Combined Analysis of Pharmacokinetic and Efficacy Data of Preclinical Studies with Statins Markedly Improves Translation of Drug Efficacy to Human Trials


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Running title: Combined Preclinical PK and Efficacy Analysis of Statin Drugs

Corresponding author: Dr. Evita van de Steeg, TNO, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist, The Netherlands, E-mail: evita.vandesteeg@tno.nl, phone: +31 88 866 2322

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Non-standard abbreviations: AUC, area under the curve; CI, confidence interval; E3L, ApoE*3Leiden; ELISA, enzyme-linked immunosorbent assay; FDA, US Food and Drug Administration; HFC diet, high fat cholesterol diet; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; NME, new molecular entity; PK, pharmacokinetics; SD, standard deviation; TC, total plasma cholesterol; TG, total plasma glycerides; VLDL, very-low-density lipoprotein.
ABSTRACT

Correct prediction of human pharmacokinetics, safety and efficacy of novel compounds based on preclinical data is essential but often fails. In the current study we aimed to further improve the predictive value of ApoE*3Leiden (E3L) transgenic mice regarding the cholesterol-lowering efficacy of various statins in humans by combining pharmacokinetic with efficacy data. The efficacy of 5 currently marketed statins (atorvastatin, simvastatin, lovastatin, pravastatin and rosuvastatin) in hypercholesterolemic patients (LDL ≥ 160 mg/dL) was ranked based on meta-analysis of published human trials. Additionally, a preclinical combined PK-efficacy dataset for these 5 statins was established in E3L mice, that were fed a high-cholesterol diet for 4 weeks, followed by 6-weeks drug intervention in which statins were supplemented to the diet. Plasma and tissue levels of the statins were determined upon administration of (radiolabeled) drugs (10 mg/kg p.o.). As expected, all statins reduced plasma cholesterol in the preclinical model, but a direct correlation between cholesterol lowering efficacy of the different statins in mice and in humans did not reach statistical significance (R²=0.11, p<0.57). Importantly, when murine data were corrected for effective liver uptake of the different statins, the correlation markedly increased (R²=0.89, p<0.05). Here we show for the first time that hepatic uptake of statins is related to their cholesterol-lowering efficacy and provide evidence that combined PK and efficacy studies can substantially improve the translational value of the E3L mouse model in case of statin treatment. This strategy may also be applicable for other classes of drugs and other preclinical models.
INTRODUCTION

The pharmaceutical industry is facing a huge challenge in marketing new medicines mainly due to decreased R&D productivity and the decreasing number of truly innovative new medicines approved by the US Food and Drug Administration (FDA) (Booth and Zemmel, 2004). For example, the number of new molecular entities (NMEs) that were approved by the FDA over 2000-2005 was 50% lower compared to the preceding 5 years. Although big variation may exist between different classes of medicines, the average investment needed to bring a NME to the market is estimated to be approximately $1.8 billion and is still rising rapidly (Paul et al., 2010). Interestingly, the number of drug candidates successfully reaching Phase 1 has increased over the past years, partly due to better preclinical characterization and improved ADMET properties. Nevertheless, attrition in late-stage drug development (Phase II and III) is still high, and therefore remains the most important determinant of the overall R&D efficiency. For example, the Phase II success rates for NMEs have fallen from 28% (2006-2007) to 18% (2008-2009), while the prospect of successfully progressing through Phase II is currently 50%. Importantly, attrition in Phase II is mainly caused by insufficient efficacy of the newly developed drug, accounting for 51% of the drug failures (Arrowsmith, 2011; Arrowsmith, 2012; Kola and Landis, 2004). The importance of correctly predicting the efficacy of novel drug candidates and demonstrating the disease-modifying potency of those candidates especially in the early stage pre-clinical phases is therefore crucial. While the translation from mouse to man proves to be difficult due to interspecies differences in efficacy and/or pharmacokinetics, humanized mouse models are being developed to improve such translation.

The ApoE*3Leiden (E3L) transgenic mouse model is a well-established humanized model for (familial) hyperlipidemia and cholesterol-induced atherosclerosis (van Vlijmen et al., 1994; Zadelaar et al., 2007). E3L mice develop a human-like lipoprotein profile upon
feeding a fat and/or cholesterol-containing diet, with elevated plasma cholesterol and triglyceride levels mainly present in very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). After long-term treatment (~14 weeks) with a fat and/or cholesterol-containing diet, E3L mice develop atherosclerotic lesions with all the characteristics of human vascular pathology (Zadelaar et al., 2007). The E3L mouse model is therefore a widely used model for studying the differential effects of cholesterol-lowering drugs (e.g. statins, fibrates, niacin) (Kleemann et al., 2003; van Vlijmen et al., 1998; Verschuren et al., 2005; Verschuren et al., 2012; Zadelaar et al., 2007).

Statins are lipid-lowering drugs that are widely prescribed to reduce the risk of primary and secondary of coronary heart disease. Statins reduce plasma cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and by enhancing hepatic uptake of LDL cholesterol via upregulation of LDL receptor expression (Liao and Laufs, 2005). It is being estimated that about 50 million people take statins everyday worldwide. The currently marketed statins used in this study belong to the second (lovastatin, simvastatin and pravastatin) and third generation (atorvastatin and rosuvastatin) statins, with the latter having a higher affinity to inhibit HMG-CoA reductase and inhibit the enzyme for a longer duration (Kleemann and Kooistra, 2005). This is also obvious from clinical studies that demonstrate that only atorvastatin and rosuvastatin can reduce plasma LDL-cholesterol by more than 40% in patients with hypercholesterolemia (Weng et al., 2010). The therapeutic equivalence of statins to reduce LDL-cholesterol in humans was recently reviewed in this meta-analysis by Weng et al., providing a ranking order from most to least potent of rosuvastatin > atorvastatin > simvastatin > lovastatin > pravastatin. Importantly, the intensity of a drug’s effect is, besides its affinity for the drug target (e.g. receptor or enzyme), mainly determined by the drug concentration at the site of action. Since HMG-CoA reductase is mainly expressed in the liver (hepatocytes), the efficacy of statins to
reduce plasma cholesterol levels is largely dependent on the pharmacokinetics and local concentration of these drugs in the liver. For example, it has been demonstrated that patients carrying a variant of the hepatic uptake transporter, OATP1B1*15 (Asn130Asp and Val174Ala) which is known to have a strongly reduced transport activity, show markedly increased plasma levels, decreased pharmacological response and even increased extra-hepatic toxicity after rosuvastatin or pravastatin treatment (Niemi et al., 2011).

The aim of this study was to test the hypothesis that intelligent combination of pharmacokinetic and efficacy data may increase the predictability of drug efficacy outcomes in pre-clinical models. To this end, we setup a large scale preclinical study with E3L transgenic mice in which we determined the PK and efficacy profile of the 5 currently marketed statins, and compared them with their efficacy in humans based on data from a literature meta-analysis. Here, we report for the first time a systematic link between the hepatic uptake of statins and their cholesterol-lowering efficacy in a valuable mouse model of hyperlipidemia, and provide evidence that PK studies combined with efficacy studies could substantially improve the translational value of the E3L mouse model in case of statin treatment.
MATERIALS & METHODS

Systematic review of the cholesterol-lowering abilities of statins in humans

To systematically review the abilities of 5 commonly used statins (atorvastatin, pravastatin, simvastatin, lovastatin and rosuvastatin) known to reduce plasma cholesterol levels in patients with hyperlipidemia, we selected all clinical trials published between 1966 and 2009 that were previously also reviewed by Weng et al. and the Oregon Health Resources Commission (OHRC) (Beth Smith et al., 2009; Weng et al., 2010). These meta-analyses used the following selection criteria to include the studies: (1) only randomized controlled trials with (2) head-to-head comparisons of at least two statins were included. Trials needed to be based on (3) human subjects older than 18 years, (4) that used statins as monotherapy to treat hyperlipidemia. The studies had to (5) report the primary data, (6) needed to last for at least 4 weeks, (7) and had to be published in English. As additional selection criteria, we only included trials in which patients with baseline plasma LDL-C levels of >160 mg/dL (i.e. cutoff for hypercholesterolemia (National Cholesterol Education Program (NCEP), 2002)) were selected, resulting in 77 selected studies (see Supplemental data 1).

Animals

ApoE*3Leiden (E3L) mice, bred by The Netherlands Organization for Applied Scientific Research TNO, were housed and handled according to institutional guidelines complying with Dutch legislation. In this study we used female heterozygous E3L transgenic mice (8–12 weeks of age), characterized by enzyme-linked immunosorbent assay (ELISA) for the presence of human apoE*3 in the serum (van Vlijmen et al., 1998). All animal experiments were approved by the Institutional Animal Care and Use Committee of TNO.
Chemicals and reagents

The following drugs were supplemented to the diet: atorvastatin (Lipitor®; atorvastatin-calcium; Pfizer), pravastatin (Selektine®; pravastatin-sodium; Bristol-Myers Squibb), lovastatin (Toronto Research Chemicals, Ontario, Canada, #L472225), simvastatin (Zocor®; simvastatin (lacton); Merck Sharp & Dohme), and rosuvastatin (Crestor®; rosvastatin-calcium; AstraZeneca). For the pharmacokinetic experiments we used atorvastatin calcium and rosuvastatin calcium from Sequoia Research Chemicals (Pangbourne, UK) and simvastatin, lovastatin and pravastatin sodium from Toronto Research Chemicals (Ontario, Canada). [3H]-atorvastatin (740 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, US). [3H]-rosuvastatin (40.7 GBq/mmol) and [3H]-pravastatin (136.9 GBq/mmol) were custom synthesized by Moravek Biochemicals (Brea, California, US).

Study design

During a 4-week week run-in period, all animals (except for the chow-control group, n=12) received a semi-synthetic high fat cholesterol (HFC) diet containing 40.5% sucrose, 15% cacao butter and 1% cholesterol (all w/w) (Abdiets, Woerden, The Netherlands #4021.04). After randomization into 11 groups (n=8 per group) matched for age, body weight, plasma cholesterol and triglyceride levels, the mice received HFC diet alone (control group) or HFC supplemented with either atorvastatin (0.003% or 0.008% w/w), pravastatin (0.03% or 0.05% w/w), lovastatin (0.05% or 0.07% w/w), simvastatin (0.03% or 0.06% w/w), or rosuvastatin (0.0025% or 0.005% w/w), for 6 weeks (Table 1). Blood samples were taken after a period of 3 and 6 weeks of treatment. Animals were kept in a temperature-controlled environment with 12-hour light/12-hour dark cycle and received food and water ad libitum. Body weight and food intake were monitored during the study.
Analysis of plasma lipids

Blood was sampled after 4 hours of fasting into EDTA-containing cups by tail bleeding, and plasma was isolated. Immediately after isolation, total plasma cholesterol (TC) and total plasma triglyceride (TG) levels were measured with commercially available enzymatic kits according to manufacturer’s protocols (Roche Diagnostics, No-1489437 for total cholesterol; Roche Diagnostics, No-1488872 for triglyceride).

Plasma and tissue pharmacokinetic experiments

The groups of mice that were treated with the highest dose of statin were used for analysis of pharmacokinetics. The evening prior to the PK study, diets of all mice were switched to the HFC control diet, to allow washout of the statin (t1/2 of orally dosed statins ≤ 5 hours in mice (Iusuf et al., 2012; Lau et al., 2006; Peng et al., 2009; Tiwari and Pathak, 2011; Zhu et al., 2011)). Subsequently, after 2-hours of fasting, mice were dosed with 10 mg/kg of the different statins (10 µL drug solution/g body weight; 1 mg/mL in saline finally containing 8.3% EtOH:polysorbate 80 (1:1, v/v)) by oral gavage. In case of [3H]-atorvastatin, [3H]-pravastatin and [3H]-rosuvastatin we used 4 µCi/mL final drug solution. Each group of 8 mice was split up into two subgroups. Mice of the first subgroup (n=4) were sacrificed after 30 minutes by CO2, blood was collected by cardiac puncture and tissues were isolated. In the other subgroup (n=4), blood was sampled by tail bleeding after 60 and 90 minutes and mice were sacrificed after 120 minutes by CO2, followed by cardiac puncture and isolation of tissues. All blood samples were centrifuged at 2700 g for 5 minutes at 4°C, plasma was collected and stored at -20°C until analysis.
Drug analysis

Levels of \(^{3}\text{H}\)-atorvastatin, \(^{3}\text{H}\)-pravastatin and \(^{3}\text{H}\)-rosuvastatin in plasma and organs were determined by scintillation counting (Tri-Carb 3100 TR; PerkinElmer, Shelton, CT). Amounts of lovastatin in plasma and liver were determined by HPLC analysis. Simvastatin in plasma and liver were determined by HPLC and LC-MS analysis, respectively (see below).

HPLC analysis of lovastatin and simvastatin

Levels of lovastatin acid and simvastatin acid in plasma and liver were measured by HPLC analysis based on the method previously described by Zhang et al. (Zhang and Yang, 2007). In brief, 50 µL methanol and 200 µL acetonitrile were added to 100 µL of plasma or liver homogenate (homogenized in ice-cold saline using a Ultra-turrax). After thoroughly vortexing, the samples were centrifuged for 10 minutes (14,000 rpm at room temperature), and 20 µL of the supernatant was injected into the column (Hypersil BDS column; 250 x 4.6 mm, 5 µm) for analysis. Samples were eluted at flow rate of 1.0 mL/minute at 20°C with 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The following mobile phase conditions were used: 0-10 min linear from 100% to 60% A and 0% to 40% B; 10-30 min linear from 60% to 30% A and 40% to 70% B; 30-40 min linear from 30% to 0% A and 70% to 100% B; 35-40 min 100% B (isocratic). UV-chromatograms were obtained at 238 nm. Peak identification and quantification was performed using Lablogic Laura software.

UPLC-MS analysis of simvastatin

Fractions of the livers (~200 mg) were sonificated twice in acetonitrile. Lysates were centrifuged 10 minutes at 14,000 rpm and 5 µL of the supernatant was injected into the
UPLC-MS for analysis (BEH Acquity column; C18, 50 x 2.1 mm). Samples were eluted at flow rate of 0.5 mL/minute at room temperature with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following mobile phase conditions were used: 0-1 min 98% A and 2% B (isocratic); 1-4 min linear 98% to 20% A and 2% to 80% B; 4-5 min: 20% A and 80% B (isocratic); 5-5.1 min linear 20% to 98% A and 80% to 2% B; 5.1-6 min: 98% A and 2% B (isocratic). The mass spectrometer was operating in selective reaction mode using turbo spray ionization in positive ion mode, with a capillary voltage of 5.5 kV and a spray temperature of 450°C. The multiple reaction monitoring (MRM) transitions were determined from MS spectra and appeared to be 437.5 m/z for the precursor ion of simvastatin acid and 285.3 m/z for the product ion. Peak identification and quantification was performed using Masslynx software version 4.1.

**Data and statistical analysis**

$\text{ED}_{30}$ values of statin efficacy in human and mouse were determined by linear regression with a 95% confidence interval (CI) (Liengme, 2010). Unless otherwise specified, the two-sided unpaired Student’s $t$-test was used throughout the study to assess the statistical significance of differences between two sets of data. Differences were considered to be statistically significant when $p < 0.05$. 
RESULTS

Comparison of cholesterol-lowering effects of statins in humans

The cholesterol-lowering effects of the different statins in patients with hypercholesterolemia are shown in Figure 1 and Supplemental data 1. There was a clear linear dose-effect relation, with exception for atorvastatin and rosuvastatin at the highest dose of 80 mg/day, which might be due to saturation of HMG-CoA reductase inhibition (Supplemental data 2). We also observed a clear and significant correlation between the reduction in plasma levels of TC and LDL-C after statin treatment ($R^2$ 0.83, p<0.001; data not shown). Based on linear regression, we calculated the ED$_{30}$ (i.e. effective dose (mg/day) to reduce plasma TC by 30%) for the individual statins, and found that these were in order from strong to weak: rosuvastatin (1.79 mg/day [CI: 0.15-3.66]), atorvastatin (16.3 mg/day [CI: 14.0 – 18.9]), simvastatin (48.5 mg/day [CI: 40.9 – 58.1]), lovastatin (75.7 mg/day [CI: 69.9 – 81.6]) and pravastatin (80.8 mg/day [CI: 77.3 – 84.2]) (Supplemental data 2).

Comparison of lipid-lowering effects of statins in E3L mice

E3L mice treated with a HFC diet containing 1% cholesterol for 4 weeks showed on average a 5.3-fold increase in plasma total cholesterol levels compared to the chow-control reference mice. After this 4-week run-in period to achieve steady state conditions, mice were randomized into 11 groups (n=8 per group) based on age, body weight, plasma cholesterol and triglyceride levels, and received the HFC diet (control group) or supplemented with different doses of statin during an intervention period of 6 weeks (Table 1). Body weights and food intake of the E3L mice during the intervention period are presented in Table 1 and Figure 2. The average body weights of the mice did not differ between the different groups. The food intake was comparable between all treatment groups (~2.6 g/day on average),
except the group receiving 1.26 mg/day lovastatin of which the food intake was 2.5 g/day (p < 0.05; one-way ANOVA followed by Dunett’s multiple comparison test; Table 1). The average food intake in the chow-control group was 3.7 g/day. The effects of the different statins on plasma TC levels after 6 weeks of treatment in E3L mice are presented in Table 2 (individual data in Supplemental data 3). Except for simvastatin, all statins decreased plasma TC levels by at least 30% at the highest tested dose tested. Based on the food intake we calculated the statin intake (mg/day) per group and plotted these against the percentage reduction in TC compared to the HFC-control group (Figure 3). Linear regression was used to calculate the ED$_{30}$ for plasma TC reduction in E3L mice for the individual statins (extrapolation of the data was used to estimate the ED$_{30}$ for simvastatin). ED$_{30}$ values (mg/day) were in order from strong to weak: rosuvastatin (0.11 mg/day [CI: 0.10 – 0.12]), atorvastatin (0.14 mg/day [CI: 0.12 – 0.16]), pravastatin (0.97 mg/day [CI: 0.90 – 1.03]), lovastatin (1.53 mg/day [CI: 1.51 – 1.54]) and simvastatin (3.34 mg/day [CI: 2.84 – 4.05]) (Figure 3). The effects of the different statins on plasma TG levels after 6 weeks of treatment in E3L mice are presented in Table 2 (individual data are presented in Supplemental data 4). Rosuvastatin, pravastatin and lovastatin decreased plasma TG levels in E3L mice (62%, 63% and 59% upon treatment of E3L mice with 0.13 mg rosuvastatin/day, 1.02 mg pravastatin/day and 1.77 mg lovastatin/day, respectively), as has been observed before (Delsing et al., 2005; Kleemann et al., 2003; van der Hoorn et al., 2007). Atorvastatin and simvastatin, however, did not statistically change plasma TG levels in E3L mice, which is also in agreement with previous observations (Delsing et al., 2003; van et al., 2001; Wang et al., 2002).

Pharmacokinetic analysis of different statins in E3L mice
To study putative differences in pharmacokinetics and tissue distribution between the various statins used in this study, E3L mice were orally dosed with a bolus injection of 10 mg/kg of the (radiolabelled) drug. Concentrations of the statin in plasma and liver are presented in Figure 4 and Figure 5, respectively. Plasma exposure as determined by the AUC was 373 min·µg/mL (pravastatin), 101 min·µg/mL (simvastatin), 36.6 min·µg/mL (atorvastatin), 9.77 min·µg/mL (rosuvastatin) and 4.78 min·µg/mL (lovastatin). Interestingly, liver levels did not correlate with the plasma levels of the different statins, and were from high to low 14.7 (atorvastatin), 9.81 (pravastatin), 4.58 (rosuvastatin), 3.49 (lovastatin) and 1.56 (simvastatin) µg/g 30 minutes after administration. Liver-to-plasma ratios of the individual statins 30 and 120 minutes after oral administration are presented in Table 3, demonstrating the highest liver-to-plasma ratios for rosuvastatin and the lowest for simvastatin.

For the three statins that were analyzed by liquid scintillation counting ([³H]-atorvastatin, [³H]-pravastatin and [³H]-rosuvastatin) we determined drug levels in stomach, intestine (tissue plus contents), colon (tissue plus contents) and kidney as well (Supplemental data 5). Drug levels in the stomach were comparable between the three statins (~50 µg/g 30 minutes after dosage). Compared with rosuvastatin and atorvastatin, pravastatin showed the lowest level of drug in the small intestine (including contents), concomitantly with the highest plasma AUC and highest level of drug present in the kidneys. These data suggest that pravastatin has the highest oral bioavailability of these statins in E3L mice.

Comparison of the efficacy of different statins between humans and E3L mice

The cholesterol-lowering efficacy of the different statins used in this study was compared between humans and E3L mice. To do so, we plotted the calculated ED₃₀ values of the statins for humans against the ED₃₀ values obtained for E3L mice, before and after
correcting for pharmacokinetic differences (plasma AUC or average liver concentration) between the statins in mice (Figure 6). Importantly, after correcting for the average liver concentrations in E3L mice, we found a significant correlation between the efficacy of statins in humans and E3L mice ($R^2$ 0.89, p<0.05; Figure 6C). The hepatic extraction of statins in humans is 80% for simvastatin, 70% for atorvastatin and lovastatin, 65% for rosuvastatin and 45% for pravastatin (Neuvonen et al., 2006). When correspondingly taking these differences into account, the correlation between human and mouse studies was even further increased ($R^2$ 0.99, p<0.001; Figure 6D).
DISCUSSION

The E3L transgenic mouse model exhibits a human-like plasma lipid profile in response to high fat diet feeding and develops atherosclerotic lesions that resemble their human counterparts (van Vlijmen et al., 1994; Verschuren et al., 2012; Zadelaar et al., 2007). This mouse model is a valid and still widely used mouse model for studying the cholesterol-lowering and pleiotropic effects of (newly developed) drugs (e.g., statins, fibrates, ezetimibe, olmesartan). There is currently a required focus of the pharmaceutical industry to correctly predict the efficacy of novel drug candidates in early stage pre-clinical phases. We therefore aimed to map the predictive value of the E3L mouse model regarding the cholesterol-lowering efficacy of a set of statins. Importantly, since the pharmacokinetics and especially the concentration at the target site co-determines the efficacy of drugs, we hypothesized that combining pharmacokinetic with efficacy data can increase the predictability of drug efficacy in pre-clinical models. To this end, the efficacy profile of 5 currently marketed statins was determined in E3L transgenic mice, and compared with their efficacy in humans based on data obtained from a comprehensive meta-analysis of human trials. Besides efficacy, we also determined the pharmacokinetics and tissue distribution of these drugs in E3L mice. These results show that oral bioavailability and target organ concentrations varied markedly between the statins. We demonstrate that consideration of these differences improves the prediction of the efficacy of the drugs in preclinical model and facilitates the translation of preclinical data to situation in humans.

Statins are being widely prescribed to reduce circulating cholesterol levels and lower the risk of cardiovascular events for more than 2 decades now. The various effects of atorvastatin, rosuvastatin and pravastatin have previously been studied in E3L transgenic mice (Delsing et al., 2003; Delsing et al., 2005; Kleemann et al., 2003; van de Poll et al., 2003; van der Hoorn et al., 2007; Verschuren et al., 2005). These studies revealed that E3L
mice, unlike other mouse models of CVD, respond to these hypolipideamic drugs by a reduction plasma cholesterol levels, and that statins can reduce atherosclerotic plaque formation in these mice, partly even beyond and independent of the cholesterol-lowering effect. This latter effect might be explained by the more pleiotropic effects of statin drugs (e.g. anti-inflammatory effects) in the vasculature and the liver, and is in line with observations in humans (Liao and Laufs, 2005). In the current study we concentrated on the cholesterol-lowering effects of the statins in the preclinical E3L model. The observed ED$_{30}$ values of atorvastatin and rosuvastatin (0.14 and 0.11 mg/day, respectively) in this study are highly comparable to those found in studies by Verschuren et al (atorvastatin: ~0.13 mg/day) and Delsing et al (rosuvastatin: ~0.09 mg/day) that were carried out more than 7 years ago using the same mouse model and comparable dietary conditions (Delsing et al., 2005; Verschuren et al., 2005). This clearly demonstrates the validity and reproducibility of the E3L transgenic mouse model for these type of research questions.

We determined plasma exposure and liver uptake of the different statins in E3L mice that were exposed to statin therapy for 6 weeks after an oral bolus injection of 10 mg/kg. It has been shown before that rosuvastatin is more efficiently taken up by hepatic cells than pravastatin and simvastatin (Nezasa et al., 2002). Here, we also observed more than 30-fold increased liver-to-plasma ratio of rosuvastatin compared to simvastatin and pravastatin, albeit that in the study by Nezasa et al. (2002) the statins were dosed intravenously at 5 mg/kg. Furthermore, in line with results from DeGorter et al. (2012), liver-to-plasma ratios of rosuvastatin were at the same order of magnitude as that of atorvastatin, with higher liver concentrations of atorvastatin compared to rosuvastatin, but more that 2-fold lower plasma exposure of rosuvastatin compared to atorvastatin. Again, in the referred study the statins were dosed differently (1 mg/kg intravenously) than in the current study, indicating that comparable studies are the lacking. In the present study, we clearly demonstrate a high
bioavailability (i.e. plasma exposure after oral administration) of pravastatin in mice compared to the other statin drugs. To the best of our knowledge, this has never been published before. Notably, this finding is substantiated by the relative low levels of remaining pravastatin in the small intestine and colon 30 and 120 minutes after dosing (Supplemental data 5). The high plasma exposure, but more importantly the relatively high liver uptake of pravastatin in E3L mice, might explain the rather high cholesterol-lowering efficacy of pravastatin compared to the other 2 second generation statins, lovastatin and atorvastatin. In contrast, simvastatin, which also has a rather high plasma exposure (101 min·µg/mL) in E3L mice, but the lowest hepatic uptake, was the least effective. These results for the first time systematically link the hepatic uptake of statins to their cholesterol-lowering efficacy in a valuable mouse model of hyperlipidemia.

The therapeutic equivalence of statins in humans was recently reviewed in a meta-analysis by Weng et al., providing a ranking order from most to least potent statins when looking at the plasma LDL-cholesterol lowering effect: rosvastatin > atorvastatin > simvastatin > lovastatin > pravastatin (Weng et al., 2010). We added 16 clinical head-to-head trials that were published between 2006 and 2009 (references from (Beth Smith et al., 2009)) and compared the total cholesterol-lowering properties of the above mentioned statins (since we also determined the total cholesterol levels in the E3L mice). We found a ranking in potency of statins in humans with hypercholesterolemia that was fully consistent with the analysis of Weng et al (Weng et al., 2010). Determination of ED₃₀ values enabled us to directly compare the efficacy of these statins in E3L mice relative to humans. Interestingly, rosvastatin and atorvastatin, that belong to the newer third generation synthetic statins (McTaggart et al., 2001), are by far the most potent statins in humans as well as in the E3L mouse model. However, the efficacy of simvastatin, pravastatin and lovastatin in humans is remarkably less well predicted by just looking at the potency of these statins in E3L mice.
Only after taking into account the differences in liver uptake of these statins in E3L mice, we found a significant correlation between the cholesterol-lowering efficacy of these statins in E3L mice and patients with hypercholesterolemia. The fact that just correcting for differences in liver accumulation of the various statins in E3L mice is already sufficient to gain a good correlation, suggests that the relative differences in liver uptake between these statins in humans are minor compared to E3L mice. Hepatic extraction of simvastatin, lovastatin, atorvastatin and rosuvastatin is reported to be comparable in humans (65-80%), whereas that of pravastatin is slightly lower (45%) (Neuvonen et al., 2006; Neuvonen et al., 2008). When taking into account these minor differences in hepatic extraction of the studied statins, we retrieve an even better correlation between the efficacy of the drugs in humans and E3L mice (Figure 6D).

In conclusion, in this study we provide evidence that a combined PK-efficacy study substantially improves the translational value of the E3L mouse model in case of the cholesterol-lowering effect of statin treatment. We hypothesize that a similar strategy could be used to increase the clinical predictability of drug treatment of other classes of drugs in various pre-clinical (disease) models.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Steeg, Kleemann, Wortelboer, DeGroot
Conducted experiments: Steeg, Duyvenvoorde, Offerman, Jansen
Performed data analysis: Steeg, Duyvenvoorde
Wrote the manuscript: Steeg
Contributed to the writing of the manuscript: Steeg, Kleemann, Wortelboer, DeGroot
REFERENCES


25
FOOTNOTES

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FIGURE LEGENDS

Figure 1. Comparison of the cholesterol-lowering effect of different statins in patients with hypercholesterolemia (plasma LDL-C > 160 mg/dL). Data are presented as the percentage reduction in TC or LDL-C after treatment compared to baseline levels (mean ± SD). The different statins and the dose studied are presented on the X-axis (A, P, L, S and R represent atorvastatin, pravastatin, simvastatin, lovastatin and rosuvastatin, respectively, with the number indicating the dose in mg/day). The value below the drug label indicates the number of head-to-head comparisons involving that specific statin and dose. LDL-C, low-density lipoprotein cholesterol; TC, total plasma cholesterol levels.

Figure 2. Body weights and food intake of E3L mice during the 6-weeks drug treatment period (t=0 is the time point of randomization after 4 weeks of run-in on HFC diet). A, P, L, S and R in the legend represent atorvastatin, pravastatin, lovastatin, simvastatin and rosuvastatin, respectively.

Figure 3. Comparison of the cholesterol-lowering effect of different statins after 6 weeks of treatment in E3L mice fed with a HFC diet. ED₃₀ values represent the effective dose (mg/day) to reduce plasma TC by 30% (in case of simvastatin the value had to be extrapolated). Data are presented as the percentage reduction of plasma TC compared to the HFC control group (mean ± SD). CI, 95% confidence interval.

Figure 4. Plasma levels of atorvastatin, pravastatin, lovastatin, simvastatin and rosuvastatin in E3L mice orally dosed with 10 mg/kg. All data are presented as means ± SD (n=4). LLQ, lower limit of quantification (i.e. 0.1 µg/mL for lovastatin).
**Figure 5.** Liver levels of atorvastatin, pravastatin, lovastatin, simvastatin and rosuvastatin in E3L mice 30 and 120 minutes after receiving an oral dose of 10 mg/kg. All data are presented as means ± SD (n=4). For very low bars the measured value is also presented above the bar.

**Figure 6.** Comparison of cholesterol-lowering effect of different statins between humans and the E3L mouse model, before (A) and after correcting for pharmacokinetic differences in plasma exposure (B) and liver uptake (C) between the different statins in E3L mice and/or hepatic extraction ratio in humans (D). ED_{30} values are defined as the effective dose (mg/day) needed to reduce plasma TC by 30%. A, P, L, S, and R represent atorvastatin, pravastatin, lovastatin, simvastatin and rosuvastatin, respectively.
Table 1. Food intake and baseline characteristics of E3L mice after randomization into different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW at t=0 (g)</th>
<th>Food intake a (g/day)</th>
<th>Statin intake (mg/day)</th>
<th>Statin intake b (mg/kg)</th>
<th>Plasma TC at t=0 (mM)</th>
<th>Plasma TG at t=0 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow-control</td>
<td>12</td>
<td>20.8 ± 0.61</td>
<td>3.72 ± 0.14</td>
<td>-</td>
<td>-</td>
<td>2.83 ± 0.21</td>
<td>2.83 ± 0.48</td>
</tr>
<tr>
<td>HFC-control</td>
<td>8</td>
<td>20.3 ± 1.11</td>
<td>2.70 ± 0.20</td>
<td>-</td>
<td>-</td>
<td>15.1 ± 3.49</td>
<td>2.41 ± 0.77</td>
</tr>
<tr>
<td>HFC-0.003% atorvastatin</td>
<td>8</td>
<td>20.9 ± 0.82</td>
<td>2.61 ± 0.21</td>
<td>0.08</td>
<td>3.76</td>
<td>15.1 ± 1.89</td>
<td>2.49 ± 0.90</td>
</tr>
<tr>
<td>HFC-0.008% atorvastatin</td>
<td>8</td>
<td>21.8 ± 1.39</td>
<td>2.65 ± 0.13</td>
<td>0.21</td>
<td>10.1</td>
<td>15.1 ± 3.52</td>
<td>2.52 ± 0.41</td>
</tr>
<tr>
<td>HFC-0.03% pravastatin</td>
<td>8</td>
<td>20.9 ± 1.57</td>
<td>2.58 ± 0.18</td>
<td>0.77</td>
<td>37.2</td>
<td>14.9 ± 2.35</td>
<td>2.28 ± 0.58</td>
</tr>
<tr>
<td>HFC-0.04% pravastatin</td>
<td>8</td>
<td>21.0 ± 1.76</td>
<td>2.55 ± 0.18</td>
<td>1.02</td>
<td>48.7</td>
<td>15.2 ± 2.81</td>
<td>2.45 ± 0.58</td>
</tr>
<tr>
<td>HFC-0.05% lovastatin</td>
<td>8</td>
<td>20.6 ± 1.21</td>
<td>2.51 ± 0.11</td>
<td>1.26</td>
<td>60.9</td>
<td>15.0 ± 2.21</td>
<td>2.37 ± 0.40</td>
</tr>
<tr>
<td>HFC-0.07% lovastatin</td>
<td>8</td>
<td>20.9 ± 1.11</td>
<td>2.53 ± 0.28</td>
<td>1.77</td>
<td>84.7</td>
<td>14.8 ± 3.03</td>
<td>2.37 ± 0.50</td>
</tr>
<tr>
<td>HFC-0.03% simvastatin</td>
<td>8</td>
<td>20.7 ± 1.03</td>
<td>2.60 ± 0.13</td>
<td>0.78</td>
<td>37.7</td>
<td>15.1 ± 2.01</td>
<td>2.41 ± 0.38</td>
</tr>
<tr>
<td>HFC-0.06% simvastatin</td>
<td>8</td>
<td>21.5 ± 0.85</td>
<td>2.60 ± 0.19</td>
<td>1.56</td>
<td>72.7</td>
<td>14.9 ± 2.71</td>
<td>2.72 ± 0.34</td>
</tr>
<tr>
<td>HFC-0.0025% rosuvastatin</td>
<td>8</td>
<td>21.5 ± 0.77</td>
<td>2.59 ± 0.09</td>
<td>0.06</td>
<td>3.01</td>
<td>15.0 ± 2.50</td>
<td>2.57 ± 0.49</td>
</tr>
<tr>
<td>HFC-0.0005% rosuvastatin</td>
<td>8</td>
<td>21.0 ± 0.89</td>
<td>2.55 ± 0.25</td>
<td>0.13</td>
<td>6.08</td>
<td>15.2 ± 2.84</td>
<td>2.71 ± 0.63</td>
</tr>
</tbody>
</table>

a Average over the 6-weeks treatment period

b Based on body weight at t=0

BW, body weight; TC, total plasma cholesterol levels; TG, total plasma triglyceride levels; t=0 after 4-weeks run-in period

*p < 0.01 when compared to HFC-control group; data are presented as mean or mean ± SD
Table 2. Plasma TC and TG in E3L mice fed a HFC diet after 6 weeks of treatment with different statins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Plasma TC at t=6 (mM)</th>
<th>Reduction (%) a</th>
<th>Plasma TG at t=6 (mM)</th>
<th>Reduction (%) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>12</td>
<td>2.03 ± 0.26</td>
<td>-</td>
<td>2.59 ± 0.35</td>
<td>-</td>
</tr>
<tr>
<td>HFC</td>
<td>8</td>
<td>16.4 ± 3.03</td>
<td>-</td>
<td>2.28 ± 0.62</td>
<td>-</td>
</tr>
<tr>
<td>HFC + 0.08 mg/day atorvastatin</td>
<td>8</td>
<td>11.6 ± 1.04***</td>
<td>29.4 ± 6.39</td>
<td>2.52 ± 0.56</td>
<td>-</td>
</tr>
<tr>
<td>HFC + 0.21 mg/day atorvastatin</td>
<td>8</td>
<td>8.55 ± 1.81***</td>
<td>47.8 ± 11.1</td>
<td>2.35 ± 0.68</td>
<td>-</td>
</tr>
<tr>
<td>HFC + 0.77 mg/day pravastatin</td>
<td>8</td>
<td>13.1 ± 1.35**</td>
<td>20.1 ± 8.27</td>
<td>1.25 ± 0.18**</td>
<td>45.2 ± 8.07</td>
</tr>
<tr>
<td>HFC + 1.02 mg/day pravastatin</td>
<td>8</td>
<td>10.9 ± 1.13***</td>
<td>33.2 ± 6.87</td>
<td>0.86 ± 0.14***</td>
<td>62.5 ± 6.20</td>
</tr>
<tr>
<td>HFC + 1.26 mg/day lovastatin</td>
<td>8</td>
<td>12.9 ± 1.39**</td>
<td>21.1 ± 8.48</td>
<td>2.13 ± 0.55</td>
<td>6.60 ± 24.2</td>
</tr>
<tr>
<td>HFC + 1.77 mg/day lovastatin</td>
<td>8</td>
<td>10.5 ± 1.65***</td>
<td>36.0 ± 10.1</td>
<td>0.94 ± 0.18***</td>
<td>59.0 ± 7.82</td>
</tr>
<tr>
<td>HFC + 0.78 mg/day simvastatin</td>
<td>8</td>
<td>16.1 ± 2.59</td>
<td>7.06 ± 10.3</td>
<td>2.55 ± 0.34</td>
<td>-</td>
</tr>
<tr>
<td>HFC + 1.56 mg/day simvastatin</td>
<td>8</td>
<td>14.7 ± 2.87</td>
<td>14.1 ± 12.3</td>
<td>2.40 ± 0.96</td>
<td>-</td>
</tr>
<tr>
<td>HFC + 0.06 mg/day rosuvastatin</td>
<td>8</td>
<td>12.2 ± 0.95**</td>
<td>25.5 ± 5.77</td>
<td>1.41 ± 0.18**</td>
<td>38.1 ± 7.94</td>
</tr>
<tr>
<td>HFC + 0.13 mg/day rosuvastatin</td>
<td>8</td>
<td>10.5 ± 0.58***</td>
<td>36.1 ± 3.56</td>
<td>0.86 ± 0.12***</td>
<td>62.4 ± 5.23</td>
</tr>
</tbody>
</table>

* Compared to average TC or TG levels in the HFC-control group at t=6

HFC, high fat cholesterol diet; TC, total plasma cholesterol levels; TG, total plasma triglyceride levels; t=6, after 6-weeks intervention period
*p < 0.01, ***p < 0.001 when compared to HFC-control group; data are presented as mean ± SD
Table 3. Liver-to-plasma ratios of different statins 30 and 120 minutes after oral dosage (10 mg/kg)

<table>
<thead>
<tr>
<th>Statin</th>
<th>Liver-to-plasma ratio (t = 30 min)</th>
<th>Liver-to-plasma ratio (t = 120 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>atorvastatin</td>
<td>41.7 ± 9.76</td>
<td>21.3 ± 3.47</td>
</tr>
<tr>
<td>pravastatin</td>
<td>1.68 ± 0.30</td>
<td>1.70 ± 0.08</td>
</tr>
<tr>
<td>lovastatin</td>
<td>10.8 ± 5.95</td>
<td>na</td>
</tr>
<tr>
<td>simvastatin</td>
<td>1.29 ± 0.52</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>rosuvastatin</td>
<td>49.5 ± 6.90</td>
<td>27.4 ± 5.27</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD, n=4

na, not applicable (plasma levels were < lower limit of quantification; LLQ)
Figure 2

A

Body Weight (g)

Weeks

B

Food intake (g/day)

Weeks

Legend:
- CHOW-control
- HFC-control
- HFC-0.08 mg/day A
- HFC-0.21 mg/day A
- HFC-0.77 mg/day P
- HFC-1.02 mg/day P
- HFC-0.78 mg/day S
- HFC-1.26 mg/day L
- HFC-1.77 mg/day L
- HFC-0.06 mg/day R
- HFC-0.13 mg/day R

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Figure 3

A. Rosuvastatin
$ED_{30} = 0.11$ (CI: 0.10 - 0.12)

B. Atorvastatin
$ED_{30} = 0.14$ (CI: 0.12 - 0.16)

C. Pravastatin
$ED_{30} = 0.97$ (CI: 0.90 - 1.03)

D. Lovastatin
$ED_{30} = 1.53$ (CI: 1.51 - 1.54)

E. Simvastatin
$ED_{30} = 3.34$ (CI: 2.84 - 4.05)
Figure 4

(A) $[^3H]$-Atorvastatin

(B) $[^3H]$-Pravastatin

(C) Lovastatin (acid)

(D) Simvastatin (acid)

(E) $[^3H]$-Rosuvastatin
Figure 5

The figure shows the level of various statins in the liver, measured in micrograms per gram of tissue. The levels are as follows:

- **Atorvastatin**:
  - 30 minutes: 15 mg/g tissue
  - 120 minutes: 3 mg/g tissue

- **Pravastatin**:
  - 30 minutes: 12 mg/g tissue
  - 120 minutes: 3 mg/g tissue

- **Lovastatin**:
  - 30 minutes: 6 mg/g tissue
  - 120 minutes: 1 mg/g tissue

- **Simvastatin**:
  - 30 minutes: 1 mg/g tissue
  - 120 minutes: 0.09 mg/g tissue

- **Rosuvastatin**:
  - 30 minutes: 3 mg/g tissue
  - 120 minutes: 1 mg/g tissue
Figure 6

(A) 

ED$_{30}$ human (mg/day) vs. ED$_{30}$ ApoE*3L (mg/day)

R$^2 = 0.22$

p = 0.43

(B) 

ED$_{30}$ human (mg/day) vs. "ED$_{30}$ ApoE*3L" (corrected for plasma AUC)

R$^2 = 0.29$

p = 0.35

(C) 

ED$_{30}$ human (mg/day) vs. "ED$_{30}$ ApoE*3L" (corrected for liver concentration)

R$^2 = 0.89$

p < 0.05

(D) 

"ED$_{30}$ ApoE*3L" (corrected for hepatic extraction ratio) vs. "ED$_{30}$ ApoE*3L" (corrected for liver concentration)

R$^2 = 0.99$

p < 0.001