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Effect of Age on The Hepatocellularity Number for Wistar rats

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ABSTRACT

Recently there has been a substantial increase in the number of juvenile animal toxicity studies that are conducted to support pediatric drug safety evaluation. Adequate design of juvenile toxicity studies in rats, for instance with respect to dose levels per age group, requires an understanding of age-dependent pharmacokinetics. In vitro–in vivo extrapolation (IVIVE) and physiologically based pharmacokinetic (PBPK) modeling can help to anticipate age-dependent drug exposure in juvenile toxicity studies provided age-dependent profiles for animal physiology and scaling factors are available. For instance, when hepatocytes are used to predict hepatic drug clearance, the hepatocellularity (number of hepatocytes per gram liver, HPGL) is required as one of the scaling factors. Although HPGL is known for adult rats, information on the influence of age on HPGL is missing. The present work profiles the hepatocellularity number in male Wistar rats as a function of age. Using the NADPH–cytochrome P450 reductase (NCR) activity method, the mean HPGL for the adult rat (8 weeks) was 104 × 10^6 cells/gram liver (relative standard deviation 17%). This value was calculated as a ratio between NRC activities in liver homogenates and suspended hepatocytes. The HPGL values were significantly higher (p <0.001) for rat pups until 3 weeks of age compared with adults. Our results revealed that the HPGL value showed a rapid decrease after 3 weeks (end of weaning), essentially reaching adult values by 4 weeks. This age-dependent HPGL profile will be instrumental for hepatic drug clearance prediction when designing juvenile toxicity studies in Wistar rats.

Introduction

In recent years there has been a surge in the number of juvenile animal toxicity studies conducted specifically for evaluating drug safety in neonates and children. Rats represent the most commonly used preclinical species for evaluating drug toxicity in developing animals. Adequate design of juvenile toxicity studies in rats, for instance with respect to setting appropriate dose levels per age group, requires an understanding of age-dependent pharmacokinetics. Indeed, immaturity of drug elimination pathways in developing animals may easily result in over- or underexposure of young animals (Baldrick, 2004; Coogan, 2012), unless the particular age-dependent effects on expression and activity of enzymes and transporters and/or on renal function are taken into account. In vivo pharmacokinetic/toxicokinetic assessment at various ages prior to the actual juvenile toxicity study represents one approach to obtain insight into the required age-dependent dose-exposure relationship but is both time- and animal-consuming. In vitro–in vivo extrapolation (IVIVE) linked to physiologically based pharmacokinetic (PBPK) modeling has become an indispensable tool in predicting drug exposure profiles as well as drug-drug interactions on the basis of in vitro experimentation. The IVIVE-PBPK approach would also hold great promise for anticipating age-dependent drug exposure in juvenile toxicity studies provided age-dependent profiles for animal physiology and also for scaling factors are available.

Reliable IVIVE-PBPK–based prediction of in vivo drug exposure as a function of age further requires generating age-dependent in vitro data regarding drug elimination, especially for the liver as the major drug eliminating organ. Primary hepatocytes represent a gold-standard in vitro model for hepatic drug-clearance prediction given their biorelevant expression of metabolizing enzymes and transporters mediating hepatobiliary drug disposition pathways (Maeda and Sugiyama, 2010). Until 15 years ago, hepatic drug disposition research focused on metabolizing enzymes as the main mechanism of hepatic clearance. However, the contribution of drug transporters in the hepatic clearance of many compounds is nowadays increasingly recognized (Annaert et al., 2007). Using suspended hepatocytes as model system, we have recently shown that transporter-mediated drug clearance, as previously observed for enzyme-mediated clearance, is also subject to age-dependency (Fattah et al., 2015). Hepatic drug transporters, such as those belonging to the organic anion-transporting polypeptide (Oatp) and organic cation transporter (Oct) families, as well as the bile acid transporter Na^+-taurocholate cotransporting polypeptide (Ntcp), have been recognized as playing a crucial role in mediating the uptake clearance of many endogenous compounds as well as numerous drugs that are currently used clinically. Functional immaturity of these drug transporters together with metabolic enzymes may substantially affect drug clearance at younger ages. Importantly, hepatocytes obtained from these animals at various ages thus provide an excellent in vitro tool to generate age-dependent hepatobiliary drug disposition data.

For extrapolating in vitro data obtained in hepatocytes to in vivo intrinsic clearance estimates, the hepatocellularity (number of hepatocytes per gram liver, HPGL) is required as one of the scaling factors (Carlile et al., 1997). Barter and coworkers (2007) have studied the HPGL in humans at different ages. However, for the purpose of supporting the design and interpretation of juvenile toxicity studies in rats as outlined above, the age-dependency for the HPGL number in rats, as generated in the present study, is required.

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ABBREVIATIONS: HPGL, (number of) hepatocytes per gram liver; IVIVE, in vitro–in vivo extrapolation; MOPS, 3-(*N*-morpholino) propanesulfonic acid; NRC, NADPH-cytochrome P450 reductase; PBPK, physiologically based pharmacokinetic.

Materials and Methods

Materials

William's E Medium, L-glutamine, penicillin-streptomycin mixture (contains 10,000 IU potassium penicillin and 10,000 μg streptomycin sulfate per ml in 0.85% saline), and fetal bovine serum were purchased from Westburg (Leusden, The Netherlands). NADPH was purchased from MilliporeSigma (Billerica, MA). Triton X-100, collagenase (Type IV), MOPS, nicotinamide, EDTA, sucrose, potassium cyanide, and cytochrome C from equine heart (C7752) were sourced from Sigma-Aldrich (Schnelldorf, Germany).

Methods

Animals. Male Wistar rats were used for liver homogenization and hepatocyte isolation from whole livers. Male rats (>26 days) from the same nest were housed in the same cage, while male pups (≤26 days old) were kept with their mothers until the day of sacrifice. Rats of exactly similar age were used at same day for hepatocyte preparation and liver homogenization. Each age group consisted of three rats of approximately the same age and were used on weekly bases, i.e., rats were split as follows: 1 week (7 days ± 1 day/group, n = 2), 2 weeks (14 days ± 1 day/group, n = 3), 3 weeks (21 days ± 1 day/group, n = 3), 4 weeks (27 days ± 1 day/group, n = 3), 6 weeks (42 days ± 1 day/group, n = 2), and 8 weeks (54 days ± 1 day/group, n = 3). The rats were housed according to the relevant Belgian and European laws, guidelines, and policies for animal experiments. Housing and care were provided in the Central Animal Facilities of the University. Approval for this project was granted by the KU Leuven Institutional Ethical Committee for Animal Experimentation.

Liver Homogenization. For the preparation of the liver homogenate, whole livers from rats aged 1- to 8-weeks-old were used. Following an appropriate dose of anesthesia on the basis of body weight (Fattah et al., 2015), the livers were perfused with calcium-free buffer (sparged with 95% O_2/5% CO_2, pH 7.4) until all blood was cleared. Tissue samples were excised into ice-cold homogenization buffer (5 mM MOPS, 1 mM EDTA, and 250 mM sucrose; pH 7.4). Approximately 1 g of liver was finely chopped and homogenized in a glass/Teflon potter homogenizer with 15 up- and down-strokes (1,200 rpm) while kept on ice. Three aliquots were taken and further diluted to the desired concentration with homogenization buffer and samples were kept on ice for at least 20 minutes prior to analysis.

Hepatocyte Isolation. Hepatocytes were isolated using a two-step collagenase perfusion, as described previously (Fattah et al., 2015). After isolation, cells were centrifuged (50g) for 3 minutes at 4°C and the pellet was resuspended in William's E medium containing 5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Hepatocytes were counted using a hemocytometer, and cell viability was determined using Trypan blue (Table 1). Three aliquots of freshly isolated rat hepatocytes were resuspended in homogenization buffer, recounted, and subsequently further diluted to 1×10^6 hepatocytes/ml. The aliquots were homogenized as mentioned above and the samples were kept on ice for at least 20 minutes prior to analysis.

Biochemical Assay. HPGL was determined by the NADPH–cytochrome C reductase (NRC) assay using the method of Beaufay et al. (1974) with slight modification. Cytochrome C activity was determined in the samples of liver homogenates as well as the hepatocytes (prepared as mentioned above). The final concentrations in the assay were 40 mM potassium phosphate (pH 7.4), 25 mM nicotinamide, 0.05% (w/v) Triton X-100, 0.25 mM potassium cyanide, 50 μM cytochrome C, and 75 μM NADPH; temperature was maintained at 37°C. The reaction was initiated by adding 50 μl of NADPH solution, and absorbance was measured using UV light spectroscopy in a Tecan Infinite M200 plate reader (Männedorf, Switzerland). The absorbance at 550 nm was monitored for the following 10-15 minutes at 37°C. The rate of cytochrome C reduction was then calculated using the following equation:

$$Cyc\ c\ = \frac{\Delta A_{550}}{\Delta t} * \frac{V}{v} * \frac{1}{\varepsilon . d * h} * 1000 * F \quad (1)$$

TABLE 1

Average viability and total cell counts per age group

Age	Viability	Cell Counts
weeks	*%*	*million cells per g liver*[a]
1 (*n=2*)	79 ± 6	75 ± 31
2 (*n=3*)	83 ± 4	98 ± 27
3 (*n=3*)	79 ± 4	78 ± 37
4 (*n=3*)	90 ± 1	77 ± 25
6 (*n=2*)	88 ± 6	78 ± 19
8 (*n=3*)	85 ± 2	42 ± 6

[a]These numbers were generated based on cell counting and corresponding liver tissue weights. Actual HPGL values were obtained based on NCR activity measurements and are shown in Table 2.

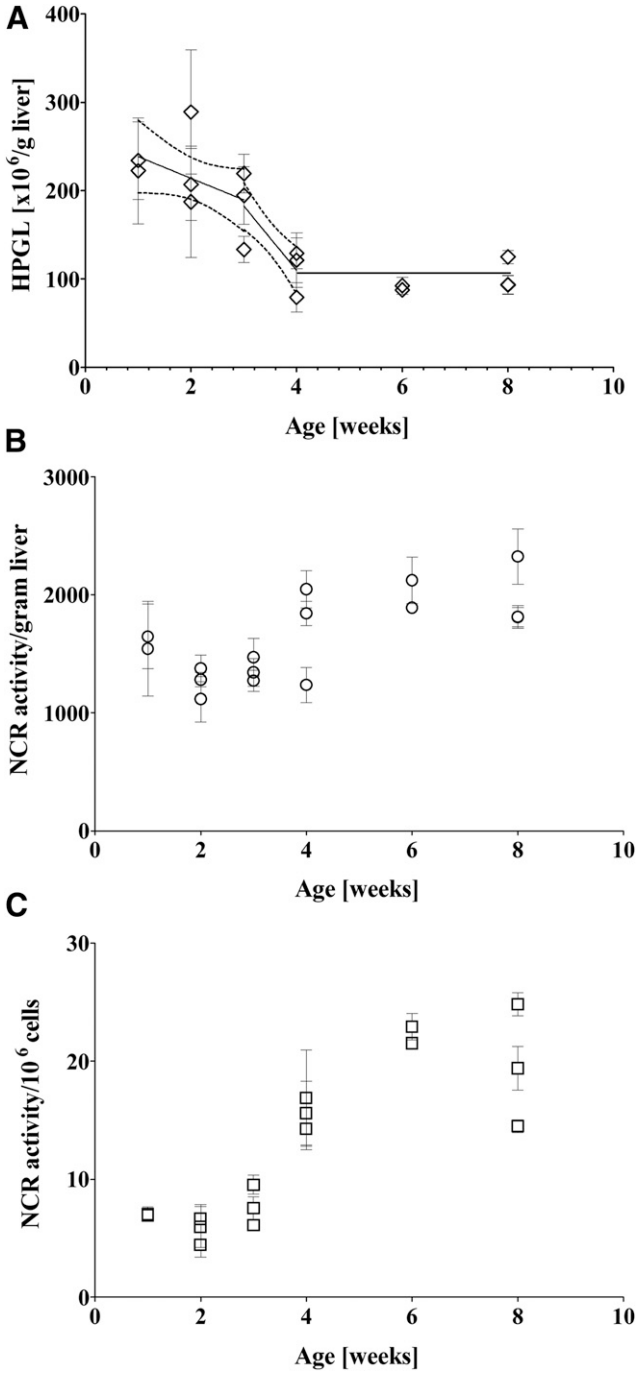

Fig. 1. (A) Correlation between HPGL and age. Points represent the ratio between NRC activity from liver homogenate (n = 3) and NRC activity in suspended rat hepatocytes (freshly isolated) (n = 3). (B) NRC activity per gram liver as a function of age. (C) NRC activity per million cells as a function of age. Each point represents an individual homogenate/hepatocyte preparation.

TABLE 2

Proposed values for HPGL at various rat ages (see also Fig. 1A)

The HPGL values are calculated according to the following equations: rat between 1 week and 2.5 weeks, HPGL = 263 – 25 * Age [week]; rat of 3.5 weeks, HPGL = 401 – 73 * Age [week]; for rats aged 3 weeks, HPGL is calculated as mean value for the outcomes from both equations. For rats 4–8 weeks, a mean HPGL value of 103 (±19) × 10^6 cells/g liver is calculated.

Age (weeks)	1	1.5	2	2.5	3	3.5	4–8
HPGL (× 10^6 cells/g liver)	239	226	214	202	186	146	103

where, ΔA_{550} is net gain of absorbance; Δt, absorbance measuring time period; V, final assay volume; v, sample aliquot; $\varepsilon.d$, extinction coefficient (19.6 mM^{-1} cm^{-1}); h, height of assay solution; and F, dilution factor.

HPGL was calculated as the ratio between NRC activities in the liver homogenates and suspended hepatocytes for corresponding ages.

Statistics. Analysis of variance and Dunnett's post-hoc analysis were used to evaluate statistical differences between hepatocellularity numbers at various age groups. The analysis was performed in GraphPad Prism v. 5.00 for Windows. A p value < 0.05 was used as the criterion for statistical significance.

Results and Discussion

In vitro intrinsic drug clearance values obtained from incubations with suspended hepatocytes are usually expressed in μl/(min*million cells). This necessitates scaling (i.e., IVIVE) to in vivo intrinsic clearance values expressed in ml/(min*kg body weight) prior to integration into PBPK models such as the well-stirred model for the estimation of whole body hepatic drug clearance (Hewitt et al., 2007). The HPGL is one of the required scaling factors used for this purpose. While HPGL is known for adult rats, the age-dependency of HPGL in rats represents a knowledge gap. Using IVIVE-PBPK, application of in vitro data generated with hepatocytes from various ages holds great promise for predicting age-dependent drug exposure in juvenile rats. Therefore, the present study aimed to profile the hepatocellularity number in rats between birth and adulthood.

Various methods are currently available to estimate the hepatocellularity number from different species. These methods typically imply measurement of hepatocyte-specific markers, such as total CYP450 content or NCR activity, intended to estimate the fractional loss during hepatocyte preparation (Barter et al., 2007). This loss of hepatocytes is essentially estimated by measuring the same marker in both the liver homogenate and the suspended hepatocytes. Although there has been some debate about the accuracy of the estimates, use of either cytochrome P450 content or NRC yielded the same value (Wilson et al., 2003; Barter et al., 2007). Hence, in the present study we have used NRC activity to estimate the HPGL with age. NRC activity was determined in both liver homogenate and hepatocyte suspensions of known density. All the data generated during this study were obtained in freshly prepared liver homogenates and suspended hepatocytes. The mean value of HPGL obtained from adult rats (8 weeks) was 104 (±18) × 10^6 cells/gram of liver, which is within the range of the hepatocellularity number (85–135 × 10^6 cells/gram of liver) previously reported for this species (Sohlenius-Sternbeck, 2006).

Interestingly, as shown in Fig. 1A, there was an inverse correlation between age and HPGL. Indeed, HPGL shows a nonlinear decrease with increasing age in rats. Mean (relative standard deviation) HPGL values were 228 (4%), 228 (24%), 183 (24%), 110 (24%), and 90 (4%) in rats of 1, 2, 3, 4, and 6 weeks of age. This outcome is in accordance with a previous study by Barter et al. (2007) showing that HPGL in humans also decreases with age. The former study explained this observation by reference to the status of nuclear polyploidization of hepatocytes. Nuclear polyploidization is associated with increasing number of chromosome sets and this could ultimately affect hepatocyte volume and density (Greengard et al., 1972; Watanabe and Tanaka, 1982; Vassy et al., 1988).

Consistently, it has been demonstrated that the volume of hepatocytes in adult individuals is larger and the liver relatively less populated compared with younger individuals. Accordingly, although the NRC activities were comparable in liver homogenates, they were lower in suspended hepatocytes, as shown in Fig. 1, B and C. The measured NRC activity in suspended hepatocytes was significantly lower ($p \leq 0.001$) during the initial weeks after birth (1–3 weeks, Fig. 1C) compared with adult rats (8 weeks).

The resulting age-dependent HPGL is represented graphically in Fig. 1A, illustrating the most pronounced change in HPGL at the end of weaning, i.e., rats aged between 3 and 4 weeks. Thus the age-dependent HPGL profile was divided in three segments: 1) rats aged 1–3 weeks; 2) rats aged between 3 and 4 weeks; 3) rats 4 weeks and older including adult rats. Accordingly, we propose HPGL values as listed in Table 2.

In conclusion, the values generated for HPGL in rats as a function of age can be used to support reliable prediction of in vivo hepatic drug clearance on the basis of data generated in suspended rat hepatocytes. The overall outcome of this study will facilitate more accurate prediction of in vivo intrinsic clearance prior to initiating studies in juvenile rodents. In future work the age-dependent HPGL profile generated in the present study should be used to predict in vivo hepatic drug clearance as a function of age in rats. Subsequent comparison of predicted values with observed in vivo PK data will support ultimate verification of the age-dependent HPGL profile.

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Drug Delivery and Disposition, KU Leuven Department of Pharmaceutical and Pharmacological Sciences, Leuven, Belgium

Sarinj Fattah
Patrick Augustijns
Pieter Annaert

Authorship Contributions

Participated in research design: Fattah, Annaert.
Conducted experiments: Fattah.
Contributed new reagents or analytic tools: Fattah.
Performed data analysis: Fattah, Annaert.
Wrote or contributed to the writing of the manuscript: Fattah, Augustijns, Annaert.

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Address correspondence to: Dr. Pieter Annaert, Drug Delivery and Disposition, KU Leuven Department of Pharmaceutical and Pharmacological Sciences, Campus Gasthuisberg, O&N2, Herestraat 49 – Bus 921, 3000 Leuven, Belgium. E-mail: pieter.annaert@pharm.kuleuven.be