Changes in Rosuvastatin Pharmacokinetics During Postnatal Ontogenesis in Rats

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ABSTRACT -- Purpose: Statin therapy should be considered in children with familial hypercholesterolemia and sustained high LDL-C levels. There are no data on rosuvastatin exposure in patients <6 years and efficacy/safety can only be derived from case reports. Our aim was to examine developmental changes in pharmacokinetics of rosuvastatin in rats *in vivo* as a basis for clinical development of formulations for patients < 6 years. **Methods:** Rosuvastatin pharmacokinetics was examined in rats aged 1, 4, 7, 10, 14, 21, 28, 35 and 42 days (from birth to sexual maturity). After intraperitoneal dose of 5 mg/kg, blood samples to determine serum rosuvastatin levels were taken at 0.5, 3 and 5 hours. Pharmacokinetic parameters (Vd, CL, AUC_{last}, $AUC_{0-\infty}$) were calculated using pharmacokinecic simulations. **Results:** Both rosuvastatin CL and Vd started to increase systematically between 2 - 3 weeks of age, which was reflected by decreased total drug exposure. The AUC was up to 13 times higher in the age groups ≤14 days compared with the value at 42 days. **Conclusions:** Based on interspecies scaling, a dose reduction could be a feasible way, how to develop appropriate dosing schedule and formulations for children aged 2 - 6 years. However, confirmation in clinical development studies will be needed.

INTRODUCTION

Familial hypercholesterolemia (FH) represents a frequent genetic disorder phenotypically characterized by increased serum cholesterol and low-density lipoprotein (LDL) levels (1). The prevalence of FH is estimated to range between 1/200 – 1:300 in western societies, while the prevalence of homozygous FH is approximately 1:160,000 – 300,000 (2).

FH is, in most cases, caused by mutations in the LDL-receptor gene. Genetic variants of other genes encoding apolipoprotein B, protein convertase subtilisin/kexin type 9 or other targets affecting cholesterol fate in the organism lead to the disease in up to 10% of affected patients. All these variants result in deficient LDL uptake and clearance and predispose patients to premature atherosclerotic cardiovascular diseases. An early diagnosis, adjustment of lifestyle and pharmacotherapy should be utilized as soon as possible to mitigate the risk of clinical complications of early atherosclerosis development (1).

Statins, i.e., 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, are considered the drugs of choice in the therapeutic armamentarium for FH. Especially the highly effective analogs such as rosuvastatin or atorvastatin are recommended for this patient population (3). Most statins have been approved for the use in patients aged 10 years or more by the major drug regulation authorities worldwide, while rosuvastatin may be used in children older than 6 years in the USA and the EU (4-6). The therapeutic guidelines recommend that off label drug therapy should be considered in younger children with sustained LDL-C levels of ≥ 200 mg/dL (7).

However, there are only a few case reports describing beneficial efficacy and safety of statin off-label treatment in younger children. Constantin et al. described the case of a 5-year-old boy, in whom rosuvastatin treatment for 2.5 years has led to the reduction of LDL and total cholesterol levels by 42.4 and 36.4%, respectively. No concurrent adverse effects were observed in this patient (2). Miyagi et al. came with similar findings in 4-year-old twins treated with pitavastatin combined with ezetimibe. In both patients, the reduction by 50 to 60% of LDL levels was achieved, and no adverse effects appeared (8).

Rosuvastatin is a long-acting hydrophilic statin possessing mean steady-state volume of distribution of 134 L in adults, which indicates considerable tissue distribution. Its binding to plasmatic proteins (especially albumin) is about

90%. Metabolism of rosuvastatin is minimal and majority of the drug is excreted unchanged. Approximately 28% of the dose is eliminated via kidneys, while major part (about 72%) is eliminated by hepatic and biliary excretion. Multiple active transporters are involved in the hepatic uptake of rosuvastatin (9, 10).

The drug pharmacokinetics in young children and infants is likely to be altered during postnatal maturation due to the extensive ontogeny of body composition, water content changes and increments of activity of drug related transporters (11). It can be expected that rosuvastatin doses commonly used in adults and older children may lead to overexposure (with potential occurrence of adverse effects) in younger pediatric populations.

FH represents a serious disorder, which early treatment should be initiated to minimize the risk of atherosclerotic cardiovascular disease. However, no clinical data allowing reliable estimate of rosuvastatin exposure, dosing or efficacy and safety are available for patients under 6 years of age. Therefore, our study aims to examine developmental changes of pharmacokinetics (PK) of rosuvastatin in rats *in vivo* as a basis for future clinical development of rosuvastatin formulations for FH patients under 6 years.

MATERIAL AND METHODS

Chemicals

Rosuvastatin calcium, acetonitrile (LC-MS grade), water (LC-MS grade), formic acid (LC-MS grade), and methanol (LC-MS grade) were purchased from Sigma-Aldrich (Saint Louis, USA). Rosuvastatin-d3 Sodium salt (purity ≥ 95%) was supplied by Toronto Research Chemicals (Toronto, Canada). Stock solution was prepared by dissolving rosuvastatin calcium in DMSO to achieve rosuvastatin concentration of 10 mg/mL. This solution was further diluted with water for injection to achieve dosing solution with concentration of 1 mg/mL. Isoflurane was used as IsoFlo 250 mL (Zoetis, Parsippany, USA).

Animals

Pregnant Wistar rats were purchased from Velaz (Prague, Czech Republic) for the purpose of this study. They were maintained under standard conditions (12-h light-dark cycle, 22±2 °C temperature and 50±10% relative humidity) and fed on water and standard granulated diet ad libitum. After delivery, the rat pups were kept with their dam until inclusion into experiment.

All experiments were performed in accordance with the "Guide to the Care and Use of Experimental Animal Care," and every effort was made to minimize animal suffering. The experimental animal project was approved by the animal care review committee of First Faculty of Medicine, Charles University and by the Ministry of Education, Youth and Sports of the Czech Republic under the number MSMT-9445/2018-8.

Experimental procedure

Rat pups 1, 4, 7, 10, 14, 21, 28, 35, and 42 days of postnatal age (PNA) were intraperitoneally dosed with 5 mg/kg of rosuvastatin (5 µL of dosing formulation per 1 g of body weight). After dosing, pups were returned to their dam until sampling. Sparse sampling had to be chosen due to low total volume of blood in rat pups. Systemic blood was drawn at 0.5, 3, and 5 hours after dosing, six rats per each dosing time were used. Blood sample was taken via cardiac puncture in terminal anesthesia (inhalation of 3% isoflurane). Immediately following the sampling, rats were sacrificed by cervical dislocation and death was confirmed by lack of a heartbeat. Blood samples were centrifuged for 10 minutes at 2500×g (4 °C) and serum was extracted. Serum samples were then stored at -80 °C before further processing.

Sample preparation

Serum samples were processed as follows. In 20 µL of serum, proteins were precipitated by the addition of 60 µL of acetonitrile (containing 40 ng/mL rosuvastatin-d_3 as an internal standard). The mixture was vortexed and centrifuged at 9800×g for 6 min. 40 µL of supernatant was transferred into a chromatographic vial. Samples whose concentration exceeded upper limit of quantitation were appropriately diluted with blank serum to reach the concentration within the linear range of the method.

Bioanalytical assay

Determination of rosuvastatin in serum samples was carried out on the Nexera X3 UHPLC coupled with a Triple Quad 8045 tandem mass spectrometer (Shimadzu, Kyoto, Japan). Poroshell 120 SB AQ column (100 × 2.1 mm; 2.6 µm particle size) from Agilent Technologies (Waldbronn, Germany), thermostatted at 40 °C, was used for the analysis. Mobile phase (A: 0.1% formic acid in deionized water, B: 0.1% formic acid in methanol) was pumped in a flow rate of 0.35 mL/min and the following optimized gradient program was applied (min/%B) 0/30, 2.5/90, 3.0/90, 3.5/30, 5.5/30. The

injection volume was 1 µL, and samples were kept at 10 °C. Effluent from the column was directed to the MS ion source between 2.0 and 4.0 min only. For the rest of the time, the effluent was directed to the waste. The tandem mass spectrometry operated in multiple reaction-monitoring mode (MRM) using positive electrospray ionization. MRM transitions of 482.2 > 258.1 (Q1 pre-bias -14 V, Q3 pre-bias -23 V and collision energy -35 V) and 485.2 > 261.1 (Q1 pre-bias -14 V, Q3 pre-bias -25 V and collision energy -35 V) were monitored for rosuvastatin and rosuvastatin-d_3 (internal standard), respectively. The ion source was set as follows: nebulizing gas flow: 3 L/min, heating gas flow: 8 L/min, interface temperature: 320 °C, desolvation line temperature: 300 °C, heat block temperature: 350 °C, and drying gas flow: 10 L/min. Calibration was performed every day before measuring samples and quality control samples were injected after each 6th sample.

Assay validation

The method was fully validated with respect to linearity, LOD, LLOQ, ULOQ, accuracy, precision, selectivity, recovery, matrix effects, dilution integrity, and stability. An eight-point calibration curve was constructed using the analyte-to-internal standard peak area ratio. Weighted least-squares linear regression ($1/x^2$ weighting factor) was used. The linearity was evaluated through the calibrations by coefficients of determination (R^2) and back-calculated concentrations of calibration points. For a succesful verification of the linear range, the back-calculated concentration values should not deviate from the nominal values by more than 15% for the whole calibration range and by 20% for the LLOQ level. LLOQ was the lowest calibration point, and ULOQ was the highest calibration point. LOD value was determined as a concentration providing a signal corresponding to 3.3 times blank matrix baseline noise. Accuracy and precision were determined via analysis of the fortified blank samples (QC samples) at three concentration levels (1, 50, and 500 ng/mL) at six replicates ($n = 6$). QC concentrations were chosen based on the expected concentration range of the majority of the rat samples. The accuracy was expressed as the relative error (RE, %; calculated as ((measured concentration – expected concentration)/expected concentration)*100), and the precision expressed by repeatability as the relative standard deviation (RSD). Recovery of the method was assessed by comparison of rosuvastatin concentration found in a serum sample spiked with the standard before precipitation of proteins and concentration found in a serum sample spiked after precipitation of proteins at three concentration levels (1, 50, and 500 ng/mL). Matrix effect was evaluated at the same concentration levels of six serum samples. It was determined by comparing the area of the rosuvastatin standard peak of the post-protein-precipitation spiked plasma sample with that of the 80% acetonitrile (without matrix effect). Method selectivity was checked by injecting six blank serum samples. Dilution integrity was investigated at six replicates of spiked serum samples with the 15 µg/mL rosuvastatin concentration. Samples were diluted 20-fold using blank serum and assayed. Serum concentrations were measured and recalculated to original concentrations. The stock solutions stability of rosuvastatin and its IS was tested at room temperature for 12 h and refrigerated (-80 °C) for 1 month. Rosuvastatin stability tests on freeze-thaw (three cycles), long-term (-80 °C for 1 month), bench-top (room temperature for 4 h), whole blood (after collection), and autosampler (processed samples in the autosampler for 24 h at 10 °C) were performed using three replicates at three concentration levels (1, 500, 15000 ng/mL).

Pharmacokinetic analysis and statistics

Median and interquartile range (IQR) serum concentration values in each sampling time and each PNA group was calculated using GraphPad Prism 8.2.1 (GraphPad Software, Inc., La Jolla, USA).

Potential differences in $C_{0.5}$, C_3 and C_5 between PNA groups were examined by the Kruscal-Wallis test using GraphPad Prism 8.2.1 (GraphPad Software, Inc., La Jolla, USA). Statistical significance was considered at $P \leq 0.05$.

Rosuvastatin pharmacokinetic parameters – area under the concentration-time curve from 0 to 5 h (AUC_{0-5}), area under the concentration-time curve from 0 to infinity ($AUC_{0-\infty}$), apparent volume of distribution (Vd) and apparent clearance (CL) were calculated in a one-compartmental pharmacokinetic model with first-order absorption and elimination kinetics based on body weight, administered rosuvastatin dose and measured rosuvastatin serum levels using MWPharm^{++} software (MediWare, Prague, Czech Republic). In this simplified model, the bioavailability was set to 100% and the absorption rate constant to 8.5 h^{-1} (corresponds to a t_{max} within half an hour) (12).The simulated rosuvastatin pharmacokinetic profile curve was individualized to maximize fitting with median rosuvastatin measured concentration points in each PNA group. The fitting was performed using Marquardt nonlinear least-square method. The goodness of fit was expressed using weighted sum of squares and root mean square values.

RESULTS

Bioanalytical assay validation

The developed method was linear in the range of 0.2-1000 ng/mL (R^2 > 0.9996). LLOQ was 0.2 ng/mL with precision and accuracy up to 14% (back-calculated). ULOQ was 1000 ng/mL with precision and accuracy up to 6% (back-calculated). The accuracy and precision of back-calculated concentrations of other calibration points were within 7% of the nominal concentration. The detection limit was 0.05 ng/mL, which was sufficient for the determination of rosuvastatin in serum samples. The accuracy of QC samples was within ±8.2 %, and interday and intraday precision were between 1.5 and 6.2 % (Table S1 in Supporting information). Recovery ranged from 97.6 to 102.1 % (Table S2 in Supporting information). The matrix effect ranged from 83 to 105%. The use of an isotope-labeled standard eliminated the matrix effect since the analyte/IS response ratio remained unaffected, even when the absolute responses of the analyte and IS were affected. The developed method was selective since no interfering peaks from endogenous serum components appeared at the retention time of rosuvastatin. The accuracy and precision of dilution integrity samples were within ±8%. No significant rosuvastatin degradation occured under the tested stability conditions. The accuracy of stability samples was within ±8 % with precision <6%. Stock solutions of rosuvastatin and its IS were stable under tested conditions. Representative MRM chromatograms of a serum blank sample, a blank serum sample spiked with rosuvastatin at the LLOQ concentration of 0.2 ng/mL, and a studied sample at the concentration of 23 ng/mL are depicted in Figure S1 (Supporting information). All validation parameters meet the criteria for bioanalytical methods and thus proved the reliability of this novel method developed for the determination of resuvastatin in serum samples.

In vivo pharmacokinetic study

Totally, 162 rat pups were enrolled into this study – six rats per sampling time (0.5, 3, and 5 hour after dosing) in each of nine PNA groups (1, 4, 7, 10, 14, 21, 28, 35, and 42 days). Among them, there were 85 males and 77 females as determined based on anogenital distance. We tried to keep the number of males and females in the PNA groups and sampling time subgroups balanced. Median body weights were 5.5, 9, 12, 17, 23, 39, 75, 125, and 174 g in 1, 4, 7, 10, 14, 21, 28, 35, and 42 PNA groups, respectively. Rosuvastain PK parameters in each PNA group are summarized in Table 1. The $C_{0.5}$, C_3, and C_5 were significantly different between PNA groups (P < 0.0001 at all sampling times). The concentration-time data as a dot plot and fitted pharmacokinetic profiles for each PNA group are presented in supplementary Figure 2 and supplementary Figure 3, respectively. Graphical illustration of rosuvastatin PK parameters against PNA is shown in Figure 1. Median (IQR) weighted sum of squares and root mean square values were 11.49 (5.53-23.90) and 0.95 (0.94-0.98), respectively.

DISCUSSION

The decision on hypolipidemic treatment onsent in patients with FH is currently mainly made based on LDL-C serum levels as this parameeter is thought to be a valid surrogate for clinical atherosclerotic cardiovascular disease and outcomes. Statin should be initiated as early as possible accordining to the current treatment guidelines for pediatric FH and the dose should be uptitrated to the maximum tolerated one (7).

The indication of rosuvastatin has been extended to children from 6 years based on the results of an open-label single arm CHARON study and a dedicated pharmacokinetic study that investigated pharmacokinetics and exposure to the drug as well as its efficacy and safety in this age group (13). Drug exposure in paediatric patients appeared to be dose proportional with only slightly increased drug accumulation observed in the pediatric population (1.8 fold) compared to the adult population (1.1-1.4 fold). This suggested that the t½ in children was only a little longer in comparison with that seen in adults (13).

The key active transporters involved in rosuvastatin hepatobiliary excretion (that is the major rosuvastatin elimination pathway) are multidrug resistance associated protein 2 (Mrp 2), bile salt export pump (Bsep), and multiple organic anion transporting polypeptides (OATP) family (14, 15). Approximate adult rat activities of these transporters may be expected after day 12 of PNA for Bsep and Mrp2 and on day 29 for OATP family based on expression levels relative to adult values achieved in the liver (16). These *in vitro* results well corresponded with the rise of rosuvastatin CL observed in our *in vivo* study. In this study, we observed that both rosuvastatin CL and Vd started to increase systematically between 2 and 3 weeks of rat PNA, which is reflected by the decreased total drug exposure.

Table 1. Pharmacokinetic parameters of rosuvastin according to PNA groups

PNA (days)	$C_{0.5}$ (mg/L)	C_3 (mg/L)	C_5 (mg/L)	AUC_{0-5} (mg.h/L)	$AUC_{0-\infty}$ (mg.h/L)	CL (L/h/kg)	Vd (L/kg)
1	4.150 (2.729-4.915)	0.417 (0.208-0.549	0.199 (0.160-0.503)	6.41	6.64	0.75	1.09
4	2.794 (2.246-3.334)	0.314 (0.240-0.530)	0.271 (0.173-0.350)	4.65	5.02	1.00	1.85
7	5.795 (5.452-8.925)	0.474 (0.322-0.788)	0.403 (0.324-0.566)	8.50	8.95	0.56	0.91
10	9.153 (5.574-11.175)	0.541 (0.212-0.906)	0.217 (0.119-0.510)	11.94	12.13	0.41	0.49
14	7.858 (6.498-9.128)	0.625 (0.526-0.800)	0.249 (0.176-0.351)	11.17	11.42	0.44	0.56
21	2.597 (0.334-2.958)	0.106 (0.867-0.130)	0.044 (0.033-0.064)	3.02	3.05	1.64	1.77
28	0.740 (0.609-0.874)	0.852 (0.387-0.105)	0.014 (0.008-0.018)	1.19	1.21	4.13	4.70
35	0.575 (0.279-0.629)	0.836 (0.380-0.867)	0.015 (0.011-0.023)	0.99	1.01	4.97	6.13
42	0.570 (0.414-0.814)	0.624 (0.332-0.776)	0.023 (0.016-0.031)	0.90	0.93	5.40	7.42

Data are presented as medians (interquartile range) or medians (PK parameters except $C_{0.5}$, C_3 and C_5 were estimated from concentration-time profiles that were simulated from levels obtained from all group probands, and therefore individual PK parameters, and thus variability cannot be calculated). PNA: postnatal age; $C_{0.5}$, C_3 and C_5: observed serum concentrations at 0.5, 3 and 5 hour after rosuvastatin administration; $AUC_{0-\infty}$: area under the curve from zero to infinity; AUC_{0-5}: area under the curve from 0 to 5 hours; CL: apparent clearance; Vd: apparent volume of distribution.

Although it is based only on an indirect comparison of the results from two studies, the ontogenic maturation of rosuvastin transport capacity is likely to explain our PK results. However, the extrapolation of our results to humans needs to be done cautiously as the knowledge about human ontogeny of drug transporter activity, which is essential for application of personalised tretment in pediatric population, is still limited. Mrp2 protein levels were significantly lower in liver specimens from children younger than 8 months in comparison with older children, while no significant age dependence of Mrp2 protein expression was observed in subjects from 7 to 63 years. For OATPs, the mRNA expression was 500 to 600-fold lower in neonates, and 90 to 100-fold lower in infants compared to adults. Similarly, mRNA expression of Bsep increased from neonates to older children and adults (17). Nevertheless, these sparse data correspond to the approximate similarity of rosuvastatin pharmacokinetics in children >6 years to that of adults seen in clinical drug development (13).

Equivalent human ages to the rat PNA ontogeny have been addopted from the study by Picut et al (18), who studied equivalent endocrinne maturational stages between rat and man. This interspecies scaling has been chosen as it has been documented that that hormonal signaling is an important factor for regulation of drug transporter activity (19). Table 2 shows approximate age comparison for developmental stages between rat and man as well as relative rosuvastatine exposure in each age group compared to the exposure in adults.

The C_{max} and AUC values at 35 and 42 days of PNA in our study were almost identical. This stage corresponds to equivalent human age of > 12 years, when the human drug pharmacokinetics is similar to that observed in adults (13, 20). Therefore, no further ontogeny of drug pharmacokinetics likely corresponds to the achievement of adult activities of the key pathways involved.

Table 2. Rosuvastatin exposure in equivalent age ranges for rat and human ontogeny

Age stage	Rat PNA	Human PNA	$AUC_{0-\infty}$ relative to adults
Newborn	0-7 days	0-28 days	540-962%
Infant	8-20 days	1-23 months	1228-1304%
Juvenile/child	21-32 days	2-12 years	109-328%
Puberty/adolescent	> 32 days	> 12 years	100%

Equivalent age ranges between rat and human was adopted from Picut *et al* (18).

At the rat age of 21 days (equivalent to human age of 2 years), the total exposure was approximately 3-times higher than in adults, while in rat pups younger than 21 days (equivalent to human neonates and infants) the drug exposure was extremely increased (from 5- to 13-fold higher) in comparison to the adult values. On the other hand, the drug exposure in rats aged 28 and 32 days have been only slightly increased in comparison to the expected adult exposures.

We acknowledge that there are some limitations of our study. Whereas this is the first exploratory study, several simplifications of the

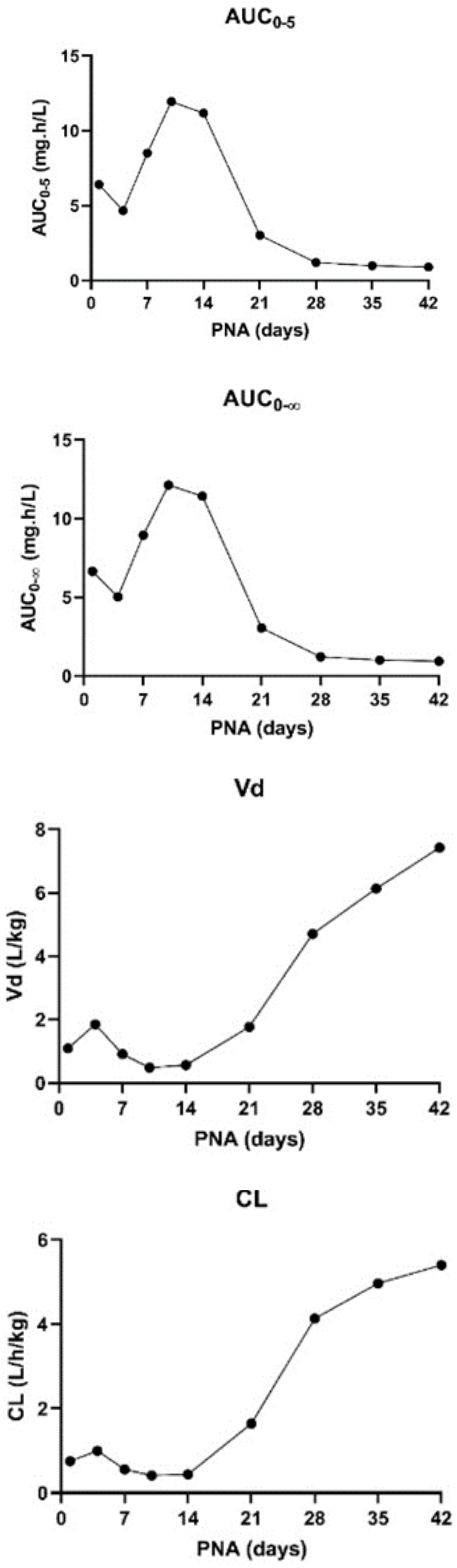

Figure 1. Development of pharmacokinetic parameters of rosuvastatin during postnatal ontogenesis. PNA: postnatal age; $AUC_{0-\infty}$: area under the curve from zero to infinity; AUC_{0-5}: area under the curve from 0 to 5 hours; CL: apparent clearance; Vd: apparent volume of distribution. Data are expressed as medians (PK parameters were estimated from concentration-time profiles that were simulated from levels obtained from all group probands, and therefore individual PK parameters, and thus variability cannot be calculated).

design have been incorporated. Although rosuvastain is intended for chronic treatment, we explored its pharmacokinetics after a single dose administration. Since both rosuvastatin plasma concentrations and AUC were described to be linear (9), this simplification shall not impact on the results and their interpretation. Rosuvastatin was administered intraperitoneally in our study, although oral application is typical in humans. Intraperitoneal administration represents a common technique in exploratory experiments with rodents as it enables reliable administration of relatively large volume of liquid medication and reduces the high variability in pharmacokinetics caused by the absorption phase. Moreover, we aimed to examine developmental changes caused especially by ontogenesis in hepatic transporters, and thus in elimination phase.

As the blood volume of such young pups is very limited, we have chosen sparse sampling approach. Limited number of measured concentrations was compensated by PK simulations to estimate the PK parameters. Since the absence of PK data for rosuvastatin in juveniles, we opted for the Marquardt nonlinear least-square method of fitting. This method fits a parameterized model to the set of data points by minimizing an objective expressed as the sum of the squares of the errors between the model function and a set of data points and thus limits the need for an initial model.

Rat as an experimental animal was chosen as traditionally the species of first choice for juvenile animal studies. Moreover, we aimed to describe mainly rosuvastatin elimination which is provided by the same transporters involved in both humans and rats (15). Both male and female rats were included in our study to mimick the real use of the drug in population. Exploration of the PK ontogeny in the age corresponding to children up to 6 years was the main aim of our study and sexual dimorphism is minor in this age group.

The typical reason for exclusion of female rats from experiments is the variability due to cyclical reproductive hormones (21). As the onset of puberty occurs in rats at PNA of 32 to 34 days, only the 2 highest age groups of rats used in our study (PNA 35 and 42) included females potentially impacted by the hormonal cycle.

CONCLUSIONS

Our study helps to understand exposure differences across age ranges from birth to sexual maturity in rats and the results may serve as a pre-clinical basis for future clinical development of rosuvastatin

containing formulations for FH treatment in pediatric patients up to 6 years of age.

Based on our data, it can be estimated, that rosuvastatin exposure may be substantially increased untill the age of 2 years. For children aged 2 - 6 years a dose reduction could be a feasible way, how to develop appropriate dosing schedule and formulations. However, since the interspecies scaling of PK data may not be fully accurate, the confirmation in clinical development studies will be needed.

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CONFLICTS OF INTEREST. The authors declare there are no competing interests.

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Supplementary

Table S1. Accuracy and precision of the LC-MS/MS method (n=6 replicates; for 2 days)

Rosuvastatin concentration (ng/mL)	**Intra-day (*n=6*)**			**Inter-day (*n=6*)**		
	Measured concentrations (ng/mL) (mean±SD)	RSD (%)	RE (%)	Measured concentrations (ng/mL) (mean±SD)	RSD (%)	RE (%)
1	0.980 ± 0.031	3.2	-2.0	1.082 ± 0.067	6.2	8.2
50	51.900 ± 1.505	2.9	3.8	52.100 ± 1.771	3.4	4.2
500	494.500 ± 7.417	1.5	-1.1	515.500 ± 14.949	2.9	3.1

SD, standard deviation; RSD, relative standard deviation; RE, relative error

Table S2. Recovery of the LC-MS/MS method (n=6 replicates)

Recovery (%) (mean)	**Recovery (%) (mean)**	**SD (%)**	**RSD (%)**
1	97.6	1.46	1.50
50	99.1	1.90	1.92
500	102.1	1.99	1.86

SD, standard deviation; RSD, relative standard deviation