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**Drug-drug interactions between rosuvastatin and oral antidiabetic drugs occurring at  
the level of OATP1B1**

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**Non-standard abbreviations:** BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; E<sub>2</sub>17 $\beta$ -G, estradiol 17 $\beta$ -D-glucuronide; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; NTCP, Na<sup>+</sup>-taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; PBS, phosphate buffered saline; SLC, solute carrier; SNP, single-nucleotide polymorphism; UPLC, ultra performance liquid chromatography

## ABSTRACT

Organic anion-transporting polypeptide 1B1 (OATP1B1) is an important hepatic uptake transporter, of which the polymorphic variant OATP1B1\*15 (Asn130Asp and Val174Ala) has been associated with decreased transport activity. Rosuvastatin is an OATP1B1 substrate and often concomitantly prescribed with oral antidiabetics in the clinic. The aim of this study was to investigate possible drug-drug interactions between these drugs at the level of OATP1B1 and OATP1B1\*15. We generated HEK293 cells stably overexpressing OATP1B1 or OATP1B1\*15, which showed similar protein expression levels of OATP1B1 and OATP1B1\*15 at the cell membrane as measured by LC-MS/MS. In HEK-OATP1B1\*15 cells, the  $V_{\max}$  for OATP1B1-mediated transport of  $E_217\beta$ -G was decreased >60%, whereas  $K_m$  values were comparable. Uptake of rosuvastatin in HEK-OATP1B1 cells ( $K_m$   $13.1 \pm 0.43 \mu\text{M}$ ) was nearly absent in HEK-OATP1B1\*15 cells. Interestingly, several oral antidiabetics (glyburide, glimepiride, troglitazone, pioglitazone, glipizide, gliclazide and tolbutamide), but not metformin, were identified as significant inhibitors of the OATP1B1-mediated transport of rosuvastatin. The  $IC_{50}$  values for inhibition of  $E_217\beta$ -G uptake were similar between OATP1B1 and OATP1B1\*15. In conclusion, these studies indicate that several oral antidiabetic drugs affect the OATP1B1-mediated uptake of rosuvastatin in vitro. The next step will be to translate these data to the clinical situation, as it remains to be established whether the studied oral antidiabetics indeed affect the clinical pharmacokinetic profile of rosuvastatin in patients.

## INTRODUCTION

The prevalence of diabetes is overwhelming nowadays, with worldwide more than 345 million people suffering from diabetes (Anonymous, 2012). Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity (Anonymous, 2012). Although life-style interventions, such as dietary adjustments and increased exercise, are initially very effective (and with low costs), long-term adherence is in general poor. Therefore, most type 2 diabetic patients will inevitably need treatment with one or more antidiabetic drugs to manage their hyperglycemia. This treatment mainly includes drugs from the class of oral antidiabetics such as metformin, sulfonylureas, and thiazolidinediones (Kalliokoski et al., 2010). The American Diabetes Association and the European Association for the Study of Diabetes recently published a consensus statement providing a guidance for treatment of hyperglycemia in type diabetic patients (Nathan et al., 2009). According to this guideline, treatment of type 2 diabetes with metformin or sulfonylureas belongs to the class 1 category of well-validated core therapies as it has been in clinical use for over 50 years now, whereas treatment with thiazolidinediones is less well validated and has been associated with serious cardiovascular side effects (Aquilante, 2010; Nathan et al., 2009). Notably, type 2 diabetic patients often concomitantly receive therapeutic drugs directed against other coincident features, such as dyslipidemia and hypertension, and it is therefore important to investigate possible drug-drug interactions.

Many oral drugs (including oral antidiabetics and the lipid-lowering statins) are extracted from the portal blood by the liver before they reach their target, which can either be intrahepatic (e.g. statins, metformin) or extrahepatic (e.g. pancreatic cells in case of sulfonylureas, or adipocytes in case of thiazolidinediones). In this study we focus on the organic anion transporting polypeptide 1B1 (OATP1B1; gene *SLCO1B1*) as one of the main hepatic uptake transporters (Hagenbuch and Meier, 2004). OATP1B1 can transport a wide

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variety of drugs, including many statins (e.g. rosuvastatin, pravastatin, pitavastatin) and some oral antidiabetics (repaglinide, troglitazone) (Fahrmayr et al., 2010; König et al., 2006; Niemi et al., 2005; Nozawa et al., 2004). The clinical importance of OATP1B1 in the pharmacokinetics of drugs has been confirmed by several studies that focused on the effect of commonly occurring single nucleotide polymorphisms in OATP1B1. In particular, the OATP1B1\*15 variant (Asn130Asp and Val174Ala), with an average haplotype frequency of 16-24% in Europe and America (Pasanen et al., 2008), is generally known to have a strongly reduced transport activity and has been associated with markedly increased plasma levels of certain OATP1B1 substrates. This might be advantageous for some drugs that do not have a target in the liver, resulting in an increased pharmacological response. However, for drugs that have a target inside the liver, decreased hepatic uptake by OATP1B1\*15 might result in decreased pharmacological response and give rise to unforeseen toxic side effects. Indeed, it has been demonstrated that patients carrying the OATP1B1\*15 variant show markedly increased plasma levels, decreased pharmacological response and even increased extra-hepatic toxicity for instance after pravastatin, pitavastatin or rosuvastatin treatment (Choi et al., 2008; Chung et al., 2005; Fahrmayr et al., 2010; Igel et al., 2006; Niemi et al., 2004; Niemi et al., 2011).

Although glyburide, troglitazone and pioglitazone have previously been reported as inhibitors of OATP1B1 (Bednarczyk, 2010; Gui et al., 2009; Hirano et al., 2006; Nozawa et al., 2004), the effects of a complete set of oral antidiabetic drugs on both OATP1B1 and OATP1B1\*15 have not been systematically studied before. In this study we therefore aimed to reveal the possible drug-drug interactions between several oral antidiabetic drugs belonging to the class of sulphonylureas and thiazolidinediones and the lipid-lowering drug rosuvastatin at the level of OATP1B1. We also included studies that explore the influence of the \*15 haplotype on this interaction.

## MATERIALS & METHODS

### *Chemicals and reagents*

[<sup>3</sup>H]-estradiol 17β-D-glucuronide ([<sup>3</sup>H]-E<sub>2</sub>17β-G; 1.85 TBq/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and [<sup>3</sup>H]-rosuvastatin (40.7 GBq/mmol) was custom synthesized by Moravek Biochemicals (California). Glyburide, glipizide, gliclazide, glimepiride, tolbutamide, pioglitazone, troglitazone, metformin, E<sub>2</sub>17β-G, cyclosporine A, rifampicin, and pravastatin were obtained from Sigma-Aldrich (St Louis, MO), rosuvastatin was from Sequoia Research Products (Pangbourne, UK).

### *Construction of HEK293 cells stably expressing OATP1B1\*1a or OATP1B1\*15*

Subcloning of human OATP1B1\*1a (NM\_006446.4 referring to wild-type; hereafter named OATP1B1) or OATP1B1\*15 cDNA into the pIRESpuro vector was performed by GeneArt (Invitrogen, Germany). HEK293 (ATTC, Germany; lot# 57954093) cells were seeded in 6-wells plates at a density of 9\*10<sup>5</sup> cells/well and grown for 24 hours, followed by co-transfection with pcDNA3.1-Ukcol (0.3 μg/well) and pIRESpuro-OATP1B1 or -OATP1B1\*15 (2.7 μg/well) using 6 equivalents of Exgen 500 (Fermentas, MBI). Parental HEK293 cells co-transfected with pcDNA3.1-Ukcol and empty pIRESpuro vector (HEK-MOCK) served as control. After 24 hours, the transfected cell lines were cultured in medium containing puromycin (1 μg/mL), and after 3-4 weeks several colonies were selected and grown into 75 cm<sup>2</sup> tissue culture flasks. Excretion of UKcol into the medium of cells of the different colonies was used to select colonies for further analyses.

### *Cell culture*

Cells were grown in 75 cm<sup>2</sup> tissue culture flasks containing culture medium consisting of Dulbecco's modified Eagle medium (DMEM) with L-GlutaMax (4.5 g of glucose per liter), supplemented with heat-inactivated fetal calf serum (10% v/v; Lonza), 100 U/mL penicillin (Invitrogen) and 100 µg/mL streptomycin (Invitrogen) at approximately 37°C in approximately 95% air/5% CO<sub>2</sub>. Near confluent cell cultures were harvested by trypsinisation, re-suspended in culture medium and the process was repeated once or twice weekly to provide sufficient cells for use.

### *Immunocytochemical staining*

HEK-MOCK, HEK-OATP1B1, and HEK-OATP1B1\*15 cells were seeded on Thermanox plastic coverslips (NUNC, Rochester) coated with 0.1 mg/mL Poly-D-Lysine (Sigma-Aldrich) in 24-well plates at a density of 4\*10<sup>5</sup> cells/well and grown for 48 hours at 37°C with 5% CO<sub>2</sub>. Cells were fixated with ice-cold methanol for 30 minutes at -20°C, followed by incubation with the mouse monoclonal primary OATP1B1 antibody (1:100; ESL clone, ab15441, AbCam) for 1 hour at RT. After removal of the primary antibody, cells were incubated for 1 hour at RT with the secondary goat anti-mouse antibody (1:100; Alexa 488, Invitrogen). Cells were washed three times with PBS and de-mineralized water, followed by step-wise dehydration with 50% and 100% methanol. Subsequently, the cover glasses were removed from the wells, mounted on slides with 4.8% Mowiol (Polysciences Inc.) mounting medium and used for analyses by fluorescent microscopy (Leica, Rijswijk, The Netherlands).

### *Membrane isolations*

To determine the active transporter protein expression levels in transfected cells, the outer cellular membranes were isolated using a sucrose density gradient centrifugation

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protocol. After trypsinisation ( $\sim 5 \times 10^7$  cells), cell pellets were re-suspended in hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, and a cocktail of protease inhibitors containing 2 mM phenylmethylsulfonylfluoride, aprotinin, leupeptin, and pepstatin). After one hour of incubation at 4°C, the homogenized cells were centrifuged at 100,000 x g for 30 minutes at 4°C using a LE-80k Centrifuge with SW28 rotor (Beckman Counter, USA). The pellet was re-suspended in hypotonic buffer followed by the addition of two volumes of isotonic buffer (10 mM Tris-Hepes and 250 mM sucrose (pH 7.4)). The extract was again homogenized using a Potter-Elvehjem homogenizer and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 100,000 x g for 30 minutes at 4°C. The pellet was re-suspended in isotonic buffer and was layered on top of a 38% sucrose solution and centrifuged at 100,000 x g for 90 minutes at 4 °C. The turbid layer at the interface, containing the plasma membranes, was recovered, diluted with isotonic buffer and centrifuged at 100,000 x g for 40 minutes at 4°C. The plasma membrane fraction was obtained from the resulting pellet, which was re-suspended in 50  $\mu$ L isotonic buffer. Protein levels were measured using the Bradford method (Biorad, USA).

*Protein digestion*

Isolated plasma membrane extracts ( $\sim 50 \mu$ g) from individual cell lines were diluted with 2 volumes of 90% methanol. The proteins were subsequently reduced with 0.01 M dithiothreitol at 37°C for 60 minutes and alkylated with 0.04 M iodoacetamide for 20 minutes at room temperature in the dark. Digestion was performed after addition of  $\text{CaCl}_2$  (final concentration 1 nM) and 0.5  $\mu$ g trypsin (Promega) in 17% methanol by diluting the solution with 50 mM  $\text{NH}_4\text{HCO}_3$ . After overnight incubation the samples were incubated for another 2 hours with 0.5  $\mu$ g trypsin. The efficiency of the tryptic digestion was checked using SDS-PAGE followed by silver stain. Finally the protein digests were evaporated by



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vacuum centrifugation (Scanvac, DK) and dissolved in 100  $\mu$ L 15% acetonitrile containing 0.1% formic acid (Merck, DE) and 5 ng/mL internal standard (AQUA peptide mix, see below).

*LC-MS/MS analysis*

The peptide analysis was performed on a UPLC coupled to a Xevo TQ-S mass spectrometer (Waters). The extract (7.5  $\mu$ L) was injected on a Acquity C18 BEH UPLC column (2.1 x 100 mm, 1.7  $\mu$ m) and was separated using gradient elution with a stable flow of 600  $\mu$ L/minute. The gradient started with 95% A (milliQ water with 0.1% formic acid) and 5% B (acetonitrile with 0.1% formic acid) followed by a linear increase to 45% B which was achieved at 5.0 minutes. Subsequently, the gradient was linearly increased within 0.1 minutes to 100% B and maintained for 2 minutes. The system subsequently switched to 5% B, which was achieved at 7.1 minutes, after which the column equilibrated at 95% A for approximately 3 minutes. The column was maintained at 50°C during analysis, and the samples were kept at 10°C. Blank injections were run after each sample and the needle and tubing's were washed with strong wash 0.1% formic acid in methanol:H<sub>2</sub>O (8:2) and weak wash 0.25% trifluoroacetic acid in acetonitrile:H<sub>2</sub>O (4:6). The MS was operating in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150°C and a desolvation temperature of 600°C. Cone voltage and collision energy were optimized for each compound individually. The multiple reaction monitoring (MRM) transitions were determined from MS/MS spectra obtained by direct infusion of 0.5  $\mu$ g/mL peptide solution. Per peptide 4 transitions were chosen (Q3-1, Q3-2, Q3-3, and Q3-4) for quantitation and confirmation. The transitions for OATP1B1 are listed in Table 1. A peptide labeled with <sup>15</sup>N and <sup>13</sup>C (AQUA peptide) was synthesized (Sigma

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Aldrich Chemie, Steinheim DE) and used as an internal standard for quantification. Peak identification and quantification was performed using Masslynx software version 4.1.

*Transport and inhibition assays*

Transport and inhibition assays were performed at different labs, with minor differences. At TNO, cells were seeded at a density of  $4 \times 10^5$  cells/well on Poly-D-Lysine coated 24-wells plates (BD biosciences) and grown to confluence for 48 hours. All incubations were performed at 37°C. For initial characterization of the cell lines and for inhibition of [ $^3\text{H}$ ]-rosuvastatin uptake by selected oral antidiabetic drugs, we used the following method. Prior to the start of the experiment cells were pre-incubated for 15-30 minutes with pre-warmed incubation medium (145 mM NaCl, 3 mM KCl, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5 mM D-glucose, 5 mM HEPES (pH 7.4)), containing appropriate concentrations of the inhibitor where applicable. The uptake was initiated after removal of the pre-incubation medium and addition of incubation medium containing substrate (and inhibitor, if applicable) concentrations as described, spiked with [ $^3\text{H}$ ]-labeled substrate (~10 kBq/ $\mu\text{L}$ ). The uptake was terminated by washing the cells twice with 1 mL ice-cold incubation medium, followed by the addition of 0.5 ml 0.5 M NaOH, of which 0.4 mL of the cell lysate was transferred to a 20 mL plastic scintillation vial for radioactivity measurement by liquid scintillation counting (Perkin Elmer).

For the inhibition of [ $^3\text{H}$ ]-E<sub>2</sub>17 $\beta$ -G uptake by selected oral antidiabetic drugs, cells were seeded at a density of  $4 \times 10^5$  cells/well on Poly-D-Lysine coated 24-wells plates (BD biosciences) and grown to confluence for 48 hours. Prior to the start of the experiment cells were washed twice with pre-warmed HBSS/HEPES (pH 7.4) buffer, followed by the addition of HBSS/HEPES containing substrate (and inhibitor) concentrations as described, spiked with [ $^3\text{H}$ ]-labeled substrate (~10 kBq/ $\mu\text{L}$ , final concentration 1  $\mu\text{M}$ ). The uptake was

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terminated by washing the cells with 0.4 ml ice-cold HBSS/HEPES + 0.5% BSA and twice with 0.4 ml ice-cold HBSS/HEPES, followed by the addition of 0.2 mL 0.5% Triton X-100, of which 0.15 mL of the cell lysate was transferred to a 5 mL plastic scintillation vial for radioactivity measurement by liquid scintillation counting (Perkin Elmer).

Experiments performed in the laboratory of Janssen Pharmaceutical Companies of Johnson & Johnson (J&J) were carried out according to the above mentioned protocol, with three minor differences: cells were seeded at a density of  $4 \times 10^5$  cells/well on collagen coated 24-wells plates (BD biosciences) and grown to confluence for 24 hours, the uptake was terminated by washing the cells with 1.5 ml ice-cold HBSS/HEPES, followed by the addition of 0.2 mL mPER-lysis (Thermo Scientific).

#### *Data analysis*

Uptake into mock-transfected cells served as a control in all experiments to correct for uptake that was not related to OATP1B1-mediated transport. Concentration-dependent uptake of compounds is expressed as pmol/mg protein/minute. Inhibition of OATP1B1-mediated uptake is expressed as the % of uptake into HEK-OATP1B1 cells incubated with vehicle and plotted against the nominal inhibitor concentration. To determine  $K_m$  and  $V_{max}$  values for radiolabeled substrates, the Michaelis-Menten model was fitted to the corrected data. To estimate  $IC_{50}$  values, a one-site binding model was fitted to the data, assuming a Hill slope of -1. To investigate whether inhibitory potency was related to compound lipophilicity, logP values of sulfonylurea-type oral antidiabetics were calculated according to the algorithms described by Cheng et al. (Cheng et al., 2011) and were obtained from the Pubchem database (National Centre for Biotechnology Information, Bethesda, MD, USA). The calculated logP values were as follows [drug (xlogP)]: glyburide (4.8), glipizide (1.9), gliclazide (1.5), glimepiride (3.9) and tolbutamide (2.3).

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The two-sided unpaired Student's *t*-test was used to assess the statistical significance of differences between two sets of data. Results are presented as the means  $\pm$  S.D. Differences were considered to be statistically significant when  $P < 0.05$ .

## RESULTS

### *Cellular localization and absolute protein expression of recombinant OATP1B1 in HEK-OATP1B1 and HEK-OATP1B1\*15 cells*

Subcellular localization of the human OATP1B1 protein in transfected HEK-OATP1B1 and HEK-OATP1B1\*15 cells was investigated by immunocytochemical staining and fluorescence microscopy. Results clearly indicate that in the HEK-OATP1B1 and HEK-OATP1B1\*15 cells the transfected protein is mainly expressed at the cell membrane (Figure 1A and 1B). No staining was observed in the vector-transfected control cells (HEK-mock; Figure 1C). The absolute protein expression of OATP1B1 at the cell membrane of HEK-OATP1B1 and HEK-OATP1B1\*15 cells was determined using a LC-MS/MS method, based on the method previously described by Kamiie et al. (Kamiie et al., 2008). Importantly, the protein expression of OATP1B1 and OATP1B1\*15 at the outer cell membrane of both cell lines was comparable ( $9.8 \pm 2.3$  and  $8.5 \pm 1.9$  pmol OATP1B1/mg membrane protein, respectively; Figure 2), justifying direct comparison of these two cell lines without further corrections.

### *Functional characterization of the HEK-OATP1B1 and HEK-OATP1B1\*15 cells*

The uptake of the control substrate [ $^3\text{H}$ ]-E<sub>2</sub>17 $\beta$ -G by HEK293 cells stably expressing OATP1B1 or OATP1B1\*15 was determined in a time- and concentration-dependent manner. Uptake of [ $^3\text{H}$ ]-E<sub>2</sub>17 $\beta$ -G was significantly higher in the transfected cell lines compared to the control cells, and was linear up to 2 minutes (data not shown). Analyses of the kinetic parameters are shown in Figure 3A and 3B and revealed similar  $K_m$  values for the transport of [ $^3\text{H}$ ]-E<sub>2</sub>17 $\beta$ -G by OATP1B1 and OATP1B1\*15 ( $7.0 \pm 0.3$  and  $10.0 \pm 0.6$   $\mu\text{M}$ , respectively), whereas the  $V_{\text{max}}$  value for OATP1B1\*15 was decreased by more than 60%

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( $798 \pm 11$  and  $311 \pm 7.2$  pmol/mg protein/minute for OATP1B1 and OATP1B1\*15, respectively). These results are consistent with a previous report of Iwai et al. (Iwai et al., 2004).

Uptake of [ $^3\text{H}$ ]-rosuvastatin was significantly higher in HEK-OATP1B1 cells compared to HEK-MOCK, but was only minimal in HEK-OATP1B1\*15 cells (Figure 3C), as published before (Choi et al., 2011; Ho et al., 2006). Due to the small difference in rosuvastatin uptake between HEK-MOCK and HEK-OATP1B1\*15 cells, the  $K_m$  and  $V_{max}$  of OATP1B1\*15-mediated rosuvastatin transport could not be determined. The  $K_m$  for uptake of [ $^3\text{H}$ ]-rosuvastatin by OATP1B1 was  $13.1 \pm 0.43$   $\mu\text{M}$  with a  $V_{max}$  of  $202 \pm 2.1$  pmol/mg protein/min (Figure 3D).

Our results were independently verified by experiments performed at J&J laboratories using the same cell lines. Despite minor protocol differences (see Materials and Methods), the  $K_m$  and  $V_{max}$  values for the OATP1B1- and OATP1B1\*15 mediated transport of [ $^3\text{H}$ ]- $\text{E}_217\beta\text{-G}$  and [ $^3\text{H}$ ]-rosuvastatin were highly comparable between the two independent laboratories (results are summarized in Table 2), demonstrating the stability and robustness of the transfected cell lines.

Interestingly, inhibition of  $\text{E}_217\beta\text{-G}$  transport by known OATP1B1 inhibitors was comparable between HEK-OATP1B1 and HEK-OATP1B1\*15 cells, with  $\text{IC}_{50}$  values for cyclosporine A (1.64 and 1.25  $\mu\text{M}$ ) and rifampicin (3.49 and 2.19) for OATP1B1 and OATP1B1\*15, respectively (Supplemental Figure 1). The uptake of the lipid-lowering drug [ $^3\text{H}$ ]-rosuvastatin was also inhibited by cyclosporine A, with an  $\text{IC}_{50}$  value of 0.89  $\mu\text{M}$  (Supplemental Figure 1).

*Inhibition of OATP1B1 and OATP1B1\*15 by several oral anti-diabetics*

In a preliminary *in vitro* inhibition screen we investigated the inhibitory effects of a library of 640 FDA-approved drugs on the transport of [<sup>3</sup>H]-E<sub>2</sub>17β-G by OATP1B1 (data not shown), and identified a set of oral anti-diabetic drugs as (novel) inhibitors of this uptake transporter. We therefore selected these drugs and determined their inhibitory effect on the uptake of [<sup>3</sup>H]-E<sub>2</sub>17β-G and [<sup>3</sup>H]-rosuvastatin by OATP1B1 and OATP1B1\*15 (Figure 4 and 5). The oral anti-diabetic drugs can be grouped into three classes and the obtained IC<sub>50</sub> values are summarized in Table 3. OATP1B1-mediated transport of [<sup>3</sup>H]-E<sub>2</sub>17β-G was significantly inhibited by glyburide (IC<sub>50</sub> 2.99 μM), glimepiride (IC<sub>50</sub> 3.55 μM), troglitazone (IC<sub>50</sub> 2.50 μM), pioglitazone (IC<sub>50</sub> 5.09 μM) and glipizide (estimated IC<sub>50</sub> ~45.3 μM, with high 95% confidence interval). These compounds were also identified as significant inhibitors of OATP1B1-mediated transport of [<sup>3</sup>H]-rosuvastatin: glyburide (IC<sub>50</sub> 1.77 μM), glimepiride (IC<sub>50</sub> 3.62 μM), troglitazone (IC<sub>50</sub> 2.84 μM), pioglitazone (IC<sub>50</sub> 32.2 μM) and glipizide (IC<sub>50</sub> 110 μM). Gliclazide, tolbutamide and metformin did not significantly affect the uptake of [<sup>3</sup>H]-E<sub>2</sub>17β-G by OATP1B1 or OATP1B1\*15 up to the highest concentration tested (100 μM). The inhibitory effect of these three compounds on wild-type OATP1B1-mediated rosuvastatin uptake was tested up to higher concentrations (1000 μM). Also in these studies, metformin did not display any effect, whereas gliclazide and tolbutamide were only weak inhibitors (IC<sub>50</sub> of 580 and 368 μM, respectively). Interestingly, the IC<sub>50</sub> values for inhibition of [<sup>3</sup>H]-E<sub>2</sub>17β-G uptake by the several oral anti-diabetics were similar for OATP1B1 and its polymorphic variant OATP1B1\*15 (Figure 4, Table 2). Notably, at low concentrations gliclazide (< 1 μM) appeared to stimulate OATP1B1\*15-mediated transport of [<sup>3</sup>H]-E<sub>2</sub>17β-G. Drug-induced stimulation of OATP1B1 (and several other transporters) *in vitro* has been reported several times (Kindla et al., 2011; Roth et al., 2011), however the underlying molecular mechanism and clinical relevance has remained unclear.

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Within the class of sulfonylureas it appeared that increased lipophilicity of these anti diabetic drugs was associated with a more potent inhibition of OATP1B1-mediated transport of rosuvastatin (Figure 6).



## DISCUSSION

Rosuvastatin belongs to the class of statins, which are widely prescribed drugs that act by inhibiting HMG-CoA reductase resulting in reduced plasma concentrations of low-density lipoprotein cholesterol (McKenney et al., 2003). After oral dosage, the rather hydrophilic rosuvastatin is efficiently and rapidly taken up from the portal vein into hepatocytes, which is predominantly mediated by the uptake transporter OATP1B1 (Choi et al., 2011; Ho et al., 2006; Kitamura et al., 2008). Since statins lower cholesterol levels by inhibiting HMG-CoA reductase within hepatocytes, and the transport processes in hepatocytes are key drivers of the clearance of statins as well, it is clear that the process of hepatic uptake is crucial for both drug efficacy and toxicity. It has been estimated that hepatic elimination of rosuvastatin in humans accounts for approximately 70% of its total clearance (Martin et al., 2003). The clinical importance of OATP1B1 in the pharmacokinetics of rosuvastatin has been demonstrated by several studies showing that subjects carrying the polymorphic variant OATP1B1\*15 (Asn130Asp and Val174Ala), which has generally been associated with decreased transport activity, have more than 2-fold higher plasma levels of rosuvastatin (Choi et al., 2008; Lee et al., 2005; Pasanen et al., 2007). Due to its wide prescription, but also due to the combined prevalence of hypercholesterolemia and type-2 diabetes (e.g. in overweight/obese patients), rosuvastatin is often concomitantly prescribed with oral antidiabetic drugs. In this study we therefore investigated the possible drug-drug interaction between rosuvastatin and several oral antidiabetics *in vitro* by generating and characterizing HEK293 cells stably overexpressing OATP1B1 or its polymorphic variant OATP1B1\*15.

Immunohistochemical characterization of the HEK-OATP1B1 and HEK-OATP1B1\*15 cells demonstrated similar cellular surface expression patterns of the transfected proteins in both cell lines, as was previously reported (Iwai et al., 2004). The absolute protein expression levels of OATP1B1 in the outer cell membrane were determined

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by LCMS/MS. The importance of determining the absolute expression levels of transfected transport proteins in different cell lines has been demonstrated before by Iwai et al. (Iwai et al., 2004), who found that in their generated HEK293 transfected cell lines the protein expression of OATP1B1\*15 at the cell membrane was 11-times higher than the expression of wild-type OATP1B1 as measured by Western blotting. Without correction for this increased expression of OATP1B1\*15, the  $V_{\max}$  of E<sub>2</sub>17 $\beta$ -G uptake by OATP1B1\*15 was comparable to that of OATP1B1, whereas after correction for the increased expression the  $V_{\max}$  of OATP1B1\*15 was only 10% of that of wild-type OATP1B1. Importantly, in the current study similar absolute protein expression levels of OATP1B1 and OATP1B1\*15 at the cell surface membrane in both cell lines were measured, justifying direct comparison of these two cell lines. This direct comparison prevents overcorrection for increased expression levels, and we observed that the  $V_{\max}$  of E<sub>2</sub>17 $\beta$ -G uptake by OATP1B1\*15 was <40% of its wild-type variant, while the  $K_m$  values were comparable. Together, our results demonstrate that the reduced transport of E<sub>2</sub>17 $\beta$ -G by OATP1B1\*15 is most likely caused by a reduction in translocation ability, instead of a reduced intrinsic affinity or reduced protein expression in the cell membrane.

To assess whether the established IC<sub>50</sub> values in the current study are of clinical relevance, we compared the obtained IC<sub>50</sub> values with reported C<sub>max</sub> values of the studied oral antidiabetics after the maximum dose in patients. Since the concentration of orally taken drugs is the highest in the portal vein (resulting from intestinal absorption), we estimated the drug concentration in portal venous blood by the method described by Ito et al. (Ito et al., 1998). The C<sub>max</sub> in patients taking glyburide (1.25 mg), glipizide (5 mg), glimepiride (5 mg) or troglitazone (600 mg) has been reported at 0.77, 1.04, 0.73 and 6.4  $\mu$ M (Brunton et al., 2011; Spencer and Markham, 1997; Zheng et al., 2009), respectively, with predicted portal vein concentrations of 0.95, 1.88, 1.15 and 100  $\mu$ M. These concentrations are within the same

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(low) micromolar range as some of the  $IC_{50}$  values for OATP1B1-mediated uptake of rosuvastatin as measured in this study (glyburide: 1.77  $\mu\text{M}$ ; glimepiride: 3.62  $\mu\text{M}$ ) and even 35-times higher than the  $IC_{50}$  values for troglitazone (2.84  $\mu\text{M}$ ). This might suggest that these oral antidiabetic drugs can cause clinically relevant drug-drug interactions when concomitantly taken with rosuvastatin, or other OATP1B1 drug substrates. However, due the high plasma protein binding of these oral antidiabetics (>98%) (Brunton et al., 2011; Spencer and Markham, 1997; Zheng et al., 2009), the unbound concentration is expected to be only a fraction of the total plasma concentration and are mainly below the  $IC_{50}$  values observed in this study. Similar conclusions were drawn by Hirano et al (Hirano et al., 2006), who stated that although OATP1B1-mediated pitavastatin uptake was inhibited by glyburide *in vitro*, it was unlikely that - due to high plasma protein binding of glyburide - a drug-drug interaction occurs in the clinical stage. Importantly however, the plasma protein binding of the well-known OATP1B1 inhibitor cyclosporine A is high as well, with a predicted unbound portal vein concentration of cyclosporine A of  $\sim 0.5 \mu\text{M}$  ( $C_{\text{max}}$  1900 ng/ml at 400 mg dose with 98.6% plasma protein binding (Akhlaghi et al., 1997; Falck et al., 2008). Whereas this value is also below the determined  $IC_{50}$  value for inhibition of rosuvastatin uptake into HEK-OATP1B1 cells (1.64  $\mu\text{M}$ ), co-administration of rosuvastatin with cyclosporine A in the clinic resulted in 5- to 10-fold higher systemic exposure to rosuvastatin, which could mainly be explained by inhibition of OATP1B1-mediated hepatic uptake of rosuvastatin (Asberg, 2003; Neuvonen et al., 2006; Simonson et al., 2004). This indicates that a straightforward comparison of the unbound maximum plasma concentration of a drug with *in vitro* measured  $IC_{50}$  parameters alone might not always be predictive for potential drug-drug interactions *in vivo*. The clinical relevance of the drug-drug interactions between rosuvastatin and oral antidiabetic drugs therefore needs to be established, as there are no clinical trials present in the literature today. Results of the present study can well be used to setup an intelligent drug

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development strategy to predict the clinical relevance of drug-drug interactions, as the combination with physiologically based pharmacokinetic (PBPK) models enables a prediction of pharmacokinetic profiles of the (unbound) drug over time and whether drug-drug interactions are to be expected in patients. In addition to *in vitro* studies and PBPK modeling, preclinical animal studies (such as humanized mice) could also contribute to an efficient preclinical phase in drug development leading to an optimized clinical trial.

The frequency of the low-activity OATP1B1\*15 differs between various geographical populations, ranging from only 2% and 9% in Sub-Saharan African and South/Central Asian population, respectively, to 15-24% in populations from North Africa, Europe, Middle East and America (Pasanen et al., 2008). This rather high frequency of the OATP1B1\*15 haplotype in selected populations demonstrates the importance of studying its effect on the disposition of drugs. Importantly, the effect of the \*15 variation on drug-drug interaction at the level of OATP1B1 has never been systematically studied *in vitro* before. We found similar IC<sub>50</sub> values for inhibition of E<sub>2</sub>17β-G uptake into HEK-OATP1B1 and HEK-OATP1B1\*15 cells for the different studied oral antidiabetics and the known inhibitors cyclosporine A, rifampicin and pravastatin. This indicates that the intrinsic affinity of OATP1B1 for these inhibitors is not affected by the amino acid changes in the \*15 variant. Further studies are needed to explore whether this is a general finding, or whether it is dependent on the substrate/inhibitor combination.

It has previously been reported that the lipophilicity of compounds can determine their inhibition capacity, as was reported for the metabolic enzyme CYP3A4 (Lewis et al., 2006; Regev-Shoshani et al., 2004). We therefore explored whether this is also the case for inhibition of the transport protein OATP1B1, and indeed found that within the class of sulfonylureas an increased lipophilicity of the antidiabetic drugs was associated with an increased potency to inhibit OATP1B1-mediated transport of rosuvastatin.

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In summary, we generated and fully characterized HEK-OATP1B1 and HEK-OATP1B1\*15 cells and provide evidence for potentially important drug-drug interactions between rosuvastatin and selected sulfonylureas and thiazolidinediones. It remains to be established, however, whether the studied oral antidiabetics affect the clinical pharmacokinetic profile of rosuvastatin in patients. The *in vitro* studies as described can be considered as an important step in an intelligent pre-clinical test strategy to detect potential drug-drug interactions which need to be confirmed in subsequent (preclinical and/or clinical) studies to provide a decisive answer.

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## **AUTHORSHIP CONTRIBUTIONS**

Participated in research design:	Steeg, Greupink, Verhoeckx, Monshouwer, Vlaming, DeGroot, Verwei, Russel, Huisman, and Wortelboer
Conducted experiments:	Steeg, Schreurs, Nooijen, Verhoeckx, Hanemaaijer, and Ripken
Performed data analysis:	Steeg, Greupink, Schreurs, Nooijen, and Verhoeckx
Wrote the manuscript:	Steeg and Greupink
Contributed to the writing of the manuscript:	Verwei, Russel, Huisman and Wortelboer

## REFERENCES

Anonymous. (2012) Diabetes. WHO fact sheet 312. World Health Organization, Geneva, Switzerland. <http://www.who.int/mediacentre/factsheets/fs312/en/>

Akhlaghi F, McLachlan AJ, Keogh AM, Brown KF. (1997) Effect of simvastatin on cyclosporine unbound fraction and apparent blood clearance in heart transplant recipients. *Br J Clin Pharmacol* **44**:537-542.

Aquilante CL. (2010) Sulfonylurea pharmacogenomics in type 2 diabetes: The influence of drug target and diabetes risk polymorphisms. *Expert Rev Cardiovasc Ther* **8**:359-372.

Asberg A. (2003) Interactions between cyclosporin and lipid-lowering drugs: Implications for organ transplant recipients. *Drugs* **63**:367-378.

Bachmakov I, Glaeser H, Fromm MF, Konig J. (2008) Interaction of oral antidiabetic drugs with hepatic uptake transporters: Focus on organic anion transporting polypeptides and organic cation transporter 1. *Diabetes* **57**:1463-1469.

Bednarczyk D. (2010) Fluorescence-based assays for the assessment of drug interaction with the human transporters OATP1B1 and OATP1B3. *Anal Biochem* **405**:50-58.

Brunton LL, Chabner BA, Knollmann BC. (2011) Goodman & Gilman's the pharmacological basis of therapeutics.

Cheng F, Yu Y, Zhou Y, Shen Z, Xiao W, Liu G, Li W, Lee PW, Tang Y. (2011) Insights into molecular basis of cytochrome p450 inhibitory promiscuity of compounds. *J Chem Inf Model* **51**:2482-2495.



Choi JH, Lee MG, Cho JY, Lee JE, Kim KH, Park K. (2008) Influence of OATP1B1 genotype on the pharmacokinetics of rosuvastatin in Koreans. *Clin Pharmacol Ther* **83**:251-257.

Choi MK, Shin HJ, Choi YL, Deng JW, Shin JG, Song IS. (2011) Differential effect of genetic variants of Na<sup>+</sup>-taurocholate co-transporting polypeptide (NTCP) and organic anion-transporting polypeptide 1B1 (OATP1B1) on the uptake of HMG-CoA reductase inhibitors. *Xenobiotica* **41**:24-34.

Chung JY, Cho JY, Yu KS, Kim JR, Oh DS, Jung HR, Lim KS, Moon KH, Shin SG, Jang IJ. (2005) Effect of OATP1B1 (SLCO1B1) variant alleles on the pharmacokinetics of pitavastatin in healthy volunteers. *Clin Pharmacol Ther* **78**:342-350.

Fahrmayr C, Fromm MF, König J. (2010) Hepatic OATP and OCT uptake transporters: Their role for drug-drug interactions and pharmacogenetic aspects. *Drug Metab Rev* **42**:380-401.

Falck P, Vethe NT, Asberg A, Midtvedt K, Bergan S, Reubsæet JL, Holdaas H. (2008) Cinacalcet's effect on the pharmacokinetics of tacrolimus, cyclosporine and mycophenolate in renal transplant recipients. *Nephrol Dial Transplant* **23**:1048-1053.

Gui C, Wahlgren B, Lushington GH, Hagenbuch B. (2009) Identification, K<sub>i</sub> determination and CoMFA analysis of nuclear receptor ligands as competitive inhibitors of OATP1B1-mediated estradiol-17β-glucuronide transport. *Pharmacol Res* **60**:50-56.

Hagenbuch B and Meier PJ. (2004) Organic anion transporting polypeptides of the OATP/SLC21 family: Phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Physiol Rev* **84**:653-665.

DMD #49023

- Hirano M, Maeda K, Shitara Y, Sugiyama Y. (2006) Drug-drug interaction between pitavastatin and various drugs via OATP1B1. *Drug Metab Dispos* **34**:1229-1236.
- Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, Wang Y, Kim RB. (2006) Drug and bile acid transporters in rosuvastatin hepatic uptake: Function, expression, and pharmacogenetics. *Gastroenterology* **130**:1793-1806.
- Igel M, Arnold KA, Niemi M, Hofmann U, Schwab M, Lutjohann D, von BK, Eichelbaum M, Kivisto KT. (2006) Impact of the SLCO1B1 polymorphism on the pharmacokinetics and lipid-lowering efficacy of multiple-dose pravastatin. *Clin Pharmacol Ther* **79**:419-426.
- Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, Sugiyama Y. (1998) Prediction of pharmacokinetic alterations caused by drug-drug interactions: Metabolic interaction in the liver. *Pharmacol Rev* **50**:387-412.
- Iwai M, Suzuki H, Ieiri I, Otsubo K, Sugiyama Y. (2004) Functional analysis of single nucleotide polymorphisms of hepatic organic anion transporter OATP1B1 (OATP-C). *Pharmacogenetics* **14**:749-757.
- Kalliokoski A, Neuvonen PJ, Niemi M. (2010) SLCO1B1 polymorphism and oral antidiabetic drugs. *Basic Clin Pharmacol Toxicol* **107**:775-781.
- Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, Terasaki T. (2008) Quantitative atlas of membrane transporter proteins: Development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm Res* **25**:1469-1483.

DMD #49023

Kindla J, Muller F, Mieth M, Fromm MF, König J. (2011) Influence of non-steroidal anti-inflammatory drugs on organic anion transporting polypeptide (OATP) 1B1- and OATP1B3-mediated drug transport. *Drug Metab Dispos* **39**:1047-1053.

Kitamura S, Maeda K, Wang Y, Sugiyama Y. (2008) Involvement of multiple transporters in the hepatobiliary transport of rosuvastatin. *Drug Metab Dispos* **36**:2014-2023.

König J, Seithel A, Gradhand U, Fromm MF. (2006) Pharmacogenomics of human OATP transporters. *Naunyn Schmiedebergs Arch Pharmacol* **372**:432-443.

Lee E, Ryan S, Birmingham B, Zalikowski J, March R, Ambrose H, Moore R, Lee C, Chen Y, Schneck D. (2005) Rosuvastatin pharmacokinetics and pharmacogenetics in white and asian subjects residing in the same environment. *Clin Pharmacol Ther* **78**:330-341.

Lewis DF, Lake BG, Dickins M. (2006) Quantitative structure-activity relationships (QSars) in CYP3A4 inhibitors: The importance of lipophilic character and hydrogen bonding. *J Enzyme Inhib Med Chem* **21**:127-132.

Martin PD, Warwick MJ, Dane AL, Brindley C, Short T. (2003) Absolute oral bioavailability of rosuvastatin in healthy white adult male volunteers. *Clin Ther* **25**:2553-2563.

McKenney JM, Jones PH, Adamczyk MA, Cain VA, Bryzinski BS, Blasetto JW. (2003) Comparison of the efficacy of rosuvastatin versus atorvastatin, simvastatin, and pravastatin in achieving lipid goals: Results from the STELLAR trial. *Curr Med Res Opin* **19**:689-698.

Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B. (2009) Medical management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy: A consensus statement of the american diabetes association and the european association for the study of diabetes. *Diabetes Care* **32**:193-203.

DMD #49023

Neuvonen PJ, Niemi M, Backman JT. (2006) Drug interactions with lipid-lowering drugs: Mechanisms and clinical relevance. *Clin Pharmacol Ther* **80**:565-581.

Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, Eichelbaum M, Kivisto KT, Neuvonen PJ. (2005) Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. *Clin Pharmacol Ther* **77**:468-478.

Niemi M, Pasanen MK, Neuvonen PJ. (2011) Organic anion transporting polypeptide 1B1: A genetically polymorphic transporter of major importance for hepatic drug uptake. *Pharmacol Rev* **63**:157-181.

Niemi M, Schaeffeler E, Lang T, Fromm MF, Neuvonen M, Kyrklund C, Backman JT, Kerb R, Schwab M, Neuvonen PJ, Eichelbaum M, Kivisto KT. (2004) High plasma pravastatin concentrations are associated with single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide-C (OATP-C, SLCO1B1). *Pharmacogenetics* **14**:429-440.

Nozawa T, Sugiura S, Nakajima M, Goto A, Yokoi T, Nezu J, Tsuji A, Tamai I. (2004) Involvement of organic anion transporting polypeptides in the transport of troglitazone sulfate: Implications for understanding troglitazone hepatotoxicity. *Drug Metab Dispos* **32**:291-294.

Pasanen MK, Fredrikson H, Neuvonen PJ, Niemi M. (2007) Different effects of SLCO1B1 polymorphism on the pharmacokinetics of atorvastatin and rosuvastatin. *Clin Pharmacol Ther* **82**:726-733.

Pasanen MK, Neuvonen PJ, Niemi M. (2008) Global analysis of genetic variation in SLCO1B1. *Pharmacogenomics* **9**:19-33.

DMD #49023

Regev-Shoshani G, Shoseyov O, Kerem Z. (2004) Influence of lipophilicity on the interactions of hydroxy stilbenes with cytochrome P450 3A4. *Biochem Biophys Res Commun* **323**:668-673.

Roth M, Araya JJ, Timmermann BN, Hagenbuch B. (2011) Isolation of modulators of the liver-specific organic anion-transporting polypeptides (OATPs) 1B1 and 1B3 from *rollinia emarginata schlecht* (annonaceae). *J Pharmacol Exp Ther* **339**:624-632.

Simonson SG, Raza A, Martin PD, Mitchell PD, Jarcho JA, Brown CD, Windass AS, Schneck DW. (2004) Rosuvastatin pharmacokinetics in heart transplant recipients administered an antirejection regimen including cyclosporine. *Clin Pharmacol Ther* **76**:167-177.

Spencer CM and Markham A. (1997) Troglitazone. *Drugs* **54**:89-101.

Zheng HX, Huang Y, Frassetto LA, Benet LZ. (2009) Elucidating rifampin's inducing and inhibiting effects on glyburide pharmacokinetics and blood glucose in healthy volunteers: Unmasking the differential effects of enzyme induction and transporter inhibition for a drug and its primary metabolite. *Clin Pharmacol Ther* **85**:78-85.

## FOOTNOTES

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## FIGURES LEGENDS

**Figure 1.** Immunocytochemical staining of OATP1B1 in HEK-OATP1B1 (A), HEK-OATP1B1\*15 (B) and HEK-MOCK (C) cells. For detection the monoclonal primary OATP1B1 antibody (ESL clone) and a goat-anti-mouse Alexa488 secondary antibody were used. Note the similar membrane expression pattern in the HEK-OATP1B1 and HEK-OATP1B1\*15 cell lines.

**Figure 2.** Absolute plasma membrane protein expression levels of OATP1B1 in HEK-OATP1B1 and HEK-OATP1B1\*15 cells as measured by UPLC/MS/MS. No expression of OATP1B1 protein was detected in HEK-MOCK cells (data not shown). Data are presented as mean  $\pm$  SD (n=3).

**Figure 3.** OATP1B1- and OATP1B1\*15-mediated uptake of [ $^3$ H]-E<sub>2</sub>17 $\beta$ -G and [ $^3$ H]-rosuvastatin. **A** and **B**, concentration-dependent uptake of [ $^3$ H]-E<sub>2</sub>17 $\beta$ -G into HEK-MOCK, HEK-OATP1B1, and HEK-OATP1B1\*15 cells after 2 minutes incubation. **C**, uptake of 1  $\mu$ M [ $^3$ H]-rosuvastatin into HEK-MOCK, HEK-OATP1B1 and HEK-OATP1B1\*15 cells after 1 minute incubation. **D**, concentration-dependent uptake of [ $^3$ H]-rosuvastatin in HEK-MOCK and HEK-OATP1B1 cells after 1 minute incubation. The dotted line represents the nonlinear fit of the uptake into the transfected cells corrected for the uptake in the mock-transfected cells. The dotted line therefore represents the OATP1B1- or OATP1B1\*15-mediated uptake of [ $^3$ H]-E<sub>2</sub>17 $\beta$ -G or [ $^3$ H]-rosuvastatin. Data are presented as mean  $\pm$  SD (n=3; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  when compared with HEK-MOCK).

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**Figure 4.** Inhibition of [<sup>3</sup>H]-E<sub>2</sub>17β-G uptake (1 μM) in HEK-OATP1B1 (black closed circles) and HEK-OATP1B1\*15 (grey open circles) cells by glyburide (**A**), glipizide (**B**), gliclazide (**C**), glimepiride (**D**), tolbutamide (**E**), troglitazone (**F**), pioglitazone (**G**), and metformin (**H**). Results are presented as mean ± SEM of three independent experiments each performed in duplo).

**Figure 5.** Inhibition of [<sup>3</sup>H]-rosuvastatin (1 μM) uptake in HEK-OATP1B1 cells by glyburide (**A**), glipizide (**B**), gliclazide (**C**), glimepiride (**D**), tolbutamide (**E**), troglitazone (**F**), pioglitazone (**G**), and metformin (**H**). Results are presented as mean ± SD (n=3).

**Figure 6.** Relation between inhibition potency of selected oral antidiabetic drugs for OATP1B1-mediated rosuvastatin uptake (LogIC<sub>50</sub>) and the lipophilicity of these compounds (xlogP), r<sup>2</sup>=0.91, p < 0.05.



## TABLES

**Table 1.** Multiple reaction monitoring (MRM) transitions of the OATP1B1 peptide and the corresponding internal standard (AQUA). The peptide sequence is chosen according to the in silico peptide criteria defines by Kamiie et al. (Kamiie et al., 2008) and is exclusively present in OATP1B1.

Protein	Peptide sequence	MW	Q1	Q3-1	Q3-2	Q3-3	Q3-4
OATP1B1	LNTVGIK	814.7	408.5	588.3	487.3	702.4	388.3
OATP1B1-AQUA	LNTVGI[ <sup>13</sup> C <sup>15</sup> N]AK	822.0	411.8	595.4	494.3	709.4	395.3

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**Table 2.**  $K_m$  and  $V_{max}$  values for the transport of [ $^3H$ ]-E<sub>2</sub>17 $\beta$ -G and [ $^3H$ ]-rosuvastatin by OATP1B1 and OATP1B1\*15 as determined by independent laboratories.

Substrate	Cell line	Experiments performed by		Experiments performed by	
		TNO:		J&J:	
		$K_m$ ( $\mu M$ )	$V_{max}$ (pmol/mg protein/minute)	$K_m$ ( $\mu M$ )	$V_{max}$ (pmol/mg protein/minute)
[ $^3H$ ]-E <sub>2</sub> 17 $\beta$ -G	HEK-OATP1B1	7.0 $\pm$ 0.3	798 $\pm$ 11	8.8 $\pm$ 1.2	795 $\pm$ 39
	HEK-OATP1B1*15	10 $\pm$ 0.6	311 $\pm$ 7.2 (61% $\downarrow$ )	10 $\pm$ 1.5	203 $\pm$ 11 (74% $\downarrow$ )
[ $^3H$ ]-rosuvastatin	HEK-OATP1B1	13 $\pm$ 0.4	202 $\pm$ 2.1	12 $\pm$ 6.3	206 $\pm$ 34

Experiments were performed in triplicate and data are presented as mean  $\pm$  SD

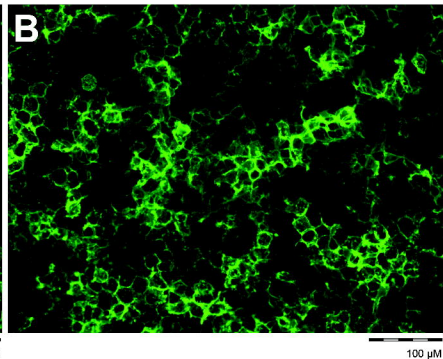
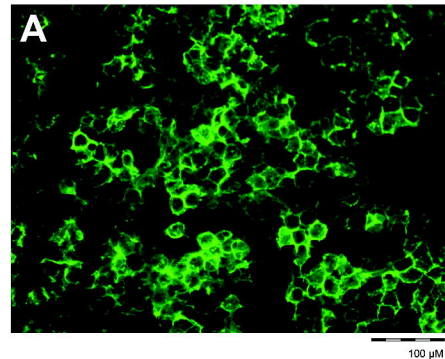
**Table 3.** Inhibition of human OATP1B1 and OATP1B1\*15 mediated transport of [<sup>3</sup>H]-E<sub>2</sub>17β-G and [<sup>3</sup>H]-rosuvastatin by selected oral anti-diabetic drugs

Class	Drug	Inhibition of [ <sup>3</sup> H]-E <sub>2</sub> 17β-G uptake				Inhibition of [ <sup>3</sup> H]-rosuvastatin uptake	
		OATP1B1		OATP1B1*15		OATP1B1	
		<i>IC</i> <sub>50</sub> (μM)	95% Confidence Intervals	<i>IC</i> <sub>50</sub> (μM)	95% Confidence Intervals	<i>IC</i> <sub>50</sub> (μM)	95% Confidence Intervals
Sulfonylurea	Glyburide	2.99	1.88 - 7.56	2.60	1.22 – 5.53	1.77	1.50 – 2.10
	Glipizide	~45.3	0.72 – 2845 <sup>a</sup>	~48.4	0.59 – 3948 <sup>a</sup>	110	54.4 - 224
	Gliclazide	>100	na	>100	na	580	335 - 1005
	Glimepiride	3.55	1.60 – 7.89	6.24	2.06 – 18.9	3.62	2.79 – 4.69
	Tolbutamide	>100	na	>100	na	368	168 - 806
Thiazolidinedione	Troglitazone	2.50	1.33 – 4.70	3.08	1.36 – 6.98	2.84	2.23 – 3.63
	Pioglitazone	5.09	1.89 – 13.7	5.32	1.34 – 21.2	32.2	17.4 – 59.8
Biguanide	Metformin	>100	na	>100	na	>1000	na

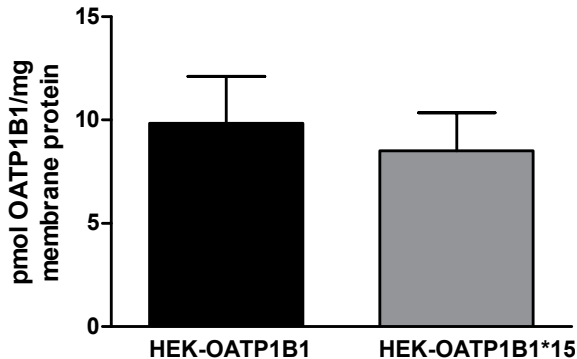
na, not applicable

<sup>a</sup> 95% confidence interval is very wide due to incomplete and non-sigmoidal fitting of the curve, *IC*<sub>50</sub> value is estimated (although >50% inhibition is observed at highest measured concentration of 100 μM)

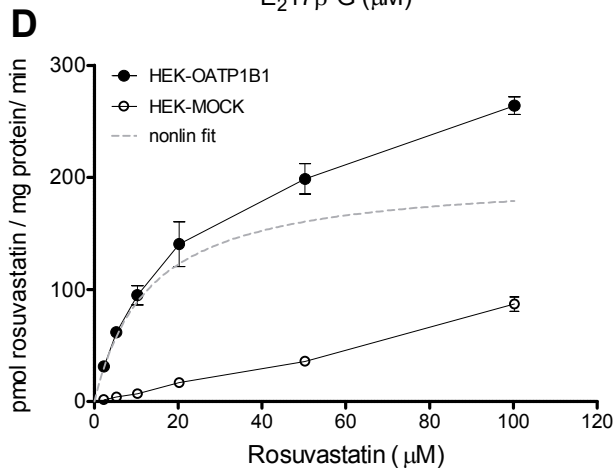
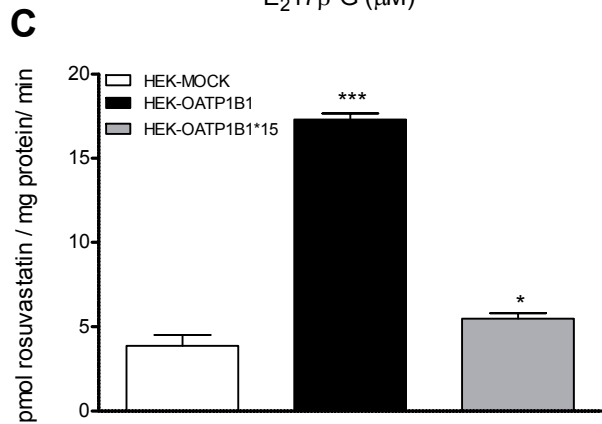
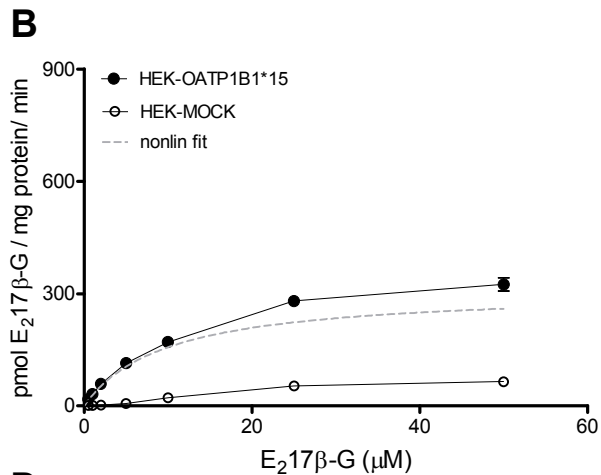
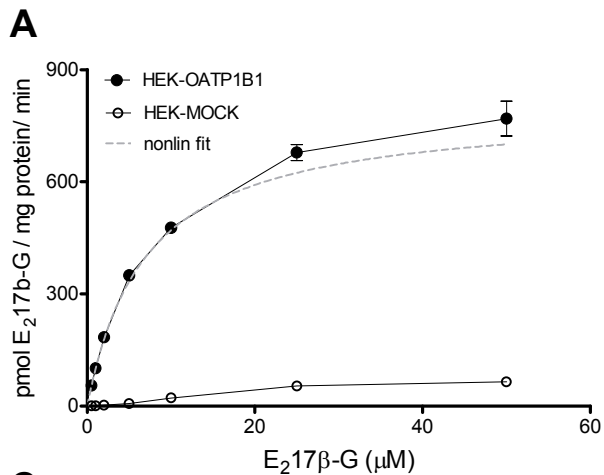
# Figure 1



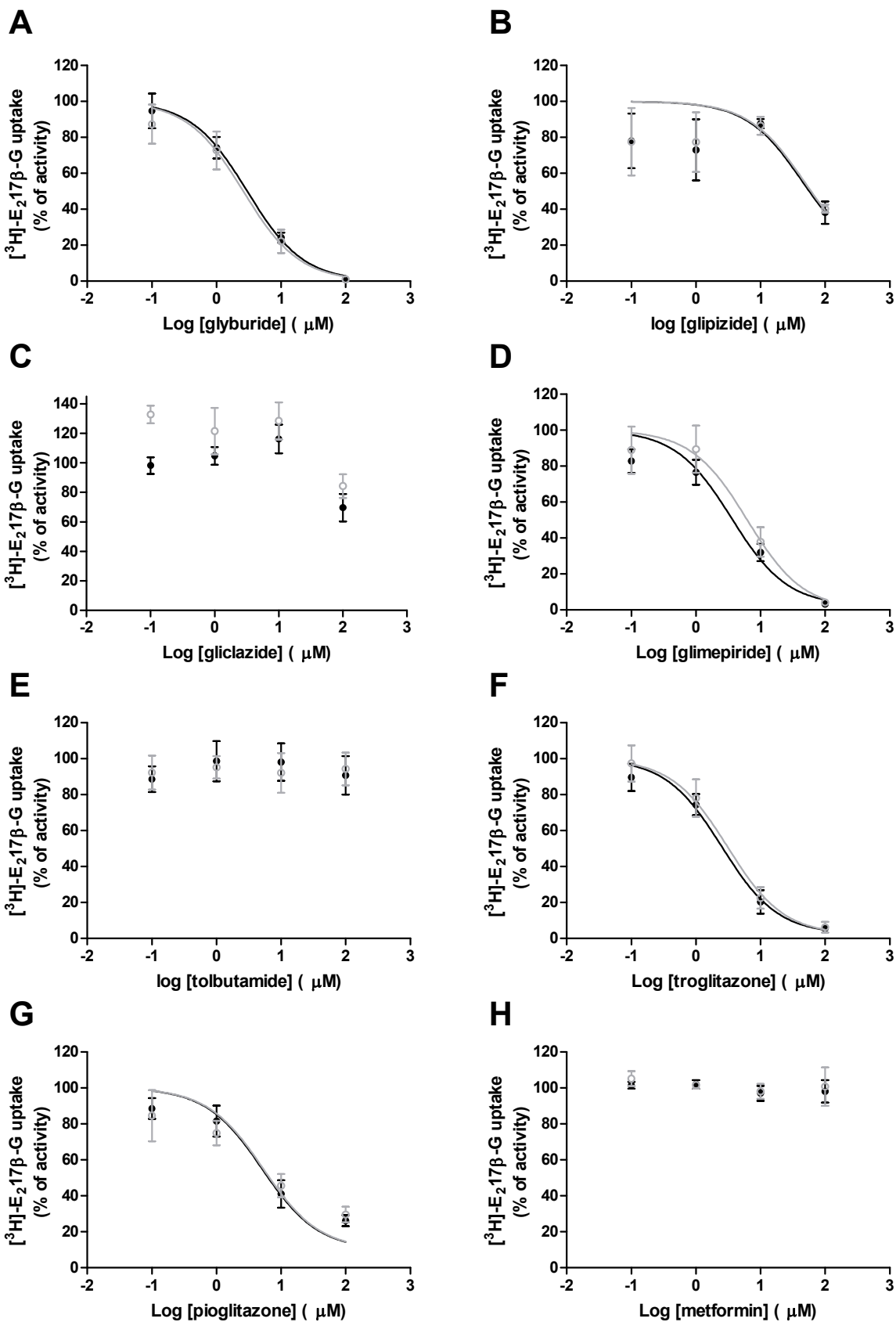
## Figure 2



**Figure 3**

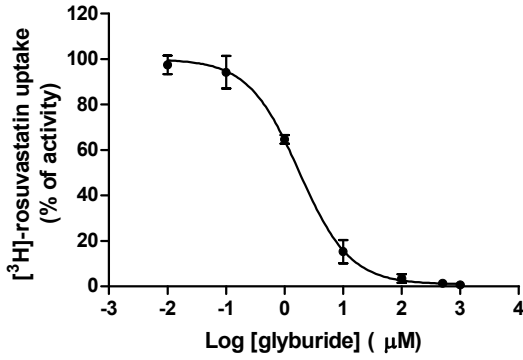


# Figure 4

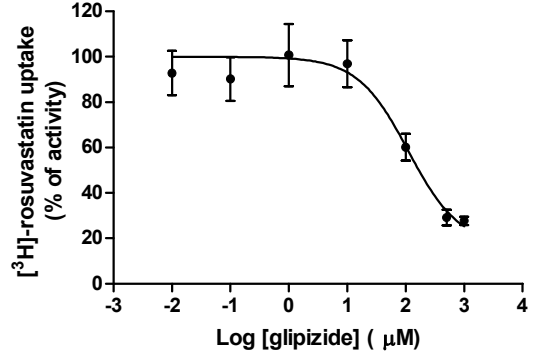


# Figure 5

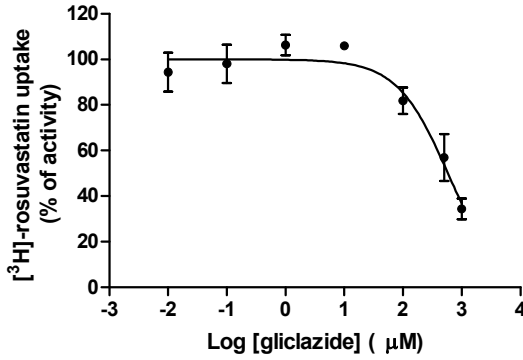
**A**



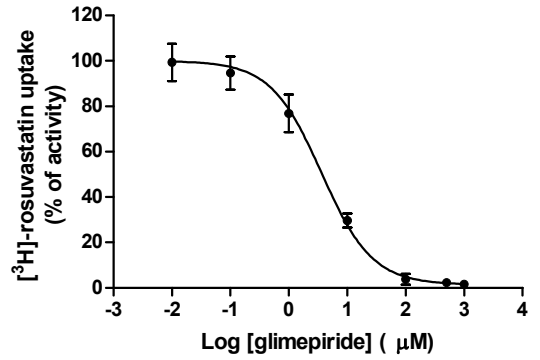
**B**



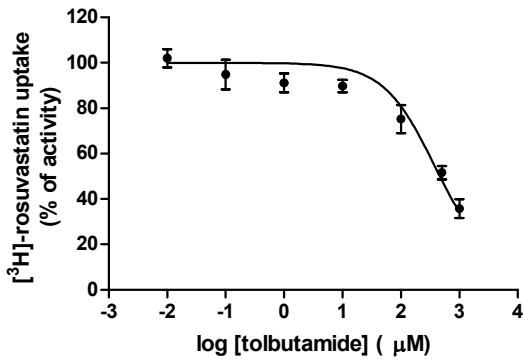
**C**



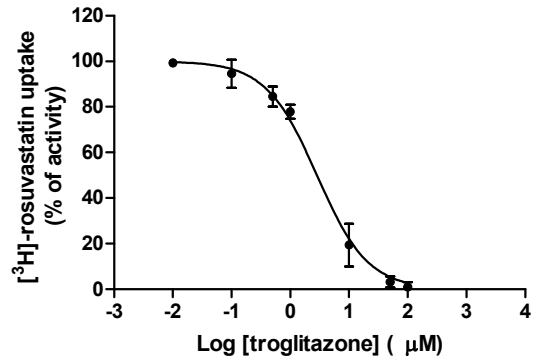
**D**



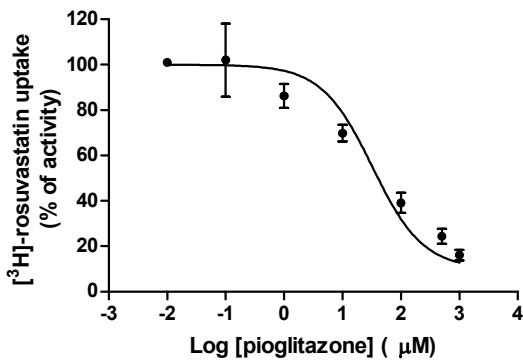
**E**



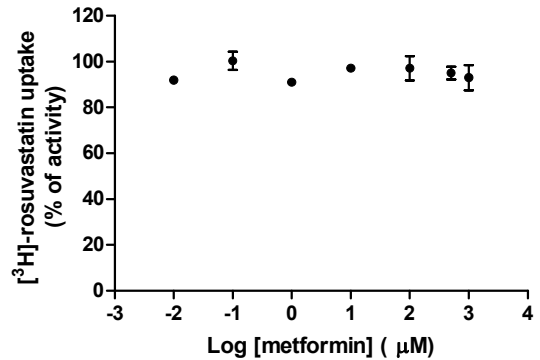
**F**



**G**



**H**





# Figure 6

