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Title Page

Comparative hepatic and intestinal metabolism and pharmacodynamics of statins[#]

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Running Title Page

a) Running title: *In vitro* metabolism and pharmacodynamics of statins

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d) Nonstandard abbreviations: CL_{int}, intrinsic clearance; $C_{max,u}$, unbound peak concentration; CYP, cytochrome P450; HIM, human intestine microsome; HLC, human liver cytosol; HLM, human liver microsome; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PMSF, phenylmethylsulfonyl fluoride; UDPGA, uridine 5'-diphospho-glucuronic acid; SULT, sulfotransferase; UGT, uridine diphosphate-glucuronosyltransferase.

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Abstract

This study aimed to comprehensively investigate the *in vitro* metabolism of statins. The metabolism of clinically relevant concentrations of atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin and their metabolites were investigated using human liver microsomes (HLMs), intestine microsomes (HIMs), liver cytosol, and recombinant cytochrome P450 (CYP) enzymes. We also determined the inhibitory effects of statin acids on their pharmacological target, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. In HLMs, statin lactones were metabolized to a much higher extent than their acid forms. Atorvastatin lactone and simvastatin (lactone) showed extensive metabolism (intrinsic clearance (CL_{int}) values of 3,700 and 7,400 µl/min/mg), while the metabolism of the lactones of 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, and pitavastatin was slower (CL_{int} 20-840 µl/min/mg). The acids had CL_{int} values in the range <0.1-80 µl/min/mg. In HIMs, only atorvastatin lactone and simvastatin (lactone) exhibited notable metabolism, with CL_{int} values corresponding to 20% of those observed in HLMs. CYP3A4/5 and CYP2C9 were the main statin-metabolizing enzymes. The majority of the acids inhibited HMG-CoA reductase with 50% inhibitory concentrations of 4-20 nM. The present comparison of the metabolism and pharmacodynamics of the various statins using identical methods provides a strong basis for further application, e.g., comparative systems pharmacology modelling.

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Significance Statement

The present comparison of the *in vitro* metabolic and pharmacodynamic properties of atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin and their metabolites using unified methodology provides a strong basis for further application. Together with *in vitro* drug transporter and clinical data, our findings are applicable for use in comparative systems pharmacology modelling to predict the pharmacokinetics and pharmacological effects of statins at different dosages.

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Introduction

3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used in the treatment of hypercholesterolemia. Although statin drugs are effective and safe in most patients, many users experience poor efficacy or adverse drug reactions (Pazzucconi *et al.*, 1995; Yebyo *et al.*, 2019). The muscle toxicity of statins is a dose- and concentration-dependent phenomenon (Bradford *et al.*, 1991; Dujovne *et al.*, 1991), and the risk of toxicity increases along with the plasma concentrations of statins. This may be caused by drug-drug interactions or inherited defects in proteins affecting statin disposition, such as cytochromes P450 (CYP) 2C9 and 3A4, organic anion transporting polypeptide (OATP) 1B1 or breast cancer resistance protein (BCRP) (Thompson *et al.*, 2003; Graham *et al.*, 2004; Neuvonen *et al.*, 2006; Pasanen *et al.*, 2006; Keskitalo *et al.*, 2009b; Hirvensalo *et al.*, 2019).

Both acid and lactone forms of statins can interact with proteins involved in drug disposition. Lovastatin and simvastatin are administered as lactone prodrugs, whereas other statins are given in the active acid form. In the body, however, significant amounts of many statins are converted to their corresponding acid/lactone form (Neuvonen *et al.*, 2006). CYP enzymes metabolize the lipophilic statin lactones more rapidly than statin acids (Fujino *et al.*, 2004). Indirectly, uridine diphosphate-glucuronosyltransferase (UGT) enzymes may participate in lactone formation by mediating glucuronidation of statin acids. The acyl glucuronides formed in this process may then undergo spontaneous cyclization to the statin lactone (Prueksaritanont *et al.*, 2002). CYP3A4 plays a key role in the elimination of atorvastatin, lovastatin, and simvastatin (Neuvonen *et al.*, 2006). In turn, the 3R,5S- and 3S,5R-enantiomers of fluvastatin are extensively biotransformed by CYP2C9 (Fischer *et al.*, 1999; Hirvensalo *et al.*, 2019). Pitavastatin, pravastatin, and rosuvastatin are excreted mainly unchanged (Neuvonen *et al.*, 2006). Hence, the available statins differ significantly in their pharmacokinetic characteristics and susceptibility to altered metabolizing enzyme function.

Interestingly, the pharmacological target of statins, HMG-CoA reductase, is expressed in the same location as the CYP enzymes, the endoplasmic reticulum of hepatocytes (Corsini *et al.*, 1995). Thus, this enzyme is also present

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in human liver microsomes (HLMs). Similar to CYPs, the active site of HMG-CoA reductase faces the cytoplasm and the enzyme uses nicotinamide adenine dinucleotide phosphate (NADPH) for its catalytic activity (Corsini *et al.*, 1995). Statin acids, which are structurally similar to its substrate HMG-CoA, competitively inhibit it in the nanomolar range, leading to a reduction of the HMG-CoA-mevalonate pathway. Also several statin metabolites have been depicted to be inhibitors of this reaction, but *in vitro* data for e.g. the 2- and 4-hydroxy metabolites of atorvastatin do not seem to be publically available.

Many published physiologically-based pharmacokinetic (PBPK) models of statins can be found in the literature, primarily aiming to evaluate their drug-drug interaction and pharmacogenetic properties. During the past two decades, PBPK modelling has evolved as an important tool in drug development, regulatory review, and clinical study design (Sager *et al.*, 2015). PBPK modelling also holds the potential to become a valuable tool to inform drug and dosing selection in clinical practice (Johnson and Rostami-Hodjegan, 2011; Jamei, 2016; Venkatakrishnan and Rostami-Hodjegan, 2019). For such purposes, it is crucial that the data used for model development of a set of drugs are comparable and high in quality. Although the metabolism of statins has been widely investigated *in vitro*, only a few substrate depletion studies comparing the microsomal metabolism of different statins have been published (Fujino *et al.*, 2004; Gertz *et al.*, 2010; Gertz *et al.*, 2011; Varma *et al.*, 2014). When appropriately applied, the substrate depletion approach results in intrinsic clearance (CL_{int}) values, which include all microsomal metabolic pathways of the tested compound (Obach, 1999) and can be scaled to hepatic metabolic clearance for PBPK modelling. Hence, to obtain comparable estimates of the metabolism of the widely used statins atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin and their metabolites, we carried out an extensive *in vitro* study in subcellular hepatic and intestinal fractions as well as in recombinant CYP enzymes using low, clinically relevant statin concentrations. Moreover, to simulate the pharmacological response of statins in PBPK models, unbound statin concentrations in hepatocytes can be linked to *in vitro* measurements of their pharmacodynamic potency. Therefore, we also compared the inhibitory effects of these statins on their pharmacological target HMG-CoA reductase.

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Materials and Methods

Chemicals and reagents

Atorvastatin (acid), atorvastatin lactone, 2-hydroxyatorvastatin (acid), 2-hydroxyatorvastatin lactone, 4-hydroxyatorvastatin (acid), 4-hydroxyatorvastatin lactone, mevalonate lactone, pravastatin (acid), rosuvastatin (acid) and all internal standards (**Supp. Table 1**) were purchased from Toronto Research Chemicals (Toronto, Canada). 3R,5S-fluvastatin (acid), 3S,5R-fluvastatin (acid), pitavastatin (acid), and pitavastatin lactone were obtained from Santa Cruz Biotechnology (Dallas, Texas), and simvastatin acid and simvastatin (lactone) from SynFine Research (Ontario, Canada). Adenosine 3-phosphate 5'-phosphosulfate triethylammmonium salt (PAPS), alamethicin, NADPH, uridine 5'-diphospho-glucuronic acid (UDPGA), and HMG-CoA reductase assay kits were obtained from Sigma-Aldrich (St. Louis, MO). HLMs (XTreme 200, a pool of 200 mixed gender donors), human liver cytosol (HLC; XTreme 200, a pool of 200 mixed gender donors), and human intestine microsomes (HIMs) both normal (a pool of 15 mixed gender donors) and free from phenylmethylsulfonyl fluoride (PMSF; optimal in carboxylesterase activity; a pool of 6 mixed gender donors) were purchased from Sekisui XenoTech (Tokai, Japan). The following recombinant EasyCYP Bactosomes were obtained from Cypex Ltd (Dundee, UK): CYP1A2R, CYP2A6BR, CYP2B6BR, CYP2C8BR, CYP2C9BHR, CYP2C19BR, CYP2D6R, CYP2E1BR, CYP2J2LR, CYP3A4BR, and CYP3A5BLR. Other chemicals were from Merck (Darmstadt, Germany).

Metabolism in human liver microsomal incubations

The metabolic depletion of statins was first measured in HLMs. With the exception for buffer controls, all incubations contained substrate, microsomes (0.2 mg/ml) in sodium phosphate buffer (0.1 M, pH 7.4) with $MgCl_2$ (5 mM). For simvastatin, the protein concentration was reduced to 0.1 mg/ml after the initial experiment. The depletion of each parent statin and its corresponding acid/lactone was studied in four different conditions: 1) addition of NADPH (1 mM) to the reaction mixture to measure the CYP-mediated metabolism, 2) addition of NADPH (1 mM), UDPGA (5 mM) to measure both CYP- and UGT-mediated metabolism, 3) no addition of cofactors (negative control), and 4) no addition of cofactors nor microsomes (buffer control). In condition 2)

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alamethicin (11 µg/ml) was also included in the incubations to allow pore formation. The metabolism of the 2- and 4-hydroxy metabolites of atorvastatin were studied using conditions 1), 3) and 4). The initial incubation concentrations of each substrate are listed in **Table 1 and Supp. Table 2**.

All incubations were carried out once in triplicate on 96-well plates. The mixtures of substrate and microsomes (or only substrate in buffer in 4)) were first preincubated for 15 min at 37°C and 350 rpm. Following preincubation, cofactors were added to initiate the reactions in 1) and 2). Samples were taken at 0, 5, 10, 20, 40, and 60 min. For atorvastatin lactone and simvastatin, the sampling times were shorter (0.5, 1, 3, 5, 7, and 10 min). Reactions were stopped by diluting samples 1:3 with of ice-cold acetonitrile containing internal standard (1:2 for rosuvastatin) (**Supp. Table 1**), and further handled as described below in the section Sample processing and analysis of metabolism samples.

Metabolism in human intestine microsomal incubations

The metabolic depletion of parent statins and their corresponding acid/lactone was also measured in HIMs. With the exception of buffer controls, all incubations contained substrate, microsomes (0.2 mg/ml) in sodium phosphate buffer (0.1 M, pH 7.4) with $MgCl_2$ (5 mM). For each substrate (incubation concentrations listed in **Table 1** and **Supp. Table 2**), the depletion was studied in four different conditions: 1) addition of NADPH (1 mM) and UDPGA (5 mM) to measure both CYP- and UGT-mediated metabolism (alamethicin 11 µg/ml also included), 2) no addition of cofactors (negative control), and 3) no addition of cofactors nor microsomes (buffer control). Furthermore, to screen for the potential effects of intestinal carboxylesterases on the metabolism of statins, each statin was also incubated with 4) 0.2 mg/ml PMSF-free HIMs (optimal in esterase activity). No cofactors were added to PMSF-free HIM incubations.

All incubations were carried out once in triplicate, preincubated and stopped in a similar manner as described for the HLM incubations above. In 1-3), samples were taken at 0, 5, 10, 20, and 45 min. In 4) only two samples were taken: at 0 and 25 min.

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Metabolism screening in human cytosolic incubations

To screen for statin metabolism mediated by cytosolic enzymes, each statin was incubated with HLC (0.2 mg/ml) in sodium phosphate buffer (0.1 M, pH 7.4) with $MgCl_2$ (5 mM) at 37°C and 350 rpm, either 1) without co-factors or 2) with the sulfotransferase (SULT) cofactor PAPS (100 µM). Two samples were taken: 0 and 25 min. These conditions were selected to match those of the HIM screening (test setting 4) above). All incubations were carried out once in triplicate and reactions were stopped as above.

Metabolism screening with recombinant CYP enzymes

The depletion of statins was also measured in recombinant CYP incubations. In the screening, the substrates were incubated with each one of 11 CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, and CYP3A5) separately at a protein concentration of 0.2 mg/ml. The substrate concentrations were the same ones as in the HLM depletion experiment (**Table 1, Supp. Table 2**). Samples were collected at 0, 30, and 60 min. All incubations were carried out once in triplicate and reactions were stopped as above.

Inhibition of metabolism in microsomal incubations

To confirm the results from the recombinant CYP screening, inhibition studies in HLMs and HIMs were carried out for selected statins (HLMs: atorvastatin and atorvastatin lactone, 3R,5S- and 3S,5R-fluvastatin, pitavastatin lactone, simvastatin and simvastatin acid; HIMs: atorvastatin lactone and simvastatin). In HLMs, inhibition of CYP3A4 and CYP isoforms causing ≥50% depletion at 60 min in the CYP screening were tested. The substrate and NADPH concentrations used were identical to those used in the microsomal depletion experiments above (**Table 1**, **Supp. Table 2**). The protein concentration was 0.2 mg/ml in atorvastatin and atorvastatin lactone experiments, 0.5 mg/ml in 3R,5S-fluvastatin and 3S,5R-fluvastatin, pitavastatin lactone, and simvastatin acid experiments, and 0.1 mg/ml in simvastatin experiments. Ketoconazole (1 µM), montelukast (5 µM), quinidine (10 µM), and sulfaphenazole (10 µM) were employed as competitive inhibitors of CYP3A4/5, CYP2C8, CYP2D6, and CYP2C9, respectively. Because of concerns regarding the selectivity of montelukast and quinidine, the effects

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of the time-dependent inhibitors gemfibrozil glucuronide (60 µM; CYP2C8) and paroxetine (15 µM; CYP2D6) were also tested. Whereas inhibitor and substrate were co-incubated in direct inhibition experiments, the experiment with paroxetine included a 15 min preincubation of inhibitor and NADPH and HLMs before addition of substrate. The experiment with gemfibrozil glucuronide was iniated by preincubating inhibitor with NADPH in HLMs. After preincubation for 15 min, 10 µl of the preincubation mix was moved to another well containing 190 µl statin and NADPH in buffer, diluting the protein concentration 20-fold. Samples in the inhibition experiments were taken at the same time points as in the HLM depletion experiment described above or at 0.5, 2, 4, 6, 8, and 10 min (atorvastatin lactone). In HIM incubations, the protein concentration was 0.2 mg/ml and only the effects of ketoconazole (1 µM) were tested. For atorvastatin lactone, samples were taken at 0, 5, 10, 20 and 45 min, and for simvastatin at 0, 3.5, 5, 7.5, and 10 min. All HLM and HIM incubations were carried out once in triplicate at 37°C and 350 rpm, and stopped as described above.

Determination of unbound fraction in microsomes

Unbound fraction in microsomes ($f_{u,mic}$) values were measured using two-chambered Rapid Equilibrium Dialysis (RED) devices (Thermo Scientific, Waltham, MA, USA). HLMs (0.2 mg/ml; 0.1 mg/ml for simvastatin) or HIMs (0.2 mg/ml) in buffer (200 µl) containing statin were transferred to one chamber and 400 µl buffer to the other, before incubation at 37°C for 4 h on a shaker (300 rpm). At the end of the incubation, 25 µl samples from the HLM and buffer chambers were transferred to a 96-well plate containing 100 µl internal standard in acetonitrile. Blank buffer or blank HLMs or HIMs (25 µl) was added to the samples from the HLM or buffer chambers, respectively, to yield identical matrices. Samples were thereafter processed as described previously. The $f_{u,mic}$ was calculated by dividing the statin concentration in buffer by that in the microsomal incubation mixture. To minimize potential metabolism by enzymes not dependent on external cofactors for their activity, old microsomes with several freeze- and thaw cycles were used. In addition, they were allowed to incubate in room temperature 8 h prior to the experiment. Denaturation was not carried out because of concerns that if would lead to conformational changes of the microsomal structure.

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Inhibition of HMG-CoA

To investigate the inhibitory effects of statins on their pharmacological target HMG-CoA reductase, we first determined the time-linearity of HMG-CoA metabolism at different enzyme concentrations (0.3-1.2 µg/ml). Herein, incubations containing HMG-CoA (30 µM) and HMG-CoA reductase in phosphate buffer (0.1 M) were first preincubated for 5 min at 37°C before addition of NADPH (4.5 mM), which initiated the reactions. Reactions were stopped by placing 50 µl samples in 150 µl ice-cold acetonitrile containing mevalonolactone-d3 as the internal standard. Based on the obtained data, we determined the enzyme kinetics of HMG-CoA to its metabolite mevalonate by preincubating HMG-CoA (5-240 µM) with HMG-CoA reductase (0.9 µg/ml) in phosphate buffer for 3 min. NADPH was added and the reactions were allowed to continue for 3 min before stopping them as described above. The same preincubation and incubation times (3+3 min) were also used in the final inhibition experiments. Herein, eight different concentrations of the statin acids (0-500 nM) were simultaneously incubated with HMG-CoA at 20 µM and HMG-CoA reductase (0.9 µg/ml) and NADPH in buffer. All incubations were performed once in triplicate. Use of recombinant enzyme was preferred over HLMs to avoid CYP-mediated metabolism of statins.

Sample processing and analysis of metabolism samples

After sample collection, metabolism samples were kept on ice for at least 10 min before centrifugation at 2,000 *g* for 10 min. All samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The drug concentrations in HLM and recombinant CYP enzyme incubations were determined using a Nexera X2 liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to an API3000 tandem mass spectrometer (AB Sciex, Toronto, Ontario, Canada), as previously described for atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin (Keskitalo *et al.*, 2009a; Keskitalo *et al.*, 2009b; Keskitalo *et al.*, 2009c; Itkonen *et al.*, 2015; Lehtisalo *et al.*, 2020). Part of the samples (HIM, HLC, RED and some inhibition incubations) were analyzed using a Sciex 5500 Qtrap LC-MS/MS system (AB Sciex) interfaced with an ESI ion source. The chromatographic separation was carried out on a Luna Omega polar C18 column (100x2.1mm I.D., 1.6µm particle size; Phenomenex, Torrance, CA) using 5 mM ammonium formate (pH 3.9, adjusted with 98% formic acid) as mobile phase A and

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acetonitrile as mobile phase B. The flow rate and the column temperature were maintained at 300 µL/min and 40 ˚C. The following gradient conditions were applied: 1 min at 20% B on hold, then a linear ramp from 20% B to 40% B over 3 min followed by a second linear ramp to 90% B over 2 min, and 1 min at 90% B before a re-equilibration step back to the initial conditions (20% B). The characteristic multiple reaction monitoring (MRM) transitions for each analyte and internal standard are presented in **Supp. Table 1**.

Sample processing and analysis of pharmacodynamic samples

Following sample collection, samples were kept on ice, and 10 µl HCl (5 M) was added to ensure the lactonization of mevalonic acid into mevalonolactone (Honda *et al.*, 2007). Samples were centrifuged at 2,000 *g* for 10 min before LC-MS/MS analysis. Analytes were separated on Kinetex C18, 2.6 µm, 100 x 2.1 mm with SecurityGuard™ ULTRA C18 2 x 2.1 mm column (Phenomenex, Torrance, CA, USA) by liquid chromatography (Nexera X2, UHPLC system, Shimadzu, Kyoto, Japan) with 0.05% formic acid as mobile phase A and acetonitrile as mobile phase B at flow rate 0.2 ml/min. The mobile phase B was kept at 30% for 0-1.5 min, increased to 100% for 1.6-3.5 min and balanced at 30% before next injection. The analytes were detected in a Shimadzu LCMS-8050 mass spectrometer (Shimadzu, Kyoto, Japan) operated in positive electrospray ionization mode (ESI+) and the multiple reaction monitoring transitions $[M+H]^+$ were m/z 130.9-43.0 for mevalonolactone and m/z 133.9 – 45.95 for the internal standard.

Data analysis and *in vitro-in vivo* extrapolation

The obtained data of the present *in vitro* experiments were analyzed using GraphPad Prism software (version 7.03; GraphPad Software, Inc., San Diego, CA). For depletion data, pseudo-first-order depletion rate constants (k_{dep}) were determined using nonlinear regression analysis ($C=C_0 \times e^{-kdep \times t}$, where C is the observed concentration, C_0 the initial concentration, and t the incubation time). Only data points in the log-linear portion of each depletion curve were included in the analyses. Statin depletions observed in incubations with cofactors were corrected for depletions in incubations lacking cofactors (negative controls) by $k_{dep,\ corrected} = k_{dep,\ incubation} - k_{dep,\ negative\ control}$. Assuming that substrate concentrations were $<<K_m$ for their metabolic pathways, their intrinsic clearance in depletion experiments was expressed as $CL_{int} = k_{dep,\ corrected}/[M]$, where [M] is the microsomal protein concentration

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or CYP concentration in recombinant enzyme incubations (Obach, 1999). Percent inhibition of statin depletion was calculated by comparing CL_{int} values of incubations containing inhibitor to those lacking inhibitor. The mean of statin CL_{int} in the presence of each inhibitor was compared with the control using the Student's *t* test in IBM SPSS Statistics (version 27.0; IBM Corp., Armonk, NY, USA). A *P* value < 0.05 was considered statistically significant. Unbound intrinsic clearance values, $CL_{int,u}$, were calculated according to $CL_{int,u} = CL_{int} / f_{u,mic}$. For pharmacodynamic data, statin concentration values producing 50% inhibition (IC_{50}) were determined using nonlinear regression in GraphPad Prism, and related to their unbound peak concentrations in plasma ($C_{max,u}$) and in the portal vein.

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Results

Metabolism in microsomal and cytosolic incubations

In HLM incubations, statin lactones were metabolized to a much higher extent than their corresponding acid forms (**Fig. 1A and 2A, Supp. Table 3**). In incubations with NADPH, atorvastatin lactone and simvastatin showed extensive metabolism (CL_{int} values of 3,700 and 7,400 µl/min/mg), while the metabolism of the lactones of 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, and pitavastatin was slower (CL_{int} 20-840 µl/min/mg). The statin acids had CL_{int} values in the range of <0.1-80 µl/min/mg. For most parent statins, the metabolism of both acid and lactone forms in NADPH+UDPGA incubations was of similar range or slightly slower than in NADPH incubations. No metabolism was observed in pitavastatin acid, pravastatin, and rosuvastatin incubations. In most HLM control incubations lacking cofactors (negative controls), the depletion of statins was <20% at 60 min (**Fig. 2A**). However, in control incubations containing simvastatin and 4-hydroxyatorvastatin lactone, there seemed to be some CYP- and UGT-independent depletion occurring; for simvastatin the depletion was 29% at 60 min and for 4-hydroxyatorvastatin lactone, it was 32%. Pitavastatin lactone exhibited large variation due to limited solubility, which was observed repeatedly across experiments. For most statins, the solubility seemed to be dependent on the presence of microsomal protein; the initial concentrations in buffer controls were often lower than in corresponding incubations containing microsomes and the solubility seemed to increase with incubation time in buffer controls (data not shown). In the HLC screening, no clear statin metabolism was evident, neither with nor without PAPS (**Fig. 2B**).

In HIM incubations with NADPH and UDPGA, only atorvastatin lactone and simvastatin showed notable metabolism (**Fig. 1B and 2C, Supp. Table 4**). The HIM CL_{int} values of atorvastatin lactone and simvastatin corresponded to approximately 20% of those obtained in HLM incubations. Following incubation for 20-25 min, no clear metabolism was observed in HIM incubations lacking cofactors, except for a slight 17% decrease in simvastatin acid concentration in PMSF-free HIM incubations (**Fig. 2C**). For atorvastatin, atorvastatin lactone, and pravastatin, the decrease was 10-12%. For pitavastatin lactone, the concentration seemed to have increased

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with time. This is likely due to solubility issues, which were also observed in other incubations with pitavastatin lactone. $f_{u,mic}$ values varied markedly between the statins (**Supp. Tables 3 and 4**)

Metabolism screening with recombinant CYP enzymes

In the recombinant CYP screening, CYP3A4, CYP3A5, CYP2D6, CYP2C9, and CYP2C8 were the most active enzymes involved in statin metabolism (**Fig. 2A**). Following incubation for 60 min, ≤50% of the initial substrate concentration remained in CYP3A4 incubations with atorvastatin lactone, 2-hydroxyatorvastatin lactone, 4-hydroxyatorvastatin lactone, pitavastatin lactone, simvastatin, and simvastatin acid. The same (<50% remaining of the initial statin concentration) was observed in CYP3A5 incubations for atorvastatin lactone, 2-hydroxyatorvastatin lactone, and simvastatin, in CYP2D6 incubations for atorvastatin lactone, 2-hydroxyatorvastatin lactone, and pitavastatin lactone, in CYP2C9 incubations for the fluvastatin enantiomers, in CYP2C8 incubations for 2-hydroxyatorvastatin lactone and simvastatin, and in CYP1A2 and CYP2A6 incubations for 2-hydroxyatorvastatin lactone. Of note, whereas the lactones of atorvastatin, 2-hydroxyatorvastatin and simvastatin were completely metabolized by both CYP3A4 and CYP3A5, their acid forms were more prone to metabolism by CYP3A4 than by CYP3A5.

Inhibition of metabolism in microsomal incubations

In HLMs, the CYP3A4 inhibitor ketoconazole completely (>95%) inhibited the metabolism (CL_{int}) of atorvastatin ($P < 0.001$) and atorvastatin lactone ($P = 0.004$), and that of simvastatin ($P = 0.002$) and simvastatin acid ($P < 0.001$) (**Fig. 3**). It had no effect (<10% inhibition) on the depletion of 3S,5R-fluvastatin ($P = 0.142$), whereas it had a moderate effect on the CL_{int} of 3R,5S-fluvastatin (35% inhibition, $P = 0.027$) and that of pitavastatin lactone (18%, $P = 0.343$). In turn, the CYP2C9 inhibitor sulfaphenazole inhibited the CL_{int} of 3R,5S-fluvastatin and 3S,5R-fluvastatin by 42% ($P < 0.001$) and 51% ($P = 0.010$). The CYP2D6 inhibitor quinidine inhibited the depletion of atorvastatin lactone by 18% ($P = 0.093$) and pitavastatin lactone by 18% ($P = 0.241$). Paroxetine inhibited the depletion of atorvastatin lactone by 16% ($P = 0.106$). The CYP2C8 inhibitor montelukast inhibited the depletion of simvastatin by 25% ($P = 0.101$), whereas gemfibrozil glucuronide had no effect on it ($P = 0.840$). In HIMs, ketoconazole inhibited the metabolism of atorvastatin lactone and simvastatin by >95% ($P < 0.001$ and $P < 0.001$).

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Interconversion between acid and lactone forms in microsomal and cytosolic incubations

Some interconversion between statin acid and lactone forms could be detected for atorvastatin, pitavastatin, and simvastatin. To examine the extent of interconversion in HIMs, we quantified the concentrations of the corresponding lactone in statin acid incubations and those of the corresponding acid in lactone incubations (**Supp. Fig. 1**). For other incubations, the data described reflect qualitative data.

In HIMs supplemented with NADPH + UDPGA, lactone concentrations marginally increased in pitavastatin but not in atorvastatin incubations (**Supp. Fig. 1**). In HLMs with NADPH and UDPGA, the lactone concentrations of both atorvastatin and pitavastatin slightly increased, but not in other incubations (NADPH, control). For simvastatin acid, there was no increase in lactone in HLMs and HIMs. In other incubations (PMSF-free HIMs, HLC ± PAPS) with these statin acids, no increase in lactone was observed.

For statin lactones, there seemed to be a trend towards increase of acid concentrations in negative control (no cofactor) HLM and HIM incubations, in PMSF-free HIMs (no cofactor added) and in HLC (±PAPS). More specifically, for atorvastatin lactone, acid concentrations increased up to 8% and 13% of the initial lactone concentration in NADPH+UDPGA and no cofactor HIM incubations, respectively (**Supp. Fig. 1**). In NADPH incubations, no increase in acid concentrations was observed. In HLMs, acid concentrations increased in negative control incubations, but not in those containing cofactors. In PMSF-free HIMs and HLC, the atorvastatin acid concentrations seemed to increase slightly.

For pitavastatin lactone, acid concentrations slightly increased in all incubations. For simvastatin, there seemed to be an increase in acid concentrations (up to 10% of the initial simvastatin concentration) in HIMs lacking cofactors but not in those containing cofactors (**Supp. Fig. 1**). In HLMs, there was no increase in acid concentrations. In PMSF-free HIMs and HLC, the acid concentrations seemed to increase slightly.

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Inhibition of HMG-CoA

In enzyme kinetic experiments, the kinetics of HMG-CoA was best described by a substrate inhibition model, with K_m, K_i, and V_{max} values corresponding to 13 µM, 105 µM, and 1,582 nmol/min/mg (**Supp. Fig. 2**). In pharmacodynamic experiments, the majority of the statin acids tested inhibited the HMG-CoA-mevalonate reaction with IC_{50} values in the range 4-20 nM (**Table 2, Fig. 4**). The IC_{50} of 3R,5S-fluvastatin was 9 nM, whereas that of its 3S,5R enantiomer approximated to 100 nM. Similarly, the IC_{50} of 2-hydroxyatorvastatin acid was 12 nM, but that of 4-hydroxyatorvastatin acid corresponded to ~100 nM. While the IC_{50} values of most statins were relatively close to their typical unbound peak concentrations in plasma (**Fig. 4**), those of 3S,5R-fluvastatin and simvastatin acid exceeded clinically relevant concentrations of 3S,5R-fluvastatin and simvastatin acid by 26-35-fold.

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Discussion

This study aimed to comprehensively investigate and compare the metabolism and pharmacodynamics of fourteen statins or statin metabolites using identical methods. Our collective data from HLM, HIM, HLC, and recombinant enzyme incubations, underlines the importance of CYP enzymes in statin metabolism. According to our findings, CYP3A4/5 (simvastatin, atorvastatin) and CYP2C9 (fluvastatin) are the main statin-metabolizing enzymes in the liver. In HLMs, statin lactones were metabolized to a much higher extent than their corresponding acids. No or very little statin depletion occurred in the liver cytosol. In HIMs, CYP3A4/5 extensively metabolized atorvastatin lactone and simvastatin. No intestinal or hepatic metabolism was observed for pitavastatin, pravastatin and rosuvastatin. In the pharmacodynamic experiment, the majority of the statin acids inhibited HMG-CoA reductase with IC_{50} values of 4-20 nM. Collectively, our findings are applicable for use in comparative systems pharmacology modelling of statins.

Although statin metabolism has been widely investigated *in vitro*, only a few depletion studies comparing the metabolism of statins have been published (Fujino *et al.*, 2004; Gertz *et al.*, 2010; Gertz *et al.*, 2011; Varma *et al.*, 2014), and none with the present palette of statins. Our HLM and HIM CL_{int} values were generally within two-fold of the previous CL_{int} values from depletion studies. In agreement with previous knowledge (Fujino *et al.*, 2004), the lactones were metabolized more extensively than their acid forms. Atorvastatin lactone and simvastatin showed extensive metabolism with HLM CL_{int} values of 3,700 and 7,400 µl/min/mg, while those of the lactones of 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, and pitavastatin were in the range 20-840 µl/min/mg. The statin acids had CL_{int} values below 80 µl/min/mg, with pitavastatin acid, pravastatin, and rosuvastatin showing negligible metabolism. In HIMs, only atorvastatin lactone and simvastatin exhibited notable metabolism, with CL_{int} values corresponding to 20% of those obtained in HLMs. As compared to incubations with NADPH as the single cofactor, UDPGA addition did not result in an increased depletion. This indicates that the role of UGTs in the overall statin metabolism is relatively small.

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To verify the CYPs involved in statin metabolism and to evaluate the potential role of the poorly characterized CYP2J2, we carried out an extensive screening in a panel of eleven recombinant CYPs. Here, CYP3A4, CYP3A5, CYP2D6, CYP2C9, and CYP2C8 were the most active enzymes. CYP2J2 exhibited some activity towards atorvastatin lactone, 2-hydroxyatorvastatin lactone, pitavastatin lactone, simvastatin, and simvastatin acid. On the other hand, these compounds were metabolized to various degrees by almost all CYPs tested. Our inhibition data in HLMs and HIMs demonstrated a major role for CYP3A4/5 in the metabolism of atorvastatin, atorvastatin lactone, simvastatin acid, and simvastatin, in line with clinical observations (Neuvonen *et al.*, 2006). For fluvastatin, CYP2C9 was of greater importance than CYP3A4. Furthermore, CYP2C9 was more prominent in the metabolism of 3S,5R-fluvastatin than in that of its enantiomer, as described earlier (Hirvensalo *et al.*, 2019). Pitavastatin lactone was markedly metabolized by CYP2D6 and CYP3A4 in the screening, but its depletion was inhibited only moderately by the corresponding inhibitors in HLMs. Overall, our data demonstrating key roles for CYP3A4 and CYP2C9 in statin metabolism are line with previous literature (Fujino *et al.*, 2004; Neuvonen *et al.*, 2006).

The interconversion between the acid and lactone forms of statins introduces an additional level of complexity into statin pharmacokinetics. The process can be enzyme-mediated, spontaneous or pH-driven, and occur in both the blood stream and hepatocytes (Jemal *et al.*, 1999; Billecke *et al.*, 2000; Prueksaritanont *et al.*, 2002; Hoffmann and Nowosielski, 2008; Li *et al.*, 2019). In line with previous studies (Prueksaritanont *et al.*, 2002; Fujino *et al.*, 2003; Yamada *et al.*, 2003), we observed slight increase in lactone concentrations in some statin acid incubations with UDPGA. The underlying mechanism is likely UGT-mediated glucuronidation, followed by spontaneous lactonization (Prueksaritanont *et al.*, 2002). For statin lactones, there was a trend towards increase of acid concentrations in negative control microsomal incubations, suggesting a role for enzymes that do not require external cofactors for their activity, such as carboxylesterases (Liederer and Borchardt, 2006). In PMSF-free HIMs, however, less than 10% of the initial lactone concentrations had been depleted at 25 min. For comparison, in HIMs fortified with NADPH and UDPGA, only 32% and <1% of the initial atorvastatin lactone and simvastatin (lactone) remained at 20 min. Thus, the role of intestinal membrane-bound esterases in lactone depletion seems to

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be negligible. There are contradictory findings on the role of human carboxylesterases in the conversion of simvastatin to its acid form; however, these enzymes seem to catalyze this process in blood (Vickers *et al.*, 1990; Wang *et al.*, 2015; Li *et al.*, 2019). Together with SULTs, carboxylesterases are also present in smaller fractions in the cytosol (Boberg *et al.*, 2017). In our HLC incubations (±PAPS), no statin showed a >10% reduction in their concentrations at 25 min. No depletion was observed for pravastatin, which has been reported to be a SULT substrate in rat liver (Kitazawa *et al.*, 1993; Watanabe *et al.*, 2009). As a longer incubation time might have shown some involvement of cytosolic enzymes in statin metabolism, our HLC data should be interpreted with caution.

We used substrate depletion to obtain comparable estimates of the microsomal metabolism of each statin. The depletion approach operates under the assumption that the initial substrate concentration is well below K_m ($C_0 << K_m$) (Obach, 1999). In our experiments, we attempted to use equal protein concentrations (0.2 mg/ml; 0.1 mg/ml for simvastatin) and low, clinically relevant statin concentrations. For most statins, an incubation concentration of 0.05 µM was used. For pitavastatin acid and lactone, and rosuvastatin the corresponding concentrations were slightly lower (0.01-0.04 µM), and for pravastatin it was higher (0.1 µM), based on initial estimations of typical unbound plasma concentrations. Regardless, in HLM incubations, the $C_0 << K_m$ criterion was fulfilled for all statins with K_m values reported in the literature (**Supp. Table 2**). Assuming a 'worst case' scenario with complete absorption of the statin into enterocytes, our initial substrate concentrations in HIMs were in general 20-fold higher than those in HLMs. For the fluvastatin enantiomers, the concentrations used did not fulfill the $C_0 << K_m$ criterion in HIMs. However, no fluvastatin metabolism was observed in HIM incubations, and also the metabolism in HLMs was limited.

The present microsomal experiments included both buffer controls and negative controls. As most statins needed protein to dissolve in the incubations, it was not possible to distinguish between potential degradation of compound in buffer and depletion in incubations lacking cofactors. However, by correcting for depletion in negative control incubations, we were able to accurately measure the NADPH- and UDPGA-dependent microsomal metabolism. Use of microsomes instead of hepatocytes allowed us to measure metabolic CL_{int} values in a system stripped from confounding factors, such as drug transporters. Interestingly, while rosuvastatin is not metabolized in microsomes,

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there are data showing (very slow) rosuvastatin metabolism in hepatocytes (McCormick et al., 2000). The statins are known substrates of drug transporters, in particular of OATP1B1 and BCRP (Pasanen *et al.*, 2006; Keskitalo *et al.*, 2009c; Giacomini *et al.*, 2013). Consequently, the hepatic clearance of statins is a product of their metabolic CL_{int} values in combination with their transporter CL_{int} values. Together with *in vitro* drug transport and pharmacodynamic data in combination with clinical data, our CL_{int} values can be applied in systems pharmacology modelling of statin pharmacokinetics and effects.

In the present study, we also tested the inhibitory effects of the statin acids on their pharmacological target in the liver, HMG-CoA reductase. While previous studies have determined these effects using radiometric assays or spectrophotometry to measure NADPH consumption (Kathawala, 1991; Holdgate *et al.*, 2003; Perchellet *et al.*, 2009), we used LC-MS/MS to measure mevalonolactone concentrations. Overall, our findings are in good agreement with literature data (**Supp. Table 5**). Our IC_{50} value obtained for pravastatin (13 nM), however, is 4-5 fold lower than previous measurements (McTaggart *et al.*, 2001; Perchellet *et al.*, 2009). The low value is supported by our preliminary experiment, in which the IC_{50} of pravastatin was 15 nM (data not shown). 3R,5S-fluvastatin (IC_{50} = 9 nM) was about 12 times more active than its 3S,5R-enantiomer (IC_{50} ~100 nM). Whereas the IC_{50} of 3R,5S-fluvastatin was between its typical unbound peak concentration in plasma and in the portal vein, that of 3S,5R-fluvastatin largely exceeded its clinically relevant unbound concentrations (**Fig. 4**). Likewise, the IC_{50} value of simvastatin acid (19.7 nM) exceeded its $C_{max,u}$ by 35-fold. On the other hand, its concentrations in the portal vein may be higher. Furthermore, simvastatin acid is a substrate of the hepatic uptake transporter OATP1B1 *in vivo* (Pasanen *et al.*, 2006), indicating that its intracellular hepatocyte concentrations are likely higher than those in the surrounding blood stream. For all other parent statins acids, the IC_{50} values obtained were close to or below their typical unbound peak concentrations in plasma and in the portal vein.

Taken together, we comprehensively investigated the *in vitro* metabolism and pharmacodynamics of statins. Together with drug transport and clinical data, our findings are applicable for use in systems pharmacology models to prospectively predict the pharmacokinetics and pharmacological effects of statins at different dosages.

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Authorship Contributions

Participated in research design: Filppula, Hirvensalo, Parviainen, Niemi

Conducted experiments or drug concentration analysis: Filppula, Hirvensalo, Parviainen, Ivaska, Lönnberg, Deng, Viinamäki, Kurkela, Neuvonen

Performed data analysis: Filppula, Hirvensalo, Parviainen, Ivaska

Wrote or contributed to the writing of the manuscript: Filppula, Hirvensalo, Parviainen, Ivaska, Lönnberg, Deng, Viinamäki, Kurkela, Neuvonen, Niemi

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Footnotes

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b) The authors declare no conflict of interest.

c) Parts of this work are included in the Master's Thesis of Heli Parviainen and Advanced Studies Thesis of Vilma E. Ivaska.

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Legends for Figures

Fig. 1. Intrinsic clearance values of the tested statins obtained in the depletion experiment in HLMs (A) and HIMs (B). The intrinsic clearance (CL_{int}) values obtained in incubations fortified with either NADPH (only in HLM incubations) or NADPH+UDPGA (both HLM and HIM incubations) are shown. The protein concentration was 0.2 mg/ml in all incubations, except for simvastatin HLM incubations (0.1 mg/ml). The data represent mean and standard deviation values of triplicate incubations, and they have been corrected for potential depletion in corresponding incubations lacking cofactors (negative controls). ATOR, atorvastatin; FLU, fluvastatin; HIM, human intestine microsome; HLM, human liver microsome; lac, lactone; PITA, pitavastatin; PRA, pravastatin; ROSU, rosuvastatin; SIM, simvastatin.

Fig. 2. Metabolism screening in recombinant CYP (A), HLC (B), and HIM (C) incubations. In the recombinant CYP screening, the mean statin concentrations remaining after a 60 min incubation with 11 recombinant CYP recombinant enzymes (0.2 mg/ml) and NADPH are shown. For comparison, the corresponding HLM data, with and without NADPH, is also included. The relative hepatic expression (%) of each CYP is shown in the panel to the left (calculated based on data available in Simcyp Simulator v.20, Certara UK Limited). In the HLC screening, no notable metabolism was observed following a 25 min incubation of statins with HLC (0.2 mg/ml), with or without PAPS. In the HIM figure, the parent statin concentrations remaining after 25 min incubation with PMSF-free HIMs (0.2 mg/ml) are shown (no cofactors included). In addition, 20 min data from the depletion experiment in normal HIMs both with and without NADPH and UDPGA are also shown. The data presented represent mean and standard deviation values of triplicate incubations. ATOR, atorvastatin; FLU, fluvastatin; HIM, human intestine microsome; HLC; human liver cytosol; HLM, human liver microsome; lac, lactone; OH, hydroxy; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PITA, pitavastatin; PMSF, phenylmethylsulfonyl fluoride; PRA, pravastatin; ROSU, rosuvastatin; SIM, simvastatin.

Fig. 3. Inhibition of statin metabolism by selected inhibitors in HLM and HIM incubations. The inhibitory effects of ketoconazole 1 µM (CYP3A4/5), gemfibrozil glucuronide 60 µM (CYP2C8), montelukast 5 µM (CYP2C8), quinidine 10 µM (CYP2D6), paroxetine 15 µM (CYP2D6), and sulfaphenazole 10 µM

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(CYP2C9) on the depletion of statins were tested in HLMs (0.5 mg/ml protein in fluvastatin, pitavastatin lactone, and simvastatin acid incubations; 0.2 mg/ml in atorvastatin acid and lactone incubations; 0.1 mg/ml in simvastatin incubations) and HIMs (0.2 mg/ml). The values indicate the remaining metabolic activity in the presence of inhibitor and are the mean and standard deviation values of triplicate incubations. The values have been corrected for potential depletion in corresponding incubations lacking cofactors (negative controls). Pitavastatin lactone exhibited large variation due to limited solubility, which was observed repeatedly across experiments. Incubation with paroxetine was also carried out for pitavastatin lactone, but its depletion following the preincubation step was too low to be measured, ATOR, atorvastatin; FLU, fluvastatin; HLM, human liver microsome; HIM, human intestine microsome; lac, lactone; PITA, pitavastatin; PRA, pravastatin; SIM, simvastatin.

Fig. 4. Pharmacodynamic IC_{50} values of statin acids in comparison to their plasma concentrations. To test the inhibitory effects of the acid forms of the statins on their pharmacological target, eight different concentrations of the test compound were incubated with HMG-CoA (20 µM) and HMG-CoA reductase (0.9 µg/ml) for 3 min. The results shown describe mean values of triplicate incubations (duplicate incubations for pitavastatin). The unbound peak concentration values in plasma ($C_{max,u}$) and the portal vein ($C_{max,u,portal}$) are from **Table 1**. As described therein, the $C_{max,u,portal}$ concentrations were calculated assuming a complete absorption scenario.

ATOR, atorvastatin; FLU, fluvastatin; IC_{50}, inhibitor concentration producing 50% inhibition; OH, hydroxy; PITA, pitavastatin; PRA, pravastatin; ROSU, rosuvastatin; SIM, simvastatin.

Tables

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Table 1

Calculated and measured *in vivo* plasma concentration data, and initial incubation concentrations (C_0) of the statins tested. Fraction unbound in plasma and peak concentration data in plasma and blood-to-plasma concentration ratios were collected from the University of Washington Drug Interaction Database (September 21, 2020) or from ADMET Predictor (version 10; Simulations Plus, Lanchester, CA). The concentration data of the fluvastatin enantiomers are from Hirvensalo *et al.* (2019) and those of the atorvastatin metabolites from Kantola *et al.* (1998) (single-dose studies).

Compound	BP	$f_{u,p}$	C_{max} (µM)	$C_{max,u}$ (µM)	C_{gut} [b] (µM)	C_{ent} [c] (µM)	$C_{ent,u}$ [c] (µM)	$C_{max,portal}$ [d] (µM)	$C_{max,u,portal}$ (µM)	C_0 in HLMs, HLC, rCYPs (µM)	C_0 in HIMs (µM)
Atorvastatin	0.667 [a]	0.02	0.31 (40 mg)	0.00062	290	8.7	0.69	2.5	0.051	0.05	1
Atorvastatin lactone	0.716 [a]	0.04 [a]	0.0070 (40 mg)	0.00028	n/a	n/a	n/a	n/a	n/a	0.05	1
2-hydroxyatorvastatin	0.673 [a]	0.05 [a]	0.017 (40 mg)	0.00085	n/a	n/a	n/a	n/a	n/a	0.05	n/d
2-hydroxyatorvastatin lactone	0.725 [a]	0.05 [a]	0.026 (40 mg)	0.0013	n/a	n/a	n/a	n/a	n/a	0.05	n/d
4-hydroxyatorvastatin	0.672 [a]	0.06 [a]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.05	n/d
4-hydroxyatorvastatin lactone	0.721 [a]	0.05 [a]	0.0032 (40 mg)	0.00016	n/a	n/a	n/a	n/a	n/a	0.05	n/d
3R,5S-Fluvastatin	0.676 [a]	0.01	0.37 (40 mg)	0.0037	97	2.9	0.74	1.9	0.019	0.05	1
3S,5R-Fluvastatin	0.676 [a]	0.01	0.38 (40 mg)	0.0038	97	2.9	0.74	1.9	0.019	0.05	1

Compound	BP	$f_{u,p}$	C_{max} (µM)	$C_{max,u}$ (µM)	C_{gut} [b] (µM)	C_{ent} [c] (µM)	$C_{ent,u}$ [c] (µM)	$C_{max, portal}$ [d] (µM)	$C_{max, u, portal}$ (µM)	C_0 in HLMs, HLC, rCYPs (µM)	C_0 in HIMs (µM)
Pitavastatin	0.695 [a]	0.04	0.16 (4 mg)	0.0064	19	0.57	0.53	0.30	0.012	0.04	1
Pitavastatin lactone	0.753 [a]	0.05 [a]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.03	1
Pravastatin	0.662 [a]	0.52	0.079 (40 mg)	0.041	380	11	11	3.0	1.6	0.1	1
Rosuvastatin	0.674 [a]	0.12	0.036 (40 mg)	0.0043	660	20	19	5.1	0.62	0.01	0.2
Simvastatin (lactone)	0.76 [a]	0.06	0.023 (40 mg)	0.0013	380	12	0.0023	2.6	0.16	0.05	0.05
Simvastatin acid	0.658 [a]	0.07 [a]	0.008 (40 mg lactone)	0.00056	n/a	n/a	n/a	n/a	n/a	0.05	0.05

BP, blood-to-plasma concentration ratio; C_{gut}, concentration in gut lumen; C_{ent}, enterocytic concentration; C_0, initial incubation concentration; C_{max}, peak concentration in plasma; $C_{max,portal}$, peak concentration in portal vein; $f_{u,p}$, unbound fraction in plasma; HLC, human liver cytosol; HIMs, human intestine microsomes; HLMs, human liver microsomes; n/a, not available; n/d, not determined; $_u$, unbound.

[a] Predicted value.

[b] C_{gut} calculated according to dose/250 ml.

[c] C_{ent} calculated according to dose x k_{abs} x f_a/Q_{ent}, where k_{abs} is the absorption rate constant, f_a, fraction absorbed into the gut wall and Q_{ent} the enterocytic blood flow (Rostami-Hodjegan and Tucker, 2004). A complete absorption scenario was assumed, using f_a=1, and standard values of k_{abs}=0.03 1/min and Q_{ent}=0.248 L/min (Obach *et al.*, 2007; Kenny *et al.*, 2012). Unbound concentrations were obtained by multiplying C_{ent} with unbound fraction in enterocytes ($f_{u,ent}$). $f_{u,ent}$ values (not shown) were predicted in Simcyp Simulator v.19 (Certara UK Limited).

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[d] $C_{max,portal}$ calculated according to C_{max} + dose x k_{abs} x f_a/Q_{hep}, where Q_{hep} is the hepatic blood flow (Ito *et al.*, 1998). A complete absorption scenario was assumed, using f_a=1, and standard values of k_{abs}=0.03 1/min and Q_{hep}=20.7 ml/min/kg (Houston and Galetin, 2008). For the calculations, the C_{max} in plasma was converted to its corresponding value in blood according to C_{max} x BP. Subsequently, the obtained $C_{max,portal}$ in blood was converted to plasma concentration by dividing it by BP.

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Table 2

The *in vitro* pharmacodynamic effects of statin acids. To test the inhibitory effects of the acid forms of the statins on their pharmacological target, eight different concentrations of the test compound were incubated with HMG-CoA (20 μM) and HMG-CoA reductase (0.9 μg/ml) for 3 min. The data represent mean and standard deviation values of triplicate incubations (duplicate incubations for pitavastatin).

Compound	IC_{50} (nM)
Atorvastatin	13.1 ± 3.2
2-hydroxyatorvastatin	12.1 ± 4.2
4-hydroxyatorvastatin	~100 [a]
3R,5S-fluvastatin	8.58 ± 2.61
3S,5R-fluvastatin	~100 [a]
Pitavastatin	12.4 ± 1.8
Pravastatin	12.6 ± 3.7
Rosuvastatin	4.37 ± 1.13
Simvastatin acid	19.7 ± 2.0

IC_{50}, inhibitor concentration producing 50% inhibition.

[a] The highest concentrations tested were 100 and 500 nM.

Figure 1

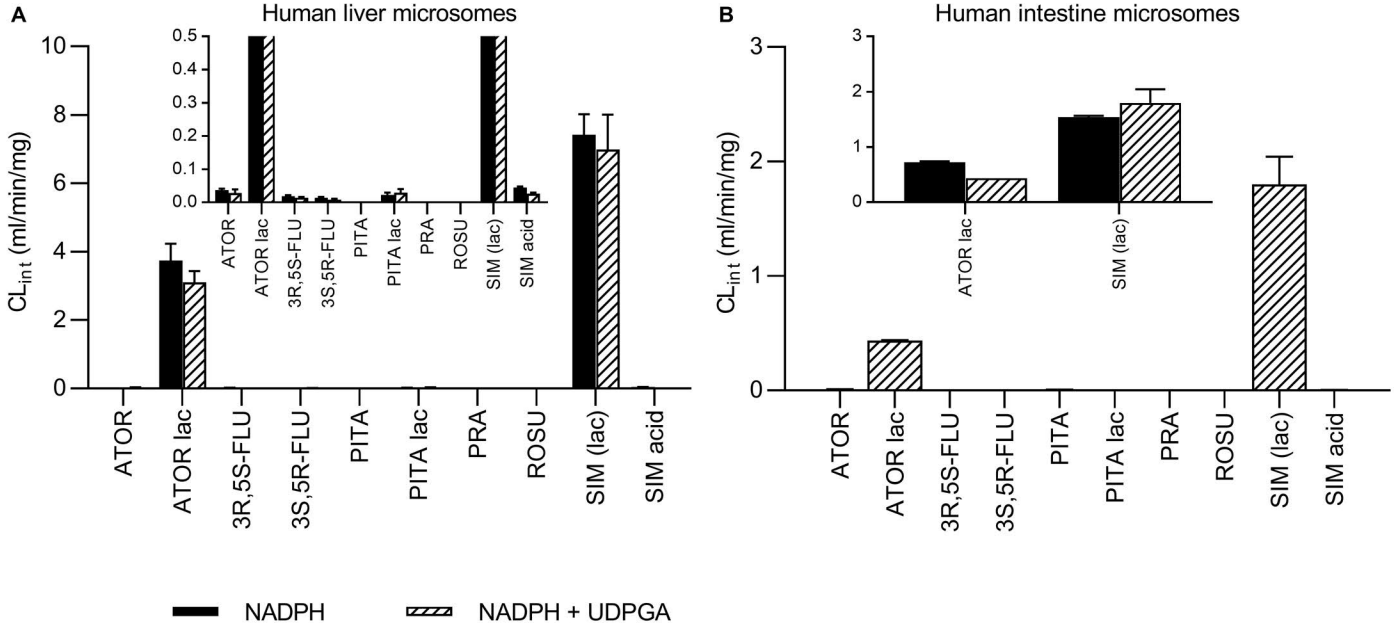
A
Human liver microsomes
CL_{int} (ml/min/mg)
ATOR
ATOR lac
3R,5S-FLU
3S,5R-FLU
PITA
PITA lac
PRA
ROSU
SIM (lac)
SIM acid
NADPH
NADPH + UDPGA
B
Human intestine microsomes
CL_{int} (ml/min/mg)

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Figure 2

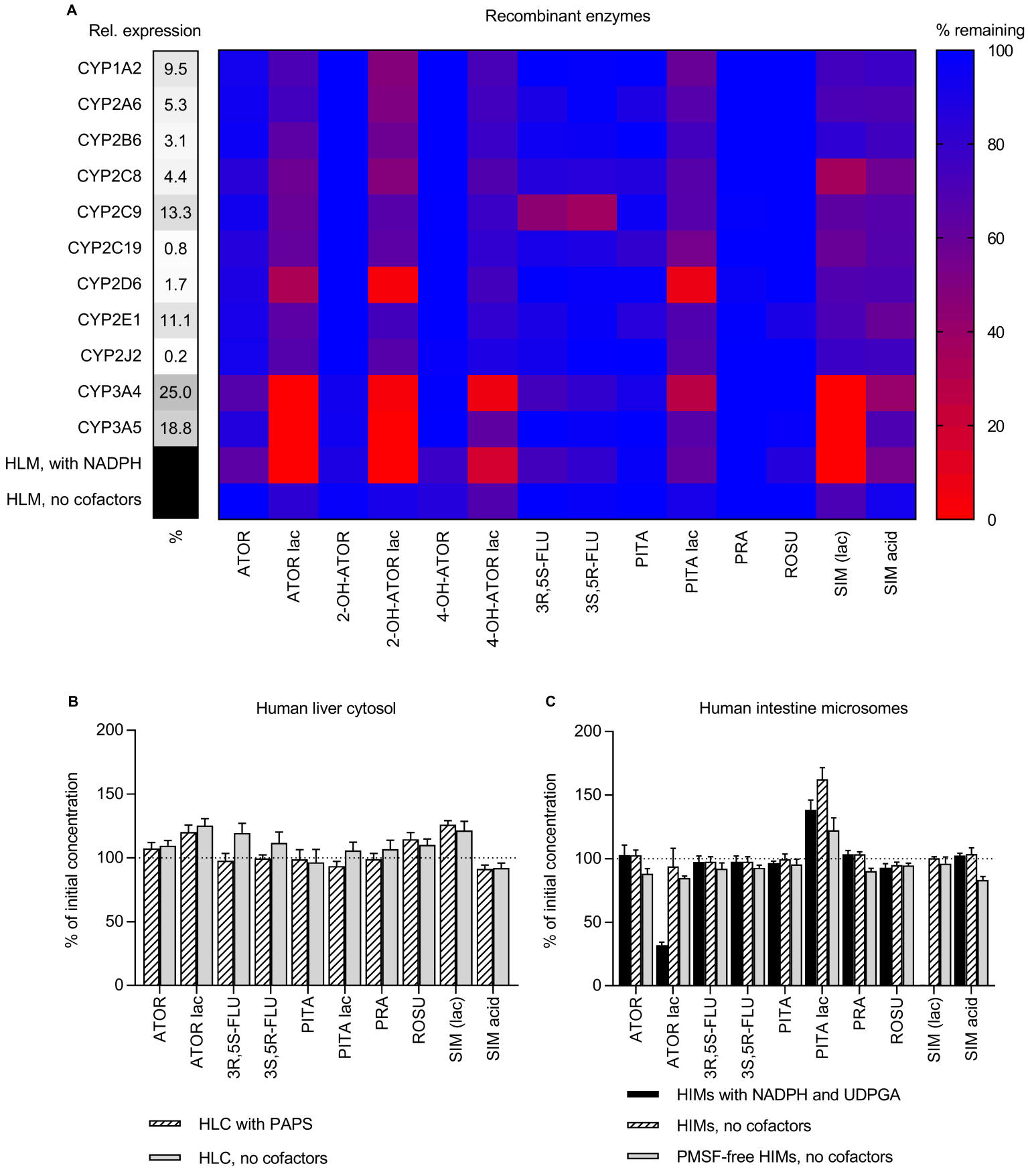

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Figure 3

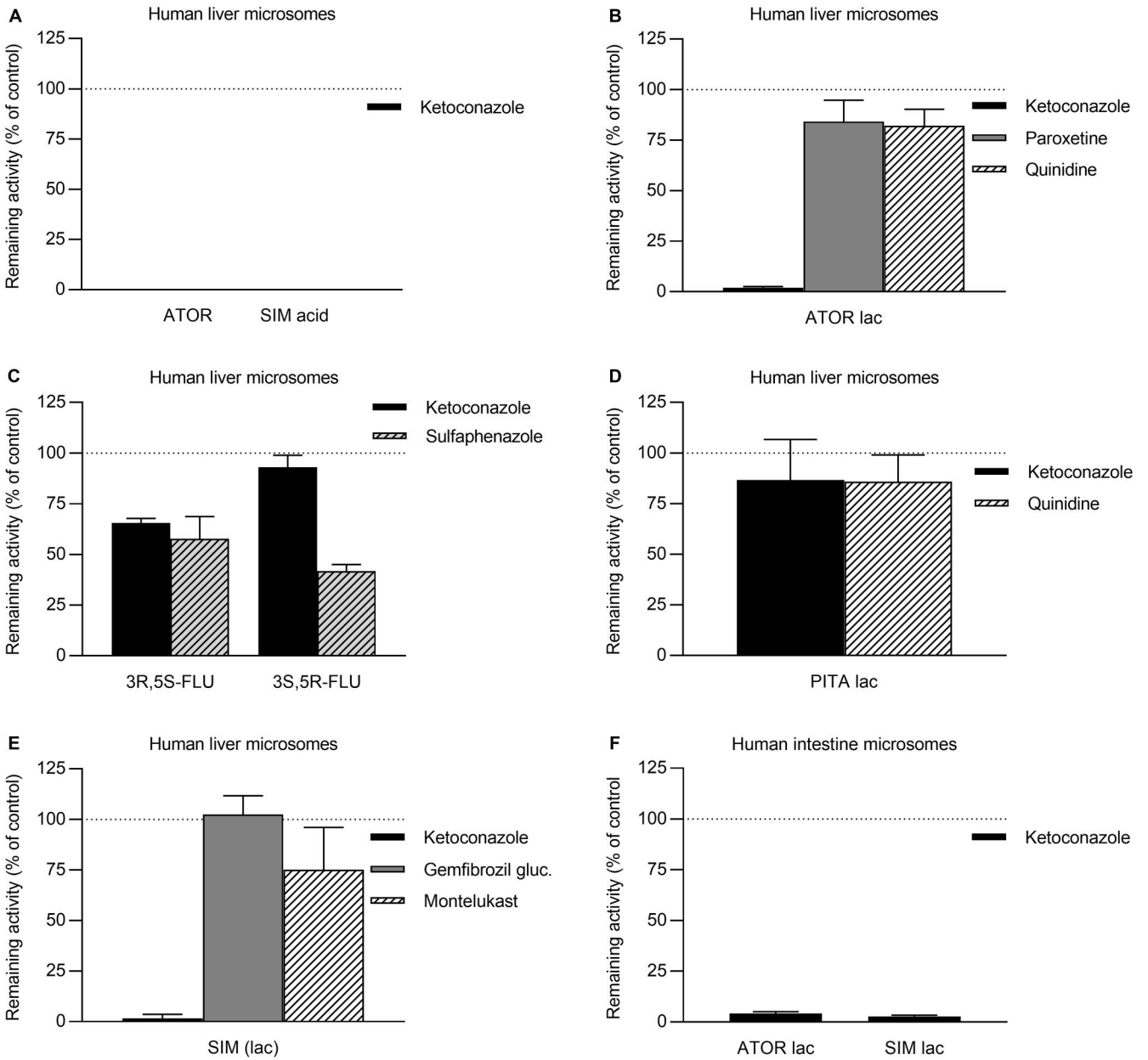
A
Human liver microsomes
Remaining activity (% of control)
125
100
75
50
25
0
Ketoconazole
ATOR
SIM acid
B
Human liver microsomes
Remaining activity (% of control)
Ketoconazole
Paroxetine
Quinidine
ATOR lac
C
Human liver microsomes
Remaining activity (% of control)
Ketoconazole
Sulfaphenazole
3R,5S-FLU
3S,5R-FLU
D
Human liver microsomes
Remaining activity (% of control)
Ketoconazole
Quinidine
PITA lac
E
Human liver microsomes
Remaining activity (% of control)
Ketoconazole
Gemfibrozil gluc.
Montelukast
SIM (lac)
F
Human intestine microsomes
Remaining activity (% of control)
Ketoconazole
ATOR lac
SIM lac

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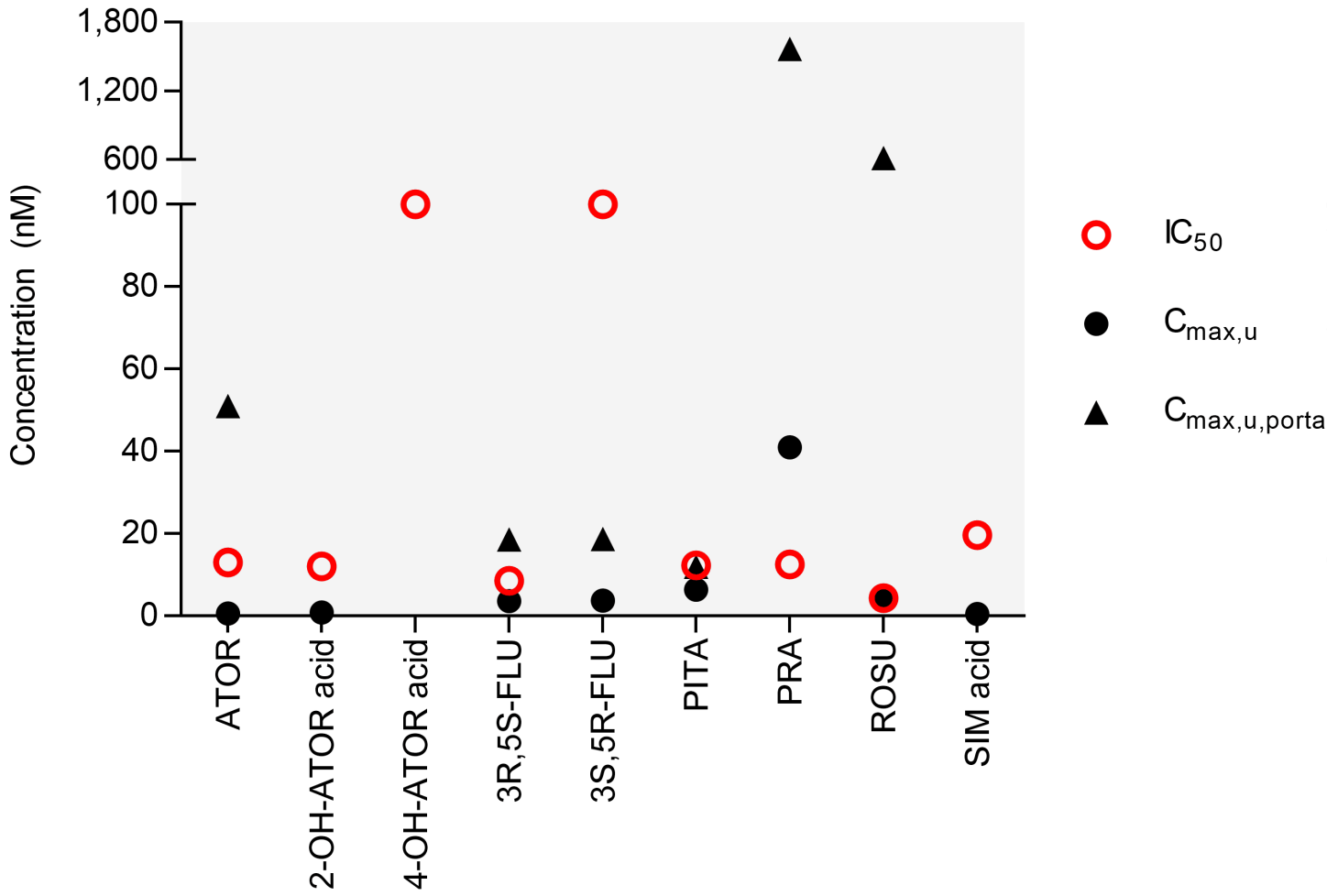
Figure 4



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