

Drug Metabolism and Pharmacokinetics (JFS, GDB, SC, AGC, DSW, MJR, JEH, JWP) and Clinical Pharmacology (EKH, RLOS, AK, WT, RLD), GlaxoSmithKline, Research Triangle Park, NC 27709
Running Title: Disposition and Drug Interaction Risk of Remogliflozin

* To whom correspondence should be addressed.

Joseph W. Polli, Ph.D.
Drug Metabolism and Pharmacokinetics
GlaxoSmithKline, Inc.
P.O. Box 13398
Room: N1.407
Research Triangle Park, NC 27709
Phone: (919) 483-3221
FAX: (919) 483-0443
Email: joseph.w.polli@gsk.com

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Nonstandard abbreviations:
remogliflozin etabonate = GSK189075 = KGT-1681 = 5-methyl-1-(1-methylethyl)-4-(4-[(1-methylethyl)oxy]phenyl)methyl)-1H-pyrazol-3-yl 6-O-[(ethyl oxy)carbonyl]-β-D-glucopyranoside;
remogliflozin = GSK189074 = KGT-1650 = 5-methyl-1-(1-methylethyl)-4-(4-[(1-methylethyl)oxy]phenyl)methyl)-1H-pyrazol-3-yl β-D-glucopyranoside;
GSK279782 = KGT-1142 = 5-methyl-4-(4-((1-methylethyl)oxy)phenyl)methyl)-1H-pyrazol-3-yl-β-D-glucopyranoside;
GSK333081 = KGT-2453 = 4-[(4-hydroxyphenyl)methyl]-5-methyl-1-(1-methylethyl)-1H-pyrazol-3-yl β-D-glucopyranoside;
GSK1997711 = 5-methyl-1-(1-methylethyl)-4-(4-[(1-methylethyl)oxy]phenyl)methyl)-1H-pyrazol-3-yl β-D-glucopyranosiduronic acid;
GSK1997714 = 5-methyl-1-(1-methylethyl)-4-(4-[(1-methylethyl)oxy]phenyl)methyl)-1H-pyrazol-3-yl 2-O-β-D-glucopyranuronosyl-β-D-glucopyranoside;
GSK355993 = 5-methyl-4-(4-[(1-methylethyl)oxy]phenyl)methyl)-1H-pyrazol-3-yl β-D-glucopyranosiduronic acid;
GSK1132678 ( remogliflozin aglycone) = 1,2-dihydro-5-methyl-4-[[4-(1-methylethoxy)phenyl]methyl]-1-(1-methylethyl)-3H-pyrazol-3-one

Pgp = P-glycoprotein; MDCK = Madin-Darby canine kidney cells; SGLT = sodium-dependent glucose co-transporter; UKPDS = United Kingdom Prospective Diabetes Study; SLC = solute carrier family; RE = remogliflozin etabonate; REM = remogliflozin
Abstract
Remogliflozin etabonate is the ester prodrug of remogliflozin, a selective sodium-dependent glucose co-transporter-2 inhibitor. This work investigated the absorption, metabolism, and excretion of [14C]remogliflozin etabonate in humans, and the influence of P-glycoprotein (Pgp) and cytochrome P450 (CYP) enzymes on the disposition of remogliflozin etabonate and its metabolites in order to understand the risks for drug interactions. After a single oral 402±1.0mg (106±0.3µCi) dose, [14C]remogliflozin etabonate is rapidly absorbed and extensively metabolized. The AUC(0-∞) of plasma radioactivity was approximately 14-fold higher than the sum of the AUC(0-∞) of remogliflozin etabonate, remogliflozin, and GSK279782, a pharmacologically active N-dealkylated metabolite. Elimination half lives of total radioactivity, remogliflozin etabonate and remogliflozin were 6.57, 0.39 and 1.57 h, respectively. Products of remogliflozin etabonate metabolism are eliminated primarily via renal excretion, with 92.8% of the dose recovered in the urine. Three glucuronide metabolites comprised the majority of the radioactivity in plasma and represent 67.1% of the dose in urine, with GSK1997711 representing 47.8% of the dose. In vitro studies demonstrated that remogliflozin etabonate and remogliflozin are Pgp substrates, and that CYP3A4 can form GSK279782 directly from remogliflozin. A ketoconazole clinical drug interaction study, along with the human mass balance findings, confirmed that CYP3A4 contributes less than 50% to remogliflozin metabolism, demonstrating that other enzyme pathways (e.g., CYPs, UGTs, glucosidases) make significant contributions to the drug’s clearance. Overall, these studies support a low clinical drug interaction risk for remogliflozin etabonate due to the availability of multiple biotransformation pathways.
Introduction

The human solute carrier 5 family comprises 12 genes, including members of the sodium-dependent glucose co-transporter (SGLT) family [Wright and Turk, 2004; Jabbour and Goldstein, 2008; Wright, 2001]. SGLT1 is a high-affinity, low-capacity glucose/galactose co-transporter primarily expressed in the intestine, but which is also present in the kidney [Bakris et al., 2009; Nishimura and Naito, 2005]. In contrast, SGLT2 is a low-affinity, high-capacity glucose transporter selectively expressed in the kidney. Together, SGLT1 and 2 are responsible for the active reabsorption of glucose across the renal luminal membrane [Wright and Turk, 2004; Idris and Donnelly, 2009]. Once reabsorbed by the renal epithelial cell, glucose is transported to the blood by facilitated diffusion via sodium-independent glucose transporters. The uptake of glucose in the proximal tubules by SGLT 1 and 2 is highly efficient, resulting in complete reabsorption. The importance of the SGLT transporters in intestinal and renal glucose absorption is evident from individuals with mutations in the SGLT genes. SGLT1 deficiency leads to glucose–galactose malabsorption syndrome, which results in severe, sometimes fatal diarrhea [Turk et al., 1991; Wright and Turk, 2004]. In contrast, genetic alterations in SGLT2 results in increased renal glucose excretion with no apparent adverse effects on carbohydrate metabolism [van den Heuvel et al., 2002; Washburn, 2009]. Therefore, based on the observation of glucosuria in subjects genetically deficient in SGLT2 activity, the SGLT2 transporter is currently a target of therapeutic interest for the treatment of diabetes and obesity [Bays, 2009; Papazafiropoulou et al., 2011; Washburn, 2012].

Remogliflozin etabonate (GSK189075; KGT-1681) is the prodrug of remogliflozin (GSK189074; KGT-1650), the active entity that inhibits SGLT2 (Figure 1) [Fujimori et al., 2008]. Remogliflozin etabonate causes a concentration dependent increase in urinary glucose excretion in humans and nonclinical species [Dobbins et al., 2012; Hussey et al., 2009]. Oral administration of remogliflozin etabonate reduces postprandial glucose excursions without inducing hypoglycemia, improves plasma glucose concentrations in subjects with diabetes, and reduces glycosylated hemoglobin (HbA1c) levels. SGLT2 inhibitors such as remogliflozin etabonate have the potential to be used in the treatment of T2DM as monotherapy and/or
in combination with existing therapies, and may also have potential efficacy as a weight loss agent [Papazafiropoulou et al., 2011]. This work investigated the absorption, metabolism, and excretion of $[^{14}\text{C}]$remogliflozin etabonate in humans and the influence of P-glycoprotein (Pgp) and cytochrome P450 (CYP) enzymes on the disposition of remogliflozin etabonate and its metabolites in order to understand the risks for drug interactions. These clinical and in vitro studies have characterized the role of metabolic enzymes and transporters involved in remogliflozin etabonate disposition, providing a mechanistic basis for predicting potential clinical drug interactions.

**Materials and Methods**

**Materials.** $[^{14}\text{C}]$Remogliflozin etabonate (25 µCi/mg) and $[^{14}\text{C}]$remogliflozin (113 µCi/mg) were prepared by Amersham Biosciences UK, Ltd (Buckinghamshire, England) with the $[^{14}\text{C}]$ label incorporated in the C3 position of the pyrazole ring (Figure 1). GlaxoSmithKline Chemical Development supplied remogliflozin etabonate, remogliflozin, GSK279782 (KGT-1142; pharmacologically active metabolite), GSK333081 (KGT-2453; metabolite), GSK1132678 (remogliflozin aglycone), GSK1997711 (glucuronide metabolite), GSK1997714 (glucuronide metabolite), and GSK355993 (glucuronide metabolite). Carbosorb E, Perma-Fluor E+, Ultima Flo M, Emulsifier-Safe, Ultima Gold, and Deepwell LumaPlate 96-well plates were obtained from PerkinElmer (Boston, MA). Pooled human liver microsomes, S9 and cytosol, and Cypex bactosomes containing individually expressed human CYPs, were obtained from Xenotech LLC (Lenexa, KS). Alamethicin, UDP-glucose dehydrogenase (type VI) from bovine liver and $\beta$-glucosidase from almond were obtained from Sigma-Aldrich (St. Louis, MO). Transwell 12-well multiwell inserts with polycarbonate membranes (0.4 µM pore size, 1.1 cm$^2$ surface area) were obtained from Corning (Cambridge, MA). All other chemicals and solvents were obtained from commercial suppliers and were analytical grade or higher.

**Clinical Study Designs.** All clinical studies were approved by their investigational review committees and were conducted according to principles of good clinical practice and the Declaration of Helsinki. All
subjects underwent an initial screening assessment following collection of written informed consent. The screening assessment was conducted within 28 days of the first dose and included a medical history, physical examination and medical and laboratory evaluations such as a 12-lead ECG, drug screen and pregnancy test (females). Exclusion criteria included a positive drug or alcohol test, past or current history of excessive alcohol or illicit drug use, recent participation in another research trial (i.e., within 30 days prior to screening), use of any prescription or non-prescription drugs, vitamins, herbal and dietary supplements or grapefruit-containing products within 14 days or 5 half-lives prior to the start of the dosing phase of the clinical study, regular use of tobacco- or nicotine-containing products within 3 months of screening visit, and any pre-existing conditions that would interfere with normal gastrointestinal anatomy or motility, hepatic and/or renal function.

The human mass balance clinical study was an open-label, single-dose study conducted with eight healthy, non-smoking males. The mean age was 42.1 years with a range of 31 to 54 years. All subjects completed the study. The in-life portion of this study was conducted at the Covance Clinical Research Unit, Inc. (Madison WI). The study protocol (KG2105264) was registered with the US NIH clinical trials database with identifier NCT00501683. Fasted subjects received 3 capsules by mouth that contained a total of 402 ± 1.0 mg of [14C]remogliflozin etabonate (106 ± 0.3 µCi; specific activity 0.27 µCi/mg) as a solution prepared in Acconon MC-8 (Abitec Corporation, Columbus, OH). Blood samples (7 ml) were collected into tubes containing potassium oxalate and sodium fluoride at times 0 (immediately pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36 and 48 h. A portion of whole blood (1 ml) was removed from each sample for the determination of blood radioactivity and the remainder was processed to provide plasma for pharmacokinetic analysis. Additional blood samples (40 ml each) were collected at 1, 4, 12 and 24 h post-dose and processed to provide plasma for metabolite profiling and structural identification. Urine was collected predose and at 0 to 6, 6 to 12, 12 to 24 h and at 24 h intervals through 144 h post dose. Urine was stored at 2 to 8°C during collection and subsequently frozen at -70°C or lower. Feces
were collected predose and at 24 h intervals through 144 h post dose and stored frozen at -70°C or lower. All eight subjects completed the study. Three AEs were reported in two subjects during the study: diarrhea, headache, and ecchymosis. No AEs were considered to be drug-related. All three AEs were mild in intensity and resolved within 2 to 10 days.

The ketoconazole drug interaction study was a single-center phase I study conducted at Ohio State University (Columbus, Ohio). The study protocol (KG2108197) was registered with the US NIH clinical trials database with identifier NCT00501397. A maximum of 22 healthy male and female subjects were to be enrolled to ensure completion of at least 16 evaluable subjects. This was an open-label, single-sequence study to investigate the effect of ketoconazole (repeat 400mg once daily for 6 days) on the pharmacokinetic profile of remogliflozin etabonate (single 250mg dose). The study consisted of 3 treatment sessions. Session 1 was a 24 h inpatient sampling period following a single dose of remogliflozin etabonate. Session 2 was a 4-day, outpatient period with 400mg oral ketoconazole administered once daily to achieve steady state plasma concentrations. Subjects returned to the clinic for Session 3 and received their daily dose of ketoconazole administered by study staff on Days 5 and 6. Session 3 was a 48h inpatient sampling period conducted after a single dose of remogliflozin etabonate administered with ketoconazole on Day 5. Subjects were to avoid food for at least 8 h before dosing for both remogliflozin etabonate and ketoconazole. Serial blood (2 mL) samples were collected into tubes containing potassium oxalate and sodium fluoride at times 0 (immediately pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36 and 48 h on Day 1 of Session 1 and on Days 5 and 6 of Session 3 for the determination of remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081 concentrations in plasma.

Of the 20 subjects enrolled in the ketoconazole study, 19 (95%) subjects completed. The mean age was 39 years with a range of 22 to 55 years. Seventeen subjects (85%) were male and 3 were female (15%). Only one adverse event, epistaxis, was reported during Session 1 when remogliflozin etabonate was
administered alone. All other adverse events occurred when ketoconazole was administered either alone (headache, nausea, eye pain) or in combination with remogliflozin etabonate (headache, nausea, vomiting, contusion, post-procedural complication, sinus congestion and furuncle). No serious adverse events were reported and no subject discontinued study medication or was withdrawn prematurely from the study as a result of an adverse event. No clinically meaningful treatment-related changes in mean chemistry or hematology values were observed during the study. No clinically relevant changes in ECG parameters were observed, and vital sign measurements were relatively stable.

**Quantification of Radioactivity.** Radioactivity in blood, plasma, urine and feces was quantified by liquid scintillation counting (LSC) by using a 2900TR liquid scintillation counter (Packard Instrument Company; Downers Grove, IL). Duplicate aliquots of plasma (0.3 ml) and triplicate aliquots of urine (1 ml) were mixed directly with Ultima Gold scintillation cocktail prior to analysis. Feces were mixed with 50% ethanol in de-ionized water (approximately 3:1 volume ethanol/water to wet weight feces) and homogenized. Duplicate aliquots of blood (0.3 ml) and triplicate aliquots of fecal homogenate (0.3 g) were combusted using a Model 307 Sample Oxidizer (Packard Instrument Company). The resulting $^{14}\text{CO}_2$ was trapped in Carbo-Sorb, mixed with Perma-Flour scintillation cocktail and analyzed. The total radioactivity in blood and plasma are reported as nanogram-equivalents remogliflozin etabonate per ml. Radioactivity in the urine and feces are reported as a percentage of the administered dose per time period. The percentage of radioactivity associated with blood cells was calculated according to the equation: 100 – [(Cp)(1-Hct)/(Cb) x 100] were Cp and Cb are the concentrations of radioactivity in plasma and blood, respectively, and Hct is the hematocrit. The blood:plasma ratio was calculated as Cb/Cp.

**Quantification of Remogliflozin Etabonate and Metabolites in Plasma.**

The plasma concentrations of remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081 were measured by using a validated LC-MS/MS method. Briefly, a 50 µl aliquot of plasma was added to 200 µl of acetonitrile containing [$^3\text{H}_2$]-labeled internal standards of each analyte and vortex mixed. The
samples were centrifuged at 1200 g for 5 min, and 150 µl of the supernatants were removed and dried under warm nitrogen. Samples were reconstituted in 75 µl of water containing 20% acetonitrile (v/v) and 0.1% formic acid (v/v), and 5 µl was injected onto an LC-MS/MS system consisting of a MDS Sciex 3000 (Applied Biosystems /MDS Sciex, Canada) equipped with a CTC HTS PAL autosampler (Leap Technologies Inc, Carrboro, NC), a Shimadzu 10AD solvent delivery system (Shimadzu Scientific Instruments Inc., Columbia, MD) and a Varian Polaris C-8-Ether column (50 x 2.0 mm, 3 µm, Varian Inc., Lake Forest CA). Mobile phase A consisted of 0.1% formic acid in water (v/v) and mobile phase B consisted of 0.1% formic acid in methanol. The gradient started at 40% B and increased linearly to 50% B from 0 to 0.6 min, to 60% B from 0.6 to 0.9 min, and to 90% B from 0.9 to 1.2 min. The gradient was held at 90% B from 1.2 to 1.8 min and returned to 40% B from 1.8 to 2.5 min. The analyses were performed at room temperature at a flow rate of 0.4 ml/min. LC-MS/MS analysis was conducted using a TurboIonSpray interface operated in the positive ion mode and a probe temperature of 450°C. The analytes were measured by multiple reaction monitoring (MRM) of the following [M+H]+ transitions: remogliflozin etabonate, m/z 523 → 289; remogliflozin, m/z 451 → 289; GSK279782, m/z 409 → 247; and GSK333081, m/z 409 → 247. The transitions monitored for the internal standards were seven mass units higher than those of the corresponding analytes. Data collection and integration were performed using Analyst software (Applied Biosystems/MDS Sciex, Canada). Quantification was based on analyte/internal standard peak area ratios and calculated using a weighted 1/x² linear regression model with SMS2000 (version 2.0, GlaxoSmithKline, Research Triangle Park, NC). The operating range of the assay was 1 to 1000 ng/ml for remogliflozin etabonate, GSK279782 and GSK333081, and 2 to 1000 ng/ml for remogliflozin.

Pharmacokinetic Analysis of Remogliflozin Etabonate and Metabolites in Plasma. The plasma pharmacokinetic parameters were estimated by noncompartmental methods with WinNonlin Version 4.1 (Pharsight, Mountain View, CA USA) using actual pharmacokinetic sampling times. The maximum
observed drug concentration (C_{max}) and the time of its occurrence (t_{max}) were taken directly from the concentration-time profile. The area under the concentration-time profile from zero time to infinity (AUC_{(0-\infty)}) was calculated using the linear up/logarithmic down trapezoidal method (AUC_{(0-\text{last})}) and extrapolation to infinite time by the addition of C_{last}/\lambda_z, where \lambda_z is the apparent terminal phase elimination rate constant estimated by linear regression of the logarithmically-transformed concentration data. A minimum of three terminal phase concentration values were used to estimate \lambda_z. Sampling of blood and plasma for total radioactivity and specific analytes was done through 48 h. Concentrations of radioactivity were below limits of quantitation (125 and 119 ng equiv/mL for blood and plasma respectively) by 36 h in all subjects. The mean %AUC extrapolated to infinity for total radioactivity in blood and plasma were 8\% and 10\% respectively (minimum=4 and 6\% and maximum=22 and 20\%). All the analytes determined by LC/MS had %AUC extrapolated to infinity that were less than 5\%.

**Statistical analyses.** Statistical analyses were performed with SAS (version 8.2, Cary, NC USA). The geometric mean and 95\% confidence intervals were calculated for each pharmacokinetic parameter, except t_{max} for which median and range were calculated. Estimation of the effect of ketoconazole on AUC_{(0-\infty)}, C_{max} and t_{1/2} of remogliflozin, GSK279782 and GSK333081; and AUC_{(0-t)}, C_{max} and t_{1/2} of remogliflozin etabonate was conducted with remogliflozin etabonate alone as the reference treatment. A mixed-effect model with loge (pharmacokinetic parameter) as the dependent variable, a fixed effect for treatment, and a random effect for subject was used to estimate treatment difference on the loge scale. The PROC MIXED from SAS® (Version 8.2, Cary, NC) was used to fit the model. The effect of co-administration of ketoconazole with remogliflozin etabonate was estimated by exponentiating the difference in least squares means [(remogliflozin etabonate +ketoconazole) - remogliflozin etabonate alone] and the corresponding 90\% CI to obtain the point estimate and CI for the ratio of geometric means. T_{max} for all analytes was analyzed nonparametrically, with point estimates and 90\% CIs for the median differences of T_{max} between treatments calculated using Hodges-Lehmann method [Hauschke et al., 1990].
Sample Preparation for Metabolite Profiling.

Plasma. Equal volumes of plasma were pooled from each subject by time point to produce representative 1, 4, 12 and 24 h post-dose samples. Pooled plasma samples (1 to 6 ml) were extracted by adding 1 volume of methanol, followed by 2 volumes of acetonitrile, vortex mixing after each addition. Samples were centrifuged at 2400 g for 5 minutes and the supernatants transferred to clean tubes. The pellets were re-extracted as above and the supernatants pooled with the initial extracts. Varying amounts of each extract (4.5 to 11.4 ml) were dried under nitrogen and reconstituted in 400 to 750 µl of water/100 mM ammonia acetate, pH 5.0/acetonitrile (8.5/1/0.5, v/v/v). The samples were centrifuged and portions of the supernatants (200 to 400 µl) of each extract profiled.

Urine. Urine was pooled proportionally by sample weight to produce a single 0-48 h sample for each subject. Pooled urine was centrifuged at 21000 g for 5 minutes and a portion (250 µl) of each supernatant was profiled.

Metabolite Profiling. Plasma and urine radiochromatograms were generated by using an HP-1100 HPLC system (Agilent, Santa Clara, CA) with a Pursuit C-18 column (4.6 mm x 250 mm, 5 µm, Varian, Palo Alto, CA). Mobile phase A consisted of water/100 mM ammonia acetate, pH 5.0/acetonitrile (8.5/1/0.5, v/v/v) and mobile phase B consisted of water/100 mM ammonia acetate, pH 5.0/acetonitrile (0.5/9/0.5, v/v/v). The gradient started at 0% B and increased linearly to 5% B from 0 to 20 min, to 6% B from 20 to 30 min, to 17% B from 30 to 60 min, to 18% B from 60 to 70 min, to 30% B from 70 to 95 min, to 45% B from 95 to 105 min, and to 80% B from 105 to 110 min. The gradient was held at 80% B for 5 minutes and returned to initial conditions at 115.1 min. The column was allowed to equilibrate for 15 min between injections. The analyses were performed at 45°C and a flow rate of 1 ml/min. On-line radiochemical detection was performed using a Flo One radio flow detector (Packard Instrument Company; Downers Grove, IL) and Ultima Flo M scintillation fluid at a flow rate of 3 ml/min. When
necessary, off-line radiochemical detection was performed by collecting column fractions into Deepwell LumaPlate 96-well plates. The plates were dried under a stream of nitrogen and analyzed on a TopCount NXT microplate scintillation counter (Perkin Elmer Life Sciences, Downers Grove, IL). Radiochemical profiles generated on-line were integrated by using Flo One for Windows software (v 3.65). The limit of detection for on-line analysis was set to 3 times background, and the lower limit of quantification was 500 cpm. For off-line analysis, TopCount data was imported into Excel and peak areas calculated. The limit of detection and quantification for off-line analysis was set to 2 times background. Profiling data was subsequently imported into Laura Radio HPLC software (LabLogic Systems, Inc, Sheffield, UK) for presentation purposes.

In vitro samples generated to determine the pathway of GSK1997711 formation were analyzed using on-line radiochemical detection in a similar fashion to plasma to urine analysis, except that the gradient started at 15% B and increased linearly to 80% from 0 to 40 min. The gradient was held at 80% B for 5 min and returned to initial conditions at 45.1 min. The column was allowed to equilibrate for 15 minutes between injections.

In vitro CYP phenotyping samples were analyzed using on-line radiochemical detection with an Agilent Zorbax Eclipse XDB-C8 column (150 x 150 mm, 5 µm). Mobile phase A consisted of 5 mM ammonium acetate in 5% acetonitrile (v/v) and 5 mM ammonium acetate in 95% acetonitrile (v/v). The gradient started at 0% B and increased linearly to 23% B from 0 to 50 min, to 33% from 50 to 57 min, and to 100% B from 57 to 60 min. The gradient was held at 100% B from 60 to 65 min and returned to initial conditions from 65 to 67 min. The column was allowed to equilibrate for 8 min between injections. The analyses were conducted at ambient temperature and a flow rate of 1 ml/min.

**Metabolite Identification.** [14C]Remogliflozin metabolites in plasma and urine were identified by mass spectrometric analysis of fractions collected after separation by using the HPLC method described for
metabolite profiling. Reconstructed radiochromatograms were used to link metabolites characterized from the LC eluent in 96 well plates to peaks in quantitative profiles. A Waters Q-TOF (Beverly, MA) with an Advion nanoelectrospray source operating in positive ion mode was used to collect MS and MS/MS data on quantifiable metabolites. Experimental parameters included a cone potential of 40V, source temperature of 90 °C, and argon collision gas. Collision energies were ramped during infusion and selected based on desired fragmentation. Leucine Enkephalin (~0.1-0.2 ug/mL) was used for full mass range acquisition accurate mass measurements, using known fragment ions as lock masses. The structural characterization of remogliflozin etabonate metabolites by nanoelectrospray mass spectral analysis was simplified by assessing MS/MS fragmentation and noting structural changes to the glucose residue, the pyrazole or the phenyl isopropyl ether group. A comparison of chromatographic retention time, accurate mass of the protonated molecular ion [M+H]^+ and MS/MS fragmentation of synthetic standards was used to characterize the majority of metabolites. The structural characterization of M13 was confirmed using a combination of 1H-NMR experiments (H-1D, TOCSY and NOESY), mass spectrometry (accurate mass and MS/MS), and comparison of the accurate mass of the protonated molecular ion [M+H]^+ and MS/MS fragmentation with GSK333081 (GSK data on file).

Identification of CYP Enzymes Involved in the Metabolism of Remogliflozin. A 1:1 blend of unlabeled and [14C]-labeled remogliflozin (50 µM) was incubated in duplicate with human liver microsomes (2 mg/ml) or Cypex bactosomes (150 pmol/ml) containing recombinantly expressed CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 or CYP3A4 for 2 h at 37°C. The reaction mixture consisted of phosphate buffer (50 mM, pH 7.4) and an NADPH regenerating system (0.44 mM β-NADP, 5.5 mM glucose-6-phosphate, and 1.2 units of glucose-6-phosphate dehydrogenase) in a final volume of 500 µL. The reactions were terminated by the addition of 500 µL of acetonitrile followed by centrifugation, and a 25 to 50 µL portion of each supernatant was profiled for metabolites as described in the “Metabolic Profiling” section. The rates of metabolite production by the expressed CYP enzymes
were normalized with respect to the content of the corresponding CYP in pooled human liver microsomes [Rodrigues, 1999]. To confirm the enzymes involved, inhibition studies were conducted using human liver microsomes as described previously in the presence or absence of the selective CYP inhibitors azamulin (CYP3A4, 1 µM) [Stratton et al., 2000; Streser et al., 2004], sulfaphenazole (CYP2C9, 10 µM) [Baldwin et al., 1995], quinidine (CYP2D6, 1 µM) [Otton et al., 1988], montelukast (CYP2C8, 1 µM) [Walsky et al., 2005], furafylline (CYP1A2, 1 µM) [Sesardic et al., 1990], and benzyl nirvanol (CYP2C19, 5 µM) [Suzuki et al., 2002]. Incubations containing azamulin and furafylline were preincubated at 37°C for 10 minutes in the presence of microsomes and the NADPH-regenerating system prior to the addition of [14C]remogliflozin. The remaining inhibitors were preincubated at 37°C for 5 minutes in the presence of microsomes and [14C]remogliflozin. All CYP phenotyping reactions were initiated by the addition of the NADPH-regenerating system.

Identification of the Pathway of GSK1997711 Formation. A role for UDP-glucose dehydrogenase in the direct conversion of remogliflozin to GSK1997711 through oxidation of the C6 hydroxyl group of the glucose group to glucuronic acid was investigated using human liver cytosol and purified UDP-glucose dehydrogenase. Human liver cytosol (1 mg/ml) and a 1:1 blend of unlabeled and [14C]-labeled remogliflozin (50 µM) were incubated in duplicate at 37°C, in a reaction mixture consisting of potassium phosphate buffer (100 mM, pH 7.4) and NAD+ (2 mM) in a final volume of 1 ml. Portions (300 µl) were removed at t = 0 and 1 h, added to an equal volume of acetonitrile, centrifuged and the supernatants profiled. Purified UDP-glucose dehydrogenase (3.8 units/ml) and a 1:1 blend of unlabeled and [14C]-labeled remogliflozin (5 µM) were incubated in duplicate at 37°C, in a reaction mixture consisting of Tris-HCl (50 mM, pH 8.7), DTT (5 mM) and NAD+ (2 mM) in a final volume of 1 ml. Control incubations were performed without NAD+. Portions (300 µl) were removed at various times up to 4 h, added to an equal volume of acetonitrile, centrifuged and the supernatant profiled.
The formation of GSK1997711 through glucuronidation of the aglycone of remogliflozin (GSK1132678) was investigated 1) by incubating [14C]remogliflozin with human liver S9 supplemented with glucuronidation reagents, and 2) by incubating [14C]remogliflozin with β-glucosidase to produce the aglycone, followed by incubation with human liver microsomes and glucuronidation reagents.

Incubations with liver S9 (1 mg/ml) and a 1:1 blend of unlabeled and [14C]-labeled remogliflozin (50 µM) were performed in duplicate at 37°C, in a reaction mixture consisting of potassium phosphate buffer (100 mM, pH 5.5 or 7.4), MgCl₂ (5 mM), alamethicin (0.025 mg/ml) and UDPGA (2 mM) in a volume of 1 ml. Portions (300 µL) were removed at various times up to 2 h, added to an equal volume of acetonitrile, centrifuged and the supernatants profiled. Incubations with purified β-glucosidase (5.25 units/ml) and 1:1 blend of unlabeled and [14C]-labeled remogliflozin (5 µM) were performed in duplicate at 37°C in potassium phosphate buffer (100 mM, pH 5.5) in a final volume of 1 ml. Portions (100 µl) were removed at various times up to 2 h, added to an equal volume of acetonitrile, and analyzed as above to confirm formation of the GSK1132678 aglycone. At the 2h time point, an additional portion (328 µl) was removed from each incubation and 40 µl of Tris-HCl (1M, pH 8.7) was added, followed by the addition (in a final volume of 400 µl) of pooled human liver microsomes (1 mg/ml), MgCl₂ (5 mM), alamethicin (0.025 mg/ml) and UDPGA (2 mM). Portions (100 µl) were removed at t = 0 and 1 h and added to an equal volume of acetonitrile, centrifuged and the supernatants profiled.

The activity of UDP-glucose dehydrogenase was confirmed spectrophotometrically, based on a previously described method [Ordman and Kirkwood, 1977] by monitoring the formation of NADH. The reaction mixture consisted of Tris-HCl (50 mM, pH 8.7) with UDP-glucose dehydrogenase (0.28 units/ml), UDP-glucose (1 mM), DTT (5 mM) and NAD+ (2 mM) in a final volume of 200 µl. Incubations were conducted in Polyfiltronics UVMax 96-well microplates (Whatman Inc., Piscataway, NJ) and the increase in absorbance at 340 nm was monitored using a Molecular Devices SpectraMax M2e plate reader (Sunnyvale, CA). Control incubations omitted either substrate or NAD+. 

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The glycolytic activity of subcellular fractions and β-glucosidase was confirmed spectrophotometrically, based on a previously described method [Berrin et al., 2002], by monitoring the cleavage of 4-nitrophenyl β-D-glucopyranoside. The reaction mixture consisted of potassium phosphate buffer (50 mM, pH 5.5) and purified enzyme (0.5 units/ml) or pooled human liver subcellular fractions (0.5 mg/ml) in a final volume of 200 µl. Incubations were conducted in Polyfiltronics UVMax 96-well microplates and the increase in absorbance at 400 nm was monitored using a Molecular Devices SpectraMax M2e platereader.

**Monolayer Efflux Studies.** The polarized Madin-Darby canine kidney (MDCKII) cells heterologously expressing human Pgp (MDCKII-MDR1 cell line) were used for the in vitro transport studies and were obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). Cell culture and transport studies were completed as previously described [Polli et al., 2008]. Stock solutions of [14C]remogliflozin etabonate, [14C]remogliflozin, GF120918 (a Pgp inhibitor), and [3H]amprenavir (Pgp substrate) were prepared in DMSO. For substrate assessment, donor solutions with and without GF120918 were prepared by diluting the stock solutions in transport medium (Dulbecco’s Modified Eagle Medium supplemented with L-glutamine, 25 mM HEPES, pyridoxine hydrochloride, 1% DMSO (v/v) but without sodium pyruvate, and phenol red). The transport of [14C]remogliflozin etabonate, [14C]remogliflozin and positive control substrate was measured in two directions (apical to basolateral [A→B] and basolateral to apical [B→A]). Drugs were quantified by LSC using a TriCarb T2900 liquid scintillation counter and Ultima Gold scintillation cocktail. For monolayer efflux studies, the flux of test articles and probe substrates was calculated using the following equation:

\[ J = \frac{V (dC/dt)}{A} \]

where \( J \) is the flux (nmol/cm²/h), \( V \) is the receptor volume (ml), \( C \) is the receiver drug concentration (nmol/ml), \( t \) is time in h, and \( A \) is the membrane surface area (cm²).
The passive permeability of test articles, positive control substrates and Lucifer yellow in the presence of GF120918 was determined using the following equation as described previously [Rautio et al., 2006]:

\[ P_{7.4} = - \left( \frac{V_D V_R}{(V_D + V_R)A t} \right) \ln \left\{ 1 - \frac{(V_D + V_R)C_R(t)}{(V_D C_D(t) + V_R C_R(t))} \right\} \times 10^7 \text{ nm/s} \]

where \( P_{7.4} \) is the permeability coefficient at pH 7.4, \( V_D, V_R \) are donor and receiver well volumes, respectively (ml), \( A \) is the membrane surface area (cm\(^2\)), \( t \) is the incubation time (seconds), \( C_R(t) \) is the measured concentration in the receiver well at time \( t \) (nmol/ml), \( C_D(t) \) is the measured concentration in the donor well at time \( t \) (nmol/ml).

**Results**

**Pharmacokinetics of Total Radioactivity, Remogliflozin Etabonate, Remogliflozin, GSK279782 and GSK333081 in Humans.** Eight, fasted healthy male subjects received a single oral dose of 400 mg of \([^{14}C]\)remogliflozin etabonate (mean dose of 402 ± 1.0 mg /106 ± 0.3 µCi). The pharmacokinetic parameters for total radioactivity, remogliflozin etabonate, remogliflozin, and GSK279782 (\(N\)-dealkylation; a pharmacologically active metabolite of remogliflozin) and GSK333081 (\(O\)-dealkylation; a metabolite of remogliflozin) are presented in Table 1 and the corresponding plasma concentration-time profiles are presented in Figure 2. Remogliflozin etabonate is rapidly absorbed (\( t_{max} 0.5 \) h; \( t_{1/2} 0.39 \) h) and converted to remogliflozin (\( t_{max} 0.64 \) h; \( t_{1/2} 1.57 \) h), followed by the formation of GSK279782 (\( t_{max} 1 \) h; \( t_{1/2} 2.68 \) h) and GSK333081 (\( t_{max} 1 \) h; \( t_{1/2} 2.84 \) h). Peak blood and plasma concentrations of total radioactivity occur at approximately 1 h post dose. The rank order of the \( C_{max} \) and \( AUC_{(0-\infty)} \) for the analytes from highest to lowest is total radioactivity >>> remogliflozin > GSK279782 >> GSK333081 >> remogliflozin etabonate (Table 1). The \( AUC_{(0-\infty)} \) of plasma radioactivity is approximately 14-fold higher than the sum of the \( AUC_{(0-\infty)} \) of remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081, indicating the presence of significant additional circulating metabolites. Total radioactivity is eliminated from blood and plasma with half-lives of 5.90 and 6.57 h, respectively, consistent with the metabolites following their own kinetics. The blood to plasma ratios for the total
radioactivity $C_{\text{max}}$ and $\text{AUC}(0-\infty)$ are 0.64 and 0.61, respectively, indicating that drug-related material does not partition extensively into blood cells (< 20%).

**Absorption and Excretion of Radioactivity.** The excretion of radioactivity in urine and feces is summarized in Table 2. The mean total recovery of radioactivity was $95.4 \pm 2.03\%$ through 144 h post-dose, with a mean of 92.8% recovered in the urine and 2.58% recovered in the feces. Elimination was rapid with 92.0% of the dose recovered in the urine within 48 h post-dose. These results indicate that there was essentially complete absorption of the $[^{14}\text{C}]$radiolabeled dose. However, the overall bioavailability of remogliflozin from remogliflozin etabonate is low, based on the observed extensive metabolism.

**Metabolite Profiles.** $[^{14}\text{C}]$Remogliflozin etabonate was stable in plasma and urine as determined by radiochemical profiles of control matrices spiked with the dosing solution. In contrast, $[^{14}\text{C}]$remogliflozin etabonate was extensively degraded in extracts of spiked fecal homogenates under the -70°C or lower storage conditions; therefore, individual fecal profiles were not determined. However, as >90% of the radiolabel was excreted in the urine, the absence of fecal profiling does not alter the understanding of remogliflozin etabonate metabolism.

**Plasma.** Metabolite profiles of plasma pooled across subjects at 1, 4, 12 and 24 h post dose are shown in Figure 3; note the different y-axis scales for each plot. Drug-related material was quantified only in 1 and 4 h samples as the amount of radioactivity upon fractionation of the 12 and 24 h samples was low (Table 3). The major component at all time points was GSK1997711 (glucuronidation of the remogliflozin aglycone GSK1132678), which represented ~ 50% of the circulating radioactivity at 1 and 4 h. GSK1997714 (direct glucuronidation of remogliflozin) and GSK355993 (glucuronidation of the GSK279782 aglycone) represented ~ 6 to 18% of the total circulating radioactivity at 1 and 4 h, while
remogliflozin and GSK279782 represented ~ 4 to 11%. Remogliflozin etabonate (~115 min) and GSK333081 (~71 min) are estimated to be <1% of the total circulating radioactivity.

**Urine.** Metabolite profiles of urine pools collected from 0 to 48 h were similar to 1 and 4 h plasma profiles (Figure 4). The most abundant metabolite in urine was GSK1997711, which represented 47.8% of the administered dose (Table 4). M13 (glucuronidation of the GSK333081 aglycone), GSK355993 and GSK1997714 each represented ~ 6 to 13% of the dose. Remogliflozin and GSK279782 represented 7.9 and 3.4% of the dose, respectively. GSK333081 was also identified in urine but was below quantifiable limits. Two uncharacterized metabolites, representing <1% of the dose each, were also present. The total recovery of radioactivity was 95.4%.

**Identification of the Pathway of GSK1997711 Formation.** GSK1997711, the glucuronide conjugate of the remogliflozin aglycone is the major circulating human metabolite, representing ~50% of the total circulating radioactivity. GSK1997711 could potentially be formed either directly from oxidation of the C6 hydroxyl of the glucose group by UDP-glucose dehydrogenase, or indirectly through glucuronidation of the remogliflozin aglycone, GSK1132678 (Figure 1). In vitro studies demonstrated that GSK1997711 is produced only after [14C]remogliflozin is first incubated with β-glucosidase to form GSK1132678 prior to incubation with human liver microsomes supplemented with glucuronidation reagents (Figure 5). In contrast, GSK1997711 is not formed directly from [14C]remogliflozin when incubated with either purified UDP-glucose dehydrogenase or liver fractions (S9, cytosol), even though the activity of UDP-glucose dehydrogenase was confirmed spectrophotometrically by monitoring the formation of NADH [Ordman and Kirkwood, 1977]

**In Vitro Pgp Substrate Assays.** To determine whether [14C]remogliflozin etabonate or [14C]remogliflozin are substrates for human Pgp, in vitro bidirectional transport across MDCKII monolayers expressing Pgp was studied. In addition, the passive permeability was determined by
inclusion of GF120918, a potent Pgp inhibitor. The efflux ratios for $[^{14}C]$remogliflozin etabonate and remogliflozin across the MDCKII-MDR1 monolayers are 76 and 17, respectively, and decreased to 0.88 and 0.98 in the presence of GF120918 (Table 5), consistent with these compounds being substrates for Pgp. The in vitro passive membrane permeability values of $[^{14}C]$remogliflozin etabonate and remogliflozin are 286 and 19 nm/s ($P_{7.4}$ B→A + GF120918), respectively.

Identification of CYP Enzymes Involved in the Metabolism of Remogliflozin. To further understand the metabolism of remogliflozin and to assess the potential for drug interactions, a CYP phenotyping study was completed. Remogliflozin etabonate was not studied as it is unstable in human liver microsomes, in the presence or absence of NADPH as the prodrug is hydrolyzed readily by esterases under these conditions (half-life <10 min). In contrast, remogliflozin is much more stable in human liver microsomes than remogliflozin etabonate. Incubation of 50 µM $[^{14}C]$remogliflozin with human liver microsomes resulted in the formation of GSK279782, GSK333081 and two oxidative metabolites, M20 and a metabolite (mw 466) where the location of the oxidation is most likely on a methyl group from either of the isopropyl moieties (Figure 6). Formation of these metabolites is substantially impaired upon co-incubation with azamulin, a selective CYP3A4 inhibitor [Stresser et al., 2004]. These four metabolites are also formed when $[^{14}C]$remogliflozin is incubated with human recombinant CYP3A4, CYP2C9 or 2C19. Unlike azamulin, which extensively inhibited the formation of the four metabolites, sulfaphenazole (a selective CYP2C9 inhibitor) and benzynirvanol (a selective CYP2C19 inhibitor) [Suzuki et al., 2002; Baldwin et al., 1995] only significantly inhibited the formation of the M20 oxidative metabolite, suggesting that CYP3A4 was the major CYP contributing to the metabolism of remogliflozin.

Effect of Ketoconazole on the Pharmacokinetics of Remogliflozin Etabonate, Remogliflozin and GSK279782. To determine the quantitative role of CYP3A4 in the metabolism of remogliflozin, a clinical drug interaction study with ketoconazole was completed. Pharmacokinetic parameter estimates for remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081 in the absence and presence of
ketoconazole are summarized in Table 6. The mean plasma exposure to remogliflozin etabonate and remogliflozin increased modestly upon co-administration with ketoconazole, yielding increases of 24%, 30% and 33% in the $C_{\text{max}}$, $\text{AUC}_{(0-\infty)}$ and $t_{1/2}$ estimates of remogliflozin etabonate, and 32%, 75% and 53% in $C_{\text{max}}$, $\text{AUC}_{(0-\infty)}$ and $t_{1/2}$ estimates of remogliflozin. In contrast, the plasma exposure to the metabolites, decreased slightly for GSK279782 and substantially for GSK333081, resulting in 28% and 16% reductions in $C_{\text{max}}$ and $\text{AUC}_{(0-\infty)}$ of GSK279782, and 71% and 66% reductions in $C_{\text{max}}$ and $\text{AUC}_{(0-\infty)}$ of GSK333081. The metabolite-to parent $\text{AUC}_{(0-\infty)}$, ratio ($\text{AUC}_{\text{ratio, m/p}}$) for remogliflozin to remogliflozin etabonate increased by about 33% after remogliflozin etabonate is co-administered with ketoconazole. In contrast, the mean $\text{AUC}_{\text{ratio, m/p}}$ for GSK279782 to remogliflozin decreased by 52%, while that for GSK333081 decreased by 81%.
Discussion

SGLT2 inhibitors are a new class of potential anti-diabetes drugs [Idris and Donnelly, 2009; Asano et al., 2004; Bays, 2009; Isaji, 2007]. Remogliflozin etabonate, the prodrug of remogliflozin, is a novel member of the β-D-glucopyranoside class of SGLT2 inhibitors. Like other members of this class, remogliflozin was developed as a prodrug to enhance oral absorption and reduce the susceptibility to glycosidase degradation [Washburn, 2009; Ellsworth et al., 2008]. Remogliflozin is the active entity with an in vitro IC₅₀ value of 12.4 nM [Fujimori et al., 2008b]. To better understand the absorption, metabolism and disposition of remogliflozin etabonate and risk for drug interactions, a series of in vitro and clinical studies were completed. The disposition of remogliflozin etabonate in humans was characterized in a mass balance study where eight healthy male volunteers each received a single 400mg oral dose of [¹⁴C]remogliflozin etabonate. In addition, the importance of CYP enzymes and Pgp transport in remogliflozin etabonate and remogliflozin disposition were determined through in vitro studies and a ketoconazole clinical drug interaction study.

Remogliflozin etabonate is rapidly absorbed and metabolized to remogliflozin and a number of metabolites after oral dosing, with a median Tₘ₉₈ ≤ 1 hr for both remogliflozin etabonate and remogliflozin. Elimination of remogliflozin etabonate and remogliflozin from blood is rapid, with half-lives of 0.39 and 1.57 h, respectively. GSK279782 and GSK333081 are two metabolites in blood formed by N- and O-dealkylation of remogliflozin, respectively, and have slightly longer half-lives than remogliflozin (Table 1). The AUC₀-∞ of plasma radioactivity is approximately 14-fold higher than the sum of the mean AUC₀-∞ of remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081. Additionally, the half-life of total radioactivity is longer (6.57 h) than the half-lives of these analytes. These data are consistent with the presence of significant additional circulating metabolites. Three glucuronide metabolites, GSK355993, GSK1997711 and GSK1997714, comprised the majority of the circulating radioactivity detected in plasma, with GSK1997711 being the most abundant (48.9% of the radioactivity).
The products of remogliflozin metabolism are eliminated primarily via renal excretion, with 92.8% of the dose recovered in the urine. Similar to plasma, the glucuronide conjugates comprised the majority of the metabolites identified in urine, with GSK1997711 accounting for the majority (47.8%) of the dose. The other glucuronide metabolites each accounted for 6 to 13% of the dose. Remogliflozin, GSK279782 and GSK333081 each accounted for 8% or less of the dose in urine. An overall view of remogliflozin etabonate absorption, disposition and metabolism is summarized in Figure 7.

The assessment of drug interactions is an essential activity in drug development. Results from the human mass balance study, along with in vitro and other clinical information, provide detailed mechanistic insights into the metabolic and transporter pathways involved in the drug’s disposition. In vitro studies demonstrated that remogliflozin etabonate and remogliflozin are Pgp substrates with high and low/moderate permeability, respectively. Based on these data, the potential for drug interactions of remogliflozin etabonate co-administered with Pgp inhibitors was also considered. The human mass balance demonstrated that following oral dosing in humans, the remogliflozin etabonate dose underwent complete oral absorption (>90%). This observation provides strong clinical evidence that efflux transporters such as Pgp and BCRP do not limit the intestinal absorption of remogliflozin etabonate, remogliflozin or metabolites; this is consistent with remogliflozin etabonate being a Biopharmaceutics Drug Disposition Classification System (BDDCS) class 1 drug displaying high permeability and solubility (GSK data on file; [Benet et al., 2011]), and remogliflozin being a BDDCS class III compound, having low permeability and high solubility. However, remogliflozin has demonstrated low central nervous system penetration in the rat, which may be related to Pgp efflux at the blood brain barrier, its low passive permeability, or other mechanisms [Polli et al., 2012]. This is not a concern in humans, as the potential for drug interactions with Pgp inhibitors at the blood brain barrier is very small [Eyal et al., 2009].
In vitro studies suggest that the formation of GSK279782 and GSK333081 appears to be mediated primarily by CYP3A4, as demonstrated by incubations of remogliflozin with human liver microsomes and recombinantly expressed CYPs. This finding has been confirmed in a clinical study with ketoconazole, which showed a reduction in the AUC\(_{(0-\infty)}\) of GSK333081 (-66%) and a modest increase in remogliflozin exposure (75%), indicating that CYP3A4 is only a minor metabolic pathway; the fraction of remogliflozin metabolized by CYP3A4 is less than 50%. In contrast, only a small reduction in AUC\(_{(0-\infty)}\) of GSK279782 (-16%) was observed, suggesting an alternative enzyme(s) can produce this metabolite in vivo. In support of this observation, both CYP2C9 and 2C19 were demonstrated to produce GSK279782 through in vitro CYP phenotyping studies (Figure 6). Overall, the in vitro CYP phenotyping, human mass balance, and clinical ketoconazole data suggest that the risk of drug interactions with CYP inhibitors is low due to the multiple CYP and non-CYP pathways for remogliflozin clearance. The modest changes in remogliflozin exposure is not clinically significant, as remogliflozin etabonate has a large therapeutic window as demonstrated in clinical studies up to 1000 mg BID per day [Kapur et al., 2009; Dobbins et al., 2012]. Therefore, these data suggest that remogliflozin etabonate can be administered with CYP inhibitors without dose adjustments.

GSK1997711, GSK355993 and M13 are glucuronide conjugates of the remogliflozin, GSK279782 and GSK333081 aglycones, respectively. Theoretically, these metabolites could be formed directly through the oxidation of the C6 hydroxyl of the glucose group or indirectly through glucuronidation of the aglycone. Glucuronic acid is generated intracellularly by UDP-glucose dehydrogenase, utilizing UDP glucose as a substrate. A possible role for UDP-glucose dehydrogenase in the direct formation of GSK1997711 was investigated by incubating \[^{14}\text{C}\]remogliflozin with purified UDP-glucose dehydrogenase, as well as with liver fractions. These studies demonstrated that GSK1997711 was not formed under these conditions by UDP-glucose dehydrogenase. However, when \[^{14}\text{C}\]remogliflozin was first incubated with \(\beta\)-glucosidase to produce the aglycone, followed by incubation with human liver microsomes supplemented with glucuronidation reagents, GSK1997711 was readily formed (Figure 5,
Panel B). These results suggest that GSK1997711, as well as GSK355993 and M13, are formed indirectly through glucuronidation of the aglycone. From a clinical drug interaction perspective, the risk of inhibition of remogliflozin and GSK279782 conversion to their respective aglycones via glucosidase inhibition appears low, as these enzymes are abundant in a number of tissues, including the intestine and liver [Day et al., 2000; Day et al., 1998].

As previously discussed, remogliflozin etabonate is rapidly absorbed and extensively metabolized in humans. It is also important to understand how the metabolism in humans compares with the nonclinical species used for safety evaluation. Overall, the biotransformation pathways were similar in the nonclinical species with some qualitative differences (GSK data on file). In mice and rats, remogliflozin etabonate was rapidly adsorbed and converted to remogliflozin and GSK279782, while in dogs, remogliflozin along with remogliflozin etabonate are the predominant circulating active components. GSK279872 plasma concentrations are low in the dog, while GSK333081 plasma concentrations are low in both the dog and rodents. A number of glucuronide conjugates have been identified in the nonclinical species, including M13, GSK355993 and GSK1997711. GSK1997714 was not detected in either the rat or dog, and was present at only low levels in mouse bile (<1% of the dose); however several glucoside–glucuronide conjugates that are positional isomers of GSK1997714 were characterized in the mouse and dog. Finally, the remogliflozin aglycone (GSK1132678), which is the step prior to formation of GSK1997711, is only readily quantifiable in dog microsomal incubations, plasma and feces (GSK data on file).

Remogliflozin and GSK279782 are both potent SGLT2 inhibitors (in vitro Ki values ~ 7 to 12 nM) and account for the majority of the pharmacological activity in vivo. In contrast, GSK333081 has an in vitro potency 3-fold lower (Ki of ~30 nM) and a 12- and 3-fold lower in vivo exposure (AUC(0-∞)) than that of remogliflozin and GSK279782; thus, it is not expected that GSK333081 contributes significantly to the pharmacological activity. The prodrug, remogliflozin etabonate, does not have any pharmacological
activity and represents <2% of the remogliflozin exposure. The glucuronide metabolites, GSK199771, GSK1997714 and GSK355993, do not inhibit the SGLT1 or 2 transporters (GSK unpublished data).

In summary, following a single dose of [14C]remogliflozin etabonate, the radioactivity is rapidly and highly absorbed following a single oral dose to humans, and dose recovery in urine is nearly complete. Remogliflozin etabonate is rapidly and extensively metabolized to remogliflozin, GSK279782 and GSK333081, which in turn are converted to their respective aglycones. The aglycones are conjugated with glucuronic acid and comprise the majority of drug-related material in both the plasma and urine. These data suggest that remogliflozin etabonate has a low risk of drug interactions due to the multiple metabolic clearance pathways that include both CYP and non-CYP pathways.
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Authorship Contributions

Participated in research design: Sigafoos, Bowers, Castellino, Culp, Wagner, Reese, Humphreys, Hussey, O’Connor-Semmes, Kapur, Tao, Dobbins and Polli

Conducted experiments: Sigafoos, Castellino, Culp, Wagner, Reese, Humphreys, Hussey, O’Connor-Semmes, Kapur, Tao, Dobbins and Polli

Contributed new reagents or analytic tools: Sigafoos, Culp, Wagner,

Performed data analysis: Sigafoos, Bowers, Castellino, Culp, Wagner, Reese, Humphreys, Hussey, O’Connor-Semmes, Kapur, Tao, Dobbins and Polli

Wrote or contributed to the writing of the manuscript: Sigafoos, Bowers, Castellino, Hussey, O’Connor-Semmes, Dobbins and Polli
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Kapur A, O'connor-Semmes RL, Hussey EK, Dobbins RL, Tao W, Hompesch M, & Nunez DJ (2009) First Human Dose Escalation Study with Remogliflozin Etabonate (RE) in Healthy Subjects and in Subjects with Type 2 Diabetes Mellitus (T2DM) *Diabetes* **58**: (Sup1), 509-P.


Legends for Figures

Figure 1. Proposed metabolic pathways of remogliflozin etabonate in humans. The * indicates the position of the [14C] label. Metabolites that contribute to the pharmacology in vivo are labeled ‘active’, and are based on the in vitro potency and in vivo plasma concentrations. Pathways of biotransformation are identified (e.g., CYP, β-glucosidases, UGTs).

Figure 2. Mean plasma concentration-time profiles of total radioactivity, remogliflozin etabonate, remogliflozin, GSK279782 and GSK333081 following a single 400 mg oral administration of [14C]remogliflozin etabonate. Sampling of blood and plasma for total radioactivity and specific analytes was done through 48 h. Concentrations of radioactivity were below limits of quantitation (125 and 119 ng equiv/mL for blood and plasma respectively) by 36 h in all subjects. The mean percentage of the AUC(0-∞) extrapolated for total radioactivity and individual analytes was equal to or lower than 10%.

Figure 3. HPLC-radiochromatogram of plasma extracts at 1, 4, 12 and 24 h following a single 400 mg oral administration of [14C]remogliflozin etabonate. Note the 10-fold difference in the y-axis scale for each plot over the 24hr time period.

Figure 4. Representative HPLC-radiochromatogram of pooled urine (0 – 48 h) following a single 400 mg oral administration of [14C] remogliflozin etabonate.

Figure 5. Radiochromatograms of [14C]remogliflozin incubations with β-glucosidase followed by incubation with human liver microsomes to produce [14C]GSK1997711. Samples were incubated with β-glucosidase for 120 minutes to generate the remogliflozin aglycone ([14C] GSK1132678) (Panel A). The pH was neutralized and glucuronidation reagents (pooled human liver microsomes, UDPGA, alamethicin, MgCl₂) added. [14C]GSK1997711 was rapidly formed (Panel B) from [14C] GSK1132678 and was shown to be cofactor dependent (Panel C). The x axis units are minutes; y axis, dpm.

Figure 6. [14C]Remogliflozin metabolite formation in human liver microsomes (HLM) and recombinant human CYP enzymes were scaled for relative CYP content in human liver microsomes.

Figure 7. Summary of the mass balance for remogliflozin etabonate in humans.
Table 1. Summary of pharmacokinetic parameters of total radioactivity, remogliflozin etabonate, remogliflozin and selected metabolites after a single oral dose of 400mg [14C]remogliflozin etabonate.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (%CV)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (Range)</th>
<th>AUC&lt;sub&gt;(0-t)&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (%CV)</th>
<th>AUC&lt;sub&gt;(0-∞)&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (%CV)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood radioactivity</td>
<td>8344 (25)</td>
<td>1.00 (0.75-3.00)</td>
<td>33395 (15)</td>
<td>37093 (16)</td>
<td>5.90 (43)</td>
</tr>
<tr>
<td>Plasma radioactivity</td>
<td>13105 (24)</td>
<td>0.88 (0.75-3.00)</td>
<td>55899 (14)</td>
<td>60966 (14)</td>
<td>6.57 (30)</td>
</tr>
<tr>
<td>Remogliflozin etabonate</td>
<td>39.0 (113)</td>
<td>0.50 (0.25-1.50)</td>
<td>25.8 (58)</td>
<td>31.1 (47)</td>
<td>0.39 (42)</td>
</tr>
<tr>
<td>Remogliflozin</td>
<td>1536 (53)</td>
<td>0.64 (0.50-2.00)</td>
<td>2586 (19)</td>
<td>2595 (18)</td>
<td>1.57 (9)</td>
</tr>
<tr>
<td>GSK279782</td>
<td>607 (28)</td>
<td>1.00 (0.75-2.00)</td>
<td>1482 (17)</td>
<td>1494 (16)</td>
<td>2.68 (10)</td>
</tr>
<tr>
<td>GSK333081</td>
<td>143 (22)</td>
<td>1.00 (0.75-2.00)</td>
<td>317 (19)</td>
<td>323 (19)</td>
<td>2.84 (12)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Geometric mean. Eight healthy male volunteers received a single 400 mg dose [14C]remogliflozin etabonate (mean 402±1.0mg (106±0.3µCi)) by mouth administered as three capsules. The mean percentage of the AUC<sub>(0-∞)</sub> extrapolated for total radioactivity and individual analytes was equal to or lower than 10%.

<sup>b</sup> Median
Table 2. Excretion of radioactivity in human urine and feces after an oral dose of 400 mg \[^{14}\text{C}]\text{remogliflozin etabonate}

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Mean</th>
<th>SDc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6</td>
<td>56.5</td>
<td>65.2</td>
<td>63.4</td>
<td>63.9</td>
<td>58.7</td>
<td>48.9</td>
<td>49.7</td>
<td>51.5</td>
<td>56.2</td>
<td>5.57</td>
</tr>
<tr>
<td>6-12</td>
<td>16.1</td>
<td>18.5</td>
<td>16.6</td>
<td>15.4</td>
<td>21.6</td>
<td>16.1</td>
<td>17.6</td>
<td>23.0</td>
<td>18.1</td>
<td>2.78</td>
</tr>
<tr>
<td>12-24</td>
<td>11.0</td>
<td>10.9</td>
<td>11.1</td>
<td>10.1</td>
<td>9.95</td>
<td>14.6</td>
<td>13.7</td>
<td>13.2</td>
<td>11.8</td>
<td>1.76</td>
</tr>
<tr>
<td>24-48</td>
<td>4.47</td>
<td>4.68</td>
<td>5.30</td>
<td>4.83</td>
<td>3.88</td>
<td>11.2</td>
<td>8.25</td>
<td>3.87</td>
<td>5.81</td>
<td>2.59</td>
</tr>
<tr>
<td>48-72</td>
<td>0.55</td>
<td>0.43</td>
<td>0.76</td>
<td>0.62</td>
<td>0.50</td>
<td>1.63</td>
<td>1.42</td>
<td>0.45</td>
<td>0.80</td>
<td>0.47</td>
</tr>
<tr>
<td>72-96</td>
<td>0.08</td>
<td>BLQ</td>
<td>0.06</td>
<td>0.05</td>
<td>0.07</td>
<td>0.22</td>
<td>0.16</td>
<td>BLQ</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>96-120</td>
<td>BLQ(^a)</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>120-144</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Subtotal</td>
<td>88.7</td>
<td>94.7</td>
<td>94.2</td>
<td>94.9</td>
<td>94.7</td>
<td>92.6</td>
<td>90.8</td>
<td>92.0</td>
<td>92.8</td>
<td>2.24</td>
</tr>
<tr>
<td>Feces</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-24</td>
<td>1.47</td>
<td>BLQ</td>
<td>NS(^b)</td>
<td>NS</td>
<td>0.24</td>
<td>BLQ</td>
<td>1.68</td>
<td>NS</td>
<td>0.42</td>
<td>0.72</td>
</tr>
<tr>
<td>24-48</td>
<td>1.05</td>
<td>1.54</td>
<td>BLQ</td>
<td>1.17</td>
<td>1.21</td>
<td>1.80</td>
<td>NS</td>
<td>NS</td>
<td>0.85</td>
<td>0.74</td>
</tr>
<tr>
<td>48-72</td>
<td>0.21</td>
<td>0.44</td>
<td>0.97</td>
<td>1.28</td>
<td>0.20</td>
<td>2.41</td>
<td>1.61</td>
<td>1.59</td>
<td>1.09</td>
<td>0.78</td>
</tr>
<tr>
<td>72-96</td>
<td>0.15</td>
<td>BLQ</td>
<td>0.41</td>
<td>0.07</td>
<td>0.11</td>
<td>0.40</td>
<td>BLQ</td>
<td>0.19</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>96-120</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.13</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.04</td>
<td>BLQ</td>
<td>0.16</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>120-144</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.03</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>0.06</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Subtotal</td>
<td>2.88</td>
<td>1.98</td>
<td>1.54</td>
<td>2.52</td>
<td>1.76</td>
<td>4.65</td>
<td>3.29</td>
<td>2.00</td>
<td>2.58</td>
<td>1.02</td>
</tr>
<tr>
<td>Total</td>
<td>91.6</td>
<td>96.7</td>
<td>95.8</td>
<td>97.4</td>
<td>96.5</td>
<td>97.3</td>
<td>94.1</td>
<td>94.0</td>
<td>95.4</td>
<td>2.03</td>
</tr>
</tbody>
</table>

\(^a\) BLQ, below quantifiable limits  
\(^b\) NS, No sample  
\(^c\) SD, standard deviation
Table 3. Relative distribution of metabolites of [14C]remogliflozin etabonate in pooled human plasma following a single 400 mg oral dose of [14C]remogliflozin etabonate.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Sample Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>GSK355993</td>
<td>6.0</td>
</tr>
<tr>
<td>GSK1997714</td>
<td>9.9</td>
</tr>
<tr>
<td>GSK279782</td>
<td>6.2</td>
</tr>
<tr>
<td>GSK1997711</td>
<td>51.8</td>
</tr>
<tr>
<td>Remogliflozin</td>
<td>10.7</td>
</tr>
<tr>
<td>Total</td>
<td>84.6</td>
</tr>
</tbody>
</table>

a NQ, not quantified

b ND, not detected
Table 4. Relative distribution of radioactive metabolites of [14C] remogliflozin etabonate in pooled human urine following a single 400 mg oral dose of [14C]remogliflozin etabonate.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean % of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>5.9</td>
</tr>
<tr>
<td>GSK333081</td>
<td>BLQ&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSK355993</td>
<td>13.3</td>
</tr>
<tr>
<td>GSK1997714</td>
<td>6.0</td>
</tr>
<tr>
<td>GSK279782</td>
<td>3.4</td>
</tr>
<tr>
<td>GSK1997711</td>
<td>47.8</td>
</tr>
<tr>
<td>Remogliflozin</td>
<td>7.9</td>
</tr>
<tr>
<td>Total</td>
<td>84.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> BLQ, below limits of quantification
Table 5. Results of transport studies for 3 μM [14C]remogliflozin etabonate and [14C]remogliflozin in MDCKII-MDR1 cell monolayers

<table>
<thead>
<tr>
<th>Test Article</th>
<th>GF120918</th>
<th>Rate A→B (pmoles/min/cm²)</th>
<th>Rate B→A (pmoles/min/cm²)</th>
<th>Apical Efflux Ratio</th>
<th>A→B Mass Balance (%)</th>
<th>B→A Mass Balance (%)</th>
<th>P_{7,4} (nm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]Remogliflozin Etabonate</td>
<td>-</td>
<td>0.16 ± 0.03</td>
<td>12 ± 0.77</td>
<td>76</td>
<td>88 ± 3.9</td>
<td>84 ± 4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.6 ± 0.19</td>
<td>3.2 ± 0.09</td>
<td>0.88</td>
<td>90 ± 3.6</td>
<td>80 ± 4.9</td>
<td>286 ± 15</td>
</tr>
<tr>
<td>[14C]Remogliflozin</td>
<td>-</td>
<td>0.15 ± 0.005</td>
<td>2.6 ± 0.04</td>
<td>17</td>
<td>97 ± 5.8</td>
<td>92 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.31 ± 0.02</td>
<td>0.30 ± 0.001</td>
<td>0.98</td>
<td>95 ± 6.2</td>
<td>90 ± 1.9</td>
<td>19 ± 0.38</td>
</tr>
</tbody>
</table>

Data are the mean ± standard deviation from three monolayers over 90 min for MDR1 expressing MDCKII monolayers. All donor compartments contained Lucifer yellow (100 μM) to determine monolayer integrity. Amprenavir served as a positive control for Pgp efflux and had an efflux ratio of 20 that dropped to 0.87 in the presence of GF120918. The measured radiochemical purity of [14C]remogliflozin etabonate and [14C]remogliflozin was >99% and no metabolic or chemical degradation was detected during the studies.

GF120918 was used in both donor and receiver compartments at 2 μM for Pgp.
<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>Remogliflozin Etabonate</th>
<th>Remogliflozin</th>
<th>Remogliflozin Etabonate</th>
<th>Remogliflozin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RE Alone</td>
<td>RE+ Ketoconazole</td>
<td>Treatment RE+K/RE</td>
<td>RE Alone</td>
</tr>
<tr>
<td>AUC(_{(0-\infty)}) (h.ng/mL)</td>
<td>27.3 (58) (^4)</td>
<td>31.6 (65) (^5)</td>
<td>na</td>
<td>1743 (38)</td>
</tr>
<tr>
<td>C(_{max}) (ng/mL)</td>
<td>31.4 (102)</td>
<td>39.0 (100)</td>
<td>1.24</td>
<td>805 (45)</td>
</tr>
<tr>
<td>t(_{1/2}) (h)</td>
<td>0.353 (61) (^4)</td>
<td>0.464 (54) (^5)</td>
<td>1.33</td>
<td>1.54 (21)</td>
</tr>
<tr>
<td>t(_{max}) (h)</td>
<td>0.50 (0.25-3.00)</td>
<td>0.50 (0.25-2.00)</td>
<td>0.00</td>
<td>0.88 (0.50-3.00)</td>
</tr>
<tr>
<td>AUC Ratio, m/p</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>90.2 (68)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GSK279782</th>
<th>GSK333081</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_{(0-\infty)}) (h.ng/mL)</td>
<td>617 (47)</td>
</tr>
<tr>
<td>C(_{max}) (ng/mL)</td>
<td>203 (48)</td>
</tr>
<tr>
<td>t(_{1/2}) (h)</td>
<td>2.22 (14)</td>
</tr>
<tr>
<td>t(_{max}) (h)</td>
<td>1.25 (0.50-4.00)</td>
</tr>
<tr>
<td>AUC Ratio, m/p</td>
<td>0.391 (50)</td>
</tr>
</tbody>
</table>

1. Values are geometric mean (%CVb) for each parameter, except for Tmax which are given as median (range). Unless stated otherwise, data from all 20 subjects were used in determining the PK parameter estimate.

2. Treatment RE: Remogliflozin etabonate 250mg; RE+K: Remogliflozin etabonate 250mg + Ketoconazole 400mg

3. For AUC, Cmax and t1/2, values are point estimates (90% CI) of the geometric least-square mean ratio between treatments on the basis of ANOVA; and for Tmax, values are point estimates (90% CI) of the median difference between treatments on the basis of Hodges-Lehmann method [Hauschke et al., 1990].

4. n=15 subjects

5. n=19 subjects

6. n=18 subjects
Fig 1

Remogliflozin etabonate → [esterase] → Remogliflozin (active)

Remogliflozin (active) → UGT → GSK1997714

Remogliflozin (active) → CYP → GSK279782 (active)

GSK333081 → [β-glycosidase] → GSK1132678

GSK1132678 → [β-glycosidase] → GSK279782 (active)

M13 → UGT → GSK355993

GSK1997711 → CYP → GSK355993
Fig 2

[Graph showing concentration over time for different substances:
- Radioactivity
- Remogliflozin etabonate
- Remogliflozin
- GSK279782
- GSK333081]
Fig 5

A

$[^{14}C]GSK189074$  $[^{14}C]GSK1132678$

0 min with UGT reagents
($=120$ min with $\beta$-glucosidase)

B

$[^{14}C]GSK1997711$

30 min with UGT reagents

C

60 min, no UDPGA cofactor
Fig 7

Radioactivity Recovery Mean % of dose
- Urine 92.8%
- Feces 2.6%
- Unaccounted 4.6%

Remogliflozin recovery Mean % of dose
- Remogliflozin 7.9%
- Metabolites 84.9%
- Not profiled

Metabolite Recovery Mean % of dose
- GSK1997711 47.8%
- GSK355993 13.3%
- GSK1997714 6.0
- M13 5.9%
- GSK279782 3.4%
- GSK333081 BLQ%
- Other 8.5%

[14C]Remogliflozin etabonate 402 mg (106 μCi) Oral Dose