispos* **25**:903-911.

Clark LH, Setzer RW and Barton HA (2004) Framework for evaluation of physiologically-based pharmacokinetic models for use in safety or risk assessment. *Risk Anal* **24**:1697-1717.

Cresteil T (1987) Regulation of drug-metabolizing enzymes during the perinatal period in rat and human liver. *Bioessays* **7**:120-124.

Dallner G, Siekevitz P and Palade GE (1966) Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *J Cell Biol* **30**:73-96.

Edginton AN, Schmitt W, Voith B and Willmann S (2006) A mechanistic approach for the scaling of clearance in children. *Clin Pharmacokinet* **45**:683-704.

Elbarbry F, Wilby K and Alcorn J (2006) Validation of a HPLC method for the determination of p-nitrophenol hydroxylase activity in rat hepatic microsomes. *J Chromatogr B Analyt Technol Biomed Life Sci* **834**:199-203.

Elbarbry FA, McNamara PJ and Alcorn J (2007) Ontogeny of hepatic CYP1A2 and CYP2E1 expression in rat. *J Biochem Mol Toxicol* **21**:41-50.

Ginsberg G, Hattis D, Miller R and Sonawane B (2004a) Pediatric pharmacokinetic data: implications for environmental risk assessment for children. *Pediatrics* **113**:973-983.

Ginsberg G, Slikker W, Jr., Bruckner J and Sonawane B (2004b) Incorporating children's toxicokinetics into a risk framework. *Environ Health Perspect* **112**:272-283.

Graeter LJ and Mortensen ME (1996) Kids are different: developmental variability in toxicology. *Toxicology* **111**:15-20.

Houston JB and Carlile DJ (1997) Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metab Rev* **29**:891-922.

Iba MM, Fung J, Cooper KR, Thomas PE, Wagner GC and Park Y (2000) Effect of gestational and lactational 2,3,7, 8-tetrachlorodibenzo-p-dioxin exposure on the level and catalytic

activities of hepatic microsomal CYP1A in prepubertal and adult rats. *Biochem Pharmacol* **59**:1147-1154.

Ito K, Iwatsubo T, Kanamitsu S, Nakajima Y and Sugiyama Y (1998) Quantitative prediction of in vivo drug clearance and drug interactions from in vitro data on metabolism, together with binding and transport. *Annu Rev Pharmacol Toxicol* **38**:461-499.

Jahn F, Reuter A, Karge E, Danz M and Klinger W (1993) Age dependent different influence of carbon tetrachloride on biotransformation of xenobiotics, glutathione content, lipid peroxidation and histopathology of rat liver. *Exp Toxicol Pathol* **45**:101-107.

Jarabek AM, Asgharian B and Miller FJ (2005) Dosimetric adjustments for interspecies extrapolation of inhaled poorly soluble particles (PSP). *Inhal Toxicol* **17**:317-334.

Johnson TN, Rostami-Hodjegan A and Tucker GT (2006) Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clin Pharmacokinet* **45**:931-956.

Johnsrud EK, Koudouritaki SB, Divakaran K, Brunengraber LL, Hines RN and McCarver DG (2003) Human hepatic CYP2E1 expression during development. *J Pharmacol Exp Ther* **307**:402-407.

Kato R and Yamazoe Y (1994) The importance of substrate concentration in determining cytochromes P450 therapeutically relevant in vivo. *Pharmacogenetics* **4**:359-362.

Kishi R, Sata F, Katakura Y, Wang RS and Nakajima T (2005) Effects of pregnancy, age and sex in the metabolism of styrene in rat liver in relation to the regulation of cytochrome P450 enzymes. *J Occup Health* **47**:49-55.

Kobayashi K, Urashima K, Shimada N and Chiba K (2002) Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. *Biochem Pharmacol* **63**:889-896.

Koren G, Kearns GL, Reed M and Pons G (2003) Use of healthy children as volunteers in drug studies: the ethical debate. *Clin Pharmacol Ther* **73**:147-152.

Lowry OH, Rosebrough NJ, Farr AL and Randall RL (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**:265-275.

Lu AY, Wang RW and Lin JH (2003) Cytochrome P450 in vitro reaction phenotyping: a re-evaluation of approaches used for P450 isoform identification. *Drug Metab Dispos* **31**:345-350.

Nong A, McCarver DG, Hines RN and Krishnan K (2006) Modeling interchild differences in pharmacokinetics on the basis of subject-specific data on physiology and hepatic CYP2E1 levels: a case study with toluene. *Toxicol Appl Pharmacol* **214**:78-87.

Obach RS (1997) Nonspecific binding to microsomes: impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* **25**:1359-1369.

Obach RS, Baxter JG, Liston TE, Silber BM, Jones BC, MacIntyre F, Rance DJ and Wastall P (1997) The prediction of human pharmacokinetic parameters from preclinical and in vitro metabolism data. *J Pharmacol Exp Ther* **283**:46-58.

Rich KJ and Boobis AR (1997) Expression and inducibility of P450 enzymes during liver ontogeny. *Microsc Res Tech* **39**:424-435.

Tang W, Wang RW and Lu AY (2005) Utility of recombinant cytochrome p450 enzymes: a drug metabolism perspective. *Curr Drug Metab* **6**:503-517.

von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Kudchadker L, Fogelman SM, Harmatz JS and Shader RI (1996) Phenacetin O-deethylation by human liver microsomes in vitro: inhibition by chemical probes, SSRI antidepressants, nefazodone and venlafaxine. *Psychopharmacology (Berl)* **128**:398-407.

Wu D and Cederbaum AI (1993) Induction of liver cytochrome P4502E1 by pyrazole and 4-methylpyrazole in neonatal rats. *J Pharmacol Exp Ther* **264**:1468-1473.

Footnotes

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Legends for Figures

Figure 1. Mean (+SD) hepatic scaling factors in male Sprague-Dawley rats (n = 8, except PD84 where n = 4) as a function of postnatal age. Hepatic scaling factors were calculated from the product of microsomal protein content (mg/g liver) and total wet liver weight (g) normalized to total body weight (g) (Equation 1). Astericks (*) indicate statistical difference from adult (112 days), $P<0.05$.

Figure 2. Michaelis-Menten constant (K_M) estimates for methoxyresorufin-O-dealkylation (MROD) activity (CYP1A2 marker) as a function of developmental age in male Sprague-Dawley rat hepatic microsomes (n=4). The asterick refers to those age groups that show significant difference from adult, $P<0.05$. Activity was detected at PD3, but the data did not allow for apparent K_M estimation.

Figure 3. V_{max} estimates for methoxyresorufin-O-dealkylase (MROD) activity (CYP1A2 marker) (A) and chlorzoxazone (CZX) hydroxylase activity (CYP2E1 marker) (B) as a function of developmental age in male Sprague-Dawley rat hepatic microsomes. The data are expressed as mean (±SD) of 4 rats. The asterick refers to those age groups that show significant difference from adult.

Figure 4. Age-dependent changes in enzyme activity (closed circle) as a function of immunoquantifiable CYP levels (open circle) in rat hepatic microsomes. (A) Average CYP1A2-mediated methoxyresorufin-O-dealkylase V_{Max} estimates as a function of average

immunoquantifiable CYP1A2 levels. (B) Average CYP2E1-mediated chlorzoxazone hydroxylase V_{Max} estimates as a function of average immunoquantifiable CYP2E1 levels.

Figure 5. Correlation between V_{Max} estimates of ρ-nitrophenol (PNP) and chlorzoxazone (CZX) for CYP2E1 (Pearson $r = 0.69$, $P<0.05$).

Figure 6. Infant scaling factor (ISF) as a function of age for CYP1A2 (closed circles) and CYP2E1 (open circles). ISF values were calculated from the product of HSF and V_{Max} values of methoxyresorufin and chlorzoxazone for CYP1A2 and CYP2E1, respectively, at each age group normalized to the adult values. Variability is represented as a coefficient of variation of ratio data (see Equation 3).

Tables

Table 1. Mean (±SD) total body and liver weights (g) and hepatic microsomal protein content (mg/g liver) for male Sprague-Dawley rats (n=4) at different pre- and postnatal ages.[a]

Age (days)	Total Body Weight (g)	Liver Weight (g)	Microsomal Protein (mg/g liver)
Fetus[b]	2.69 (0.58)[c]	0.21 (0.055)[c]	5.71 (2.3)[c]
1	7.09 (0.46)[c]	0.32 (0.077)[c]	9.40 (3.2)[c]
3	9.40 (0.82)[c]	0.42 (0.070)[c]	14.7 (3.8)[c]
5	12.9 (1.4)[c]	0.53 (0.096)[c]	15.7(6.8)[c]
7	17.4 (1.3)[c]	0.63 (0.089)[c]	20.6 (4.0)
10	26.2 (4.3)[c]	0.86 (0.34)[c]	22.3 (4.4)
14	32.7 (3.1)[c]	1.13 (0.24)[c]	27.3 (4.7)
21	59.4 (4.8)[c]	2.62 (0.24)[c]	36.4 (14.1)
28	105 (6.3)[c]	4.93 (0.54)[c]	39.2 (14.0)
42	203 (12.1)[c]	9.39 (0.75)[c]	43.1 (15.3)
63	399 (30.5)[c]	16.2 (2.6)	36.0 (10.9)
84	411 (9.6)[c]	13.8 (0.51)	41.3 (6.9)
112	507 (27.3)	15.0 (1.4)	32.4 (7.3)

[a]Significant life stages in relation to age groups reported in the table: Average gestation period = 22 days; Weaning = 21-28 days; Puberty = 42-63 days; Adult = 112 days (life stage information provided by Charles River).

[b]Fetuses were collected at 20 days gestation from 3 dams and the fetal livers obtained from a single dam were pooled for microsomal preparation.

[c]Statistically significant from adult (112 days), $P<0.05$.

Figure 1

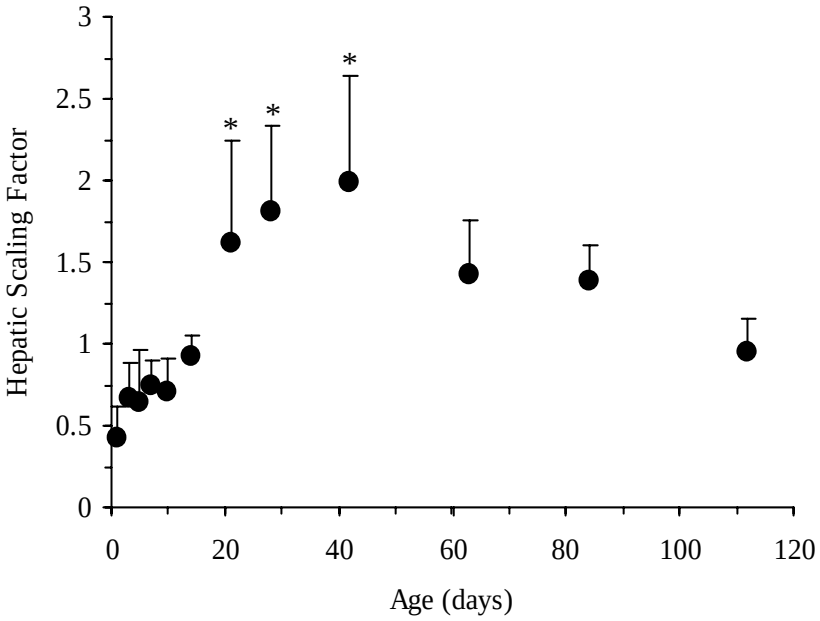


Figure 2

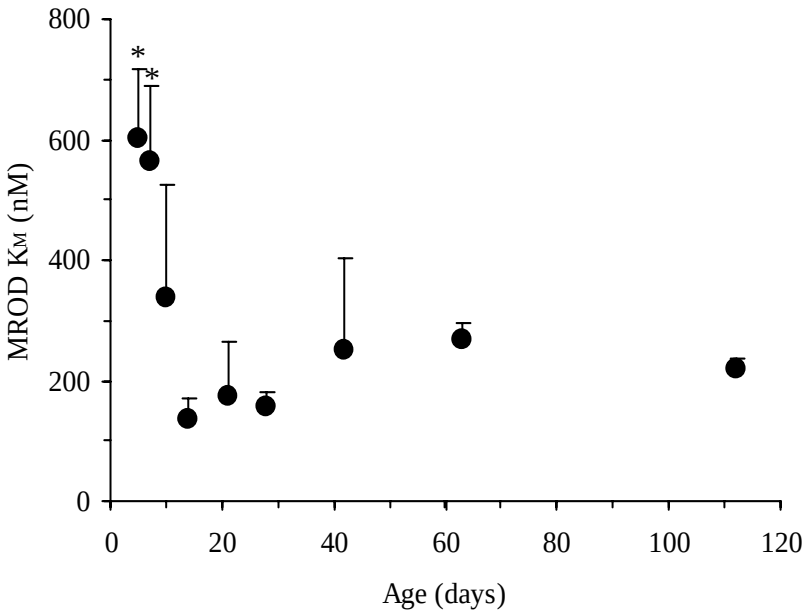


Figure 3A

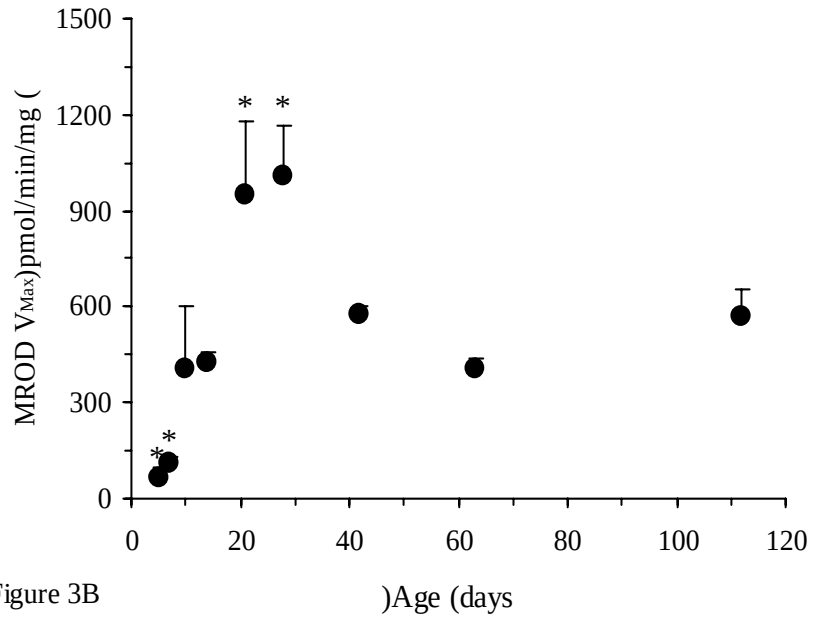


Figure 3B

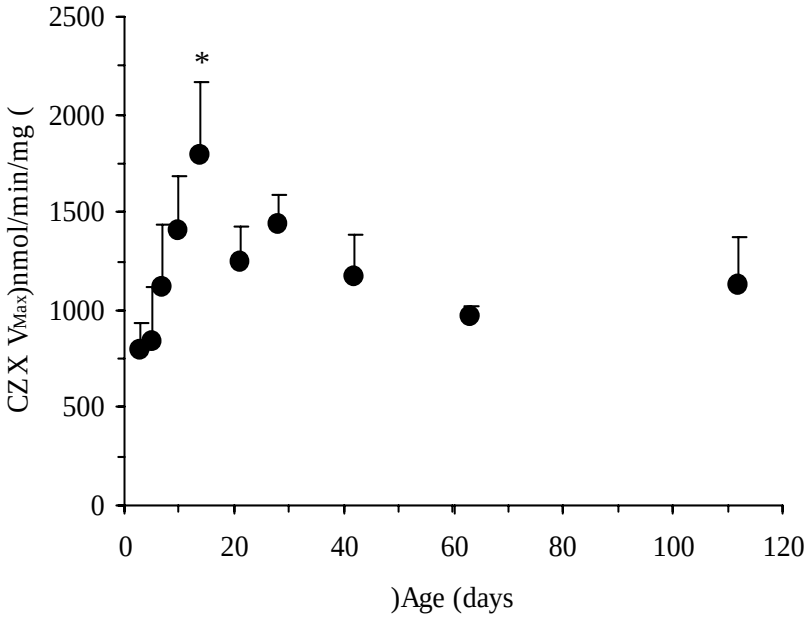
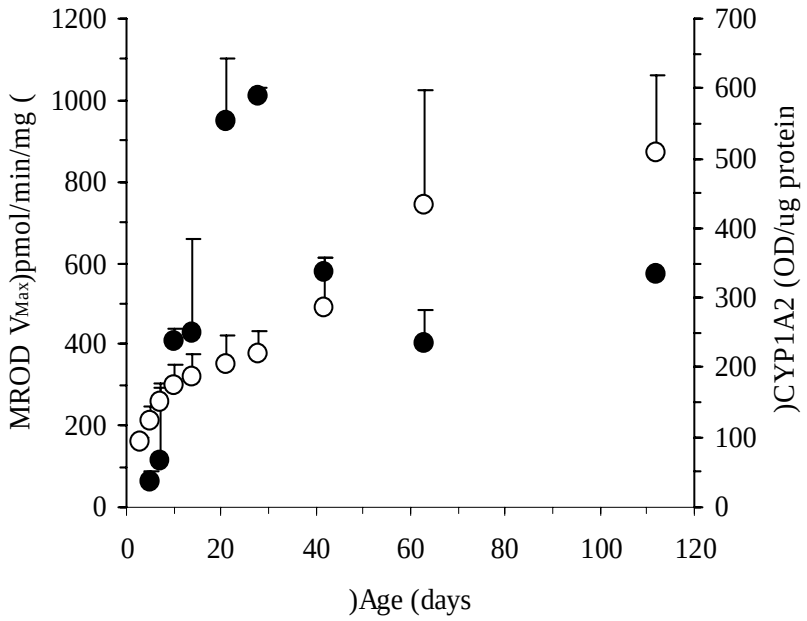


Figure 4A



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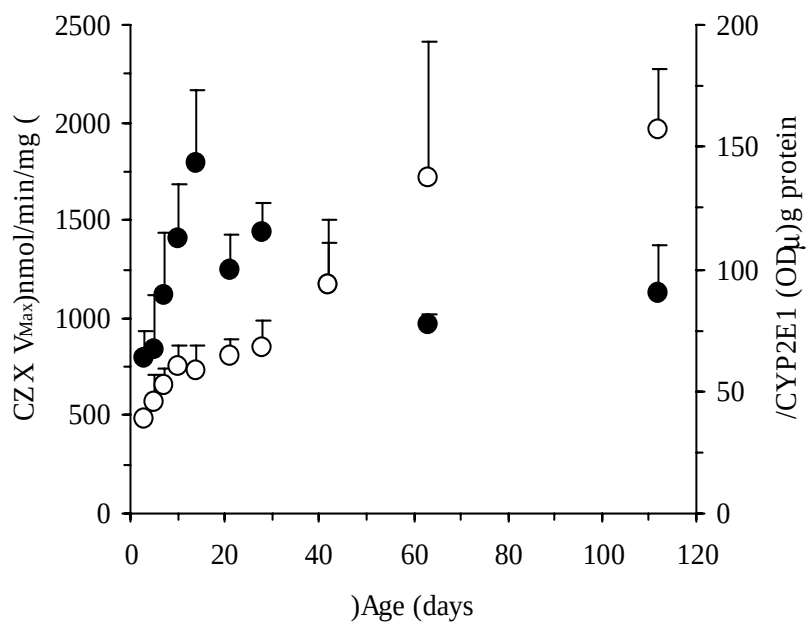

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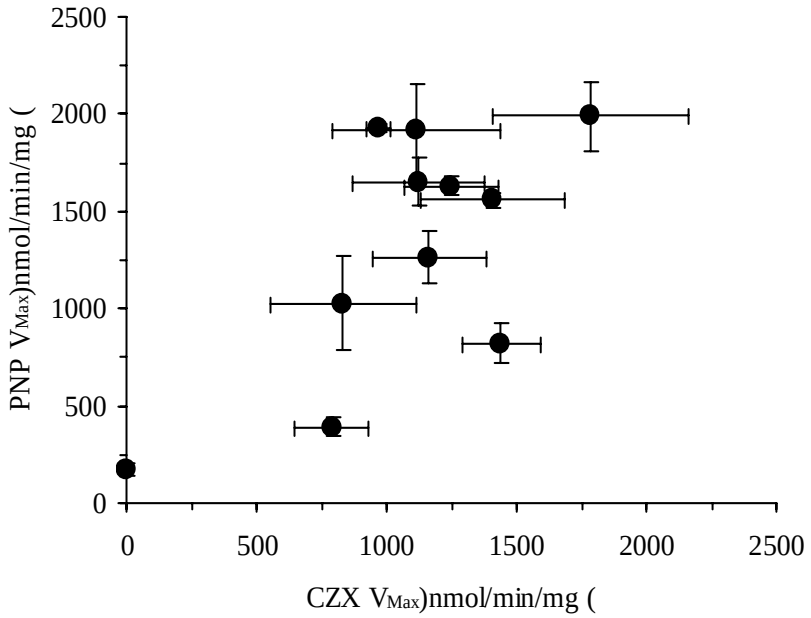

Figure 6