In Vitro Characterization of Ertugliflozin Metabolism by UDP-Glucuronosyltransferase and Cytochrome P450 Enzymes

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ABSTRACT

Ertugliflozin is primarily cleared through UDP-glucuronosyltransferase (UGT)–mediated metabolism (86%) with minor oxidative clearance (12%). In vitro phenotyping involved enzyme kinetic characterization of UGTs or cytochrome P450 enzymes catalyzing formation of the major 3-O-β-glucuronide (M5c) and minor 2-O-β-glucuronide (M5a), monohydroxylated ertugliflozin (M1 and M3), and des-ethyl ertugliflozin (M2) metabolites in human liver microsomes (HLMs). Fractional clearance (fCL) from HLM intrinsic clearance (CLint) indicated a major role for glucuronidation (fCL 0.96; CLint 375 µl/min per milligram) versus oxidative metabolism (fCL 0.04; CLint 1.64 µl/min per milligram). Substrate concentration at half-maximal velocity (Km) and maximal rate of metabolism (Vmax) for M5c and M5a formation were 10.8 µM, 375 pmol/min per milligram, and 34.7 µM, 375 pmol/min per milligram, and 34.7 µM, 375 pmol/min per milligram, respectively. Inhibition of HLM CLint with 10 µM digoxin or tranilast (UGT1A9) and 3 µM 16β-phenylnongifolol (UGT2B7/UGT2B4) resulted in fraction metabolism (fm) estimates of 0.81 and 0.19 for UGT1A9 and UGT2B7/UGT2B4, respectively. Relative activity factor scaling of recombinant enzyme kinetics provided comparable fm for UGT1A9 (0.86) and UGT2B7 (0.14). Km and Vmax for M1, M2, and M3 formation ranged 73.0–93.0 µM and 24.3–116 pmol/min per milligram, respectively, and was inhibited by ketoconazole (M1, M2, and M3) and montelukast (M2). In summary, ertugliflozin metabolism in HLMs was primarily mediated by UGT1A9 (78%) with minor contributions from UGT2B7/UGT2B4 (18%), CYP3A4 (3.4%), CYP3A5 (0.4%), and CYP2C8 (0.16%). Considering higher ertugliflozin oxidative metabolism (fCL 0.12) obtained from human mass balance, human systemic clearance is expected to be mediated by UGT1A9 (70%), UGT2B7/UGT2B4 (16%), CYP3A4 (10%), CYP3A5 (1.2%), CYP2C8 (0.5%), and renal elimination (2%).

SIGNIFICANCE STATEMENT

This manuscript describes the use of orthogonal approaches (i.e., enzyme kinetics, chemical inhibitors, and recombinant enzymes) to characterize the fraction of ertugliflozin metabolism through various UDP-glucuronosyltransferase (UGT) and cytochrome P450 (CYP) enzyme-mediated pathways. Phenotyping approaches routinely used to characterize CYP hepatic fractional metabolism (fm) to estimate specific enzymes contributing to overall systemic clearance were similarly applied for UGT-mediated metabolism. Defining the in vitro metabolic disposition and fm for ertugliflozin allows risk assessment when considering potential victim-based drug-drug interactions perpetrated by coadministered drugs.

Introduction

Ertugliflozin (Fig. 1) is an orally administered, potent, and selective sodium-dependent glucose cotransporter (SGLT) 2 inhibitor (Mascitti et al., 2011) approved for the treatment of adults with type 2 diabetes mellitus (T2DM) at single daily doses of 5 or 15 mg as either monotherapy, fixed-dose combinations with metformin or sitagliptin, or in combination with antidiabetic medications (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&applno=209803; http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/004315/human_med_002241.jsp&mid=WCOb01ac058001d124; Markham, 2018). Diabetes mellitus is a highly prevalent disease; there are approximately 463 million adults living with diabetes mellitus in 2019, a number expected to rise to 700 million by 2045 (https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html). According to the National Diabetes Statistics Report from 2020, 34.2 million people in the United States have T2DM, of whom 7.3 million are undiagnosed (https://www.cdc.gov/diabetes/data/statistics/statistics-report.html). SGLT2 inhibitors such as ertugliflozin are indicated to treat T2DM as an adjunct to diet and exercise to improve glycemic control through inhibition of glucose reabsorption in the kidneys (Terra et al., 2017). Glucose homeostasis in the kidney is maintained by SGLT1, SGLT2, and SGLT3 transporters located in the brush border of the epithelial cells of proximal renal tubules (Wright, 2001). SGLT2 is the primary transporter responsible for glucose reabsorption (80%–90%) with a lesser role of SGLT1 (10%–20%), substantiated by compelling clinical data (Plosker, 2014; Deeks and Scheen, 2017; F rampton, 2018), preclinical investigation (Freitas et al., 2008; Han et al., 2008), and in vitro human kidney experiments (Hummel et al., 2011; Ghezzi and Wright, 2012; Vrhovac et al., 2015). Ertugliflozin is >2000-fold more selective as a SGLT2 inhibitor over SGLT1 (Mascitti et al., 2011). After a 5 or 15 mg once daily dose of ertugliflozin for 26 weeks to subjects with T2DM demonstrating inadequate glycemic control despite diet and exercise, fasting plasma
Ertugliflozin Phenotyping

Fig. 1. Ertugliflozin human relevant metabolites identified from a mass-balance study.

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; BSA, bovine serum albumin; CL, clearance; CLint, apparent intrinsic clearance; CLint,scal, scaled in vivo whole organ unbound intrinsic clearance; CLint,unbound, unbound in vitro intrinsic clearance; fM, fraction of metabolism; fU, fraction unbound; HKM, human kidney microsome; HLM, human liver microsome; IS, internal standard; ISEF, intersystem extrapolation factor; Km, apparent substrate concentration at half-maximal velocity; Ksi, inhibition constant for substrates exhibiting substrate inhibition kinetics; LC-MS/MS, liquid chromatography–tandem mass spectrometry; M1, monohydroxylated ertugliflozin; M2, des-ethyl ertugliflozin; M3, monohydroxylated ertugliflozin; M5a, 2-0-β-glucuronide; M5c, 3-0-β-glucuronide; M1 and M3, M2, M5c, M5a, and 16β-phenyllongifolol were obtained from Pfizer Global Research and Development Chemistry. Alamethicin, UDP glucuronic acid (UDPGA), MgCl2, NADPH, diclofenac, terfenadine, furafylline, 2-phenyl-2-(1-piperidinyl)propane (PPP), montelukast, sulfaphenazole, benzylnirvanol, quinidine, ketoconazole, CYPC3side, tranilast, digoxin, 1 M potassium phosphate dibasic solution, 1 M potassium phosphate monobasic solution, Tris, bovine serum albumin (BSA; crude BSA product no. A7906), and DMSO were purchased from Sigma Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs) prepared from 50 mixed-gender donors and recombinantly expressed human UGT enzymes exhibited atypical kinetics; SGLT, sodium-dependent glucose cotransporter; T2DM, type 2 diabetes mellitus; UDPGA, UDP glucuronic acid; UGT, UDP-glucuronosyltransferase; Vmax, maximal rate of metabolism.
Enzyme kinetic parameters for ertugliflozin glucuronide metabolite (M5a and M5c) formation in pooled HLMs

Data are expressed as the means ± S.D. from triplicate experiments, except M5c formation with 2% BSA (n = 6).

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>K_m (µM)</th>
<th>V_max (µmol/min/µg)</th>
<th>K_m (µM)</th>
<th>V_max (µmol/min/µg)</th>
<th>K_m (µM)</th>
<th>V_max (µmol/min/µg)</th>
<th>IC_50</th>
<th>IC_50</th>
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</tr>
</thead>
<tbody>
<tr>
<td>HLMs with 2% BSA</td>
<td>379 ± 11</td>
<td>41.7</td>
<td>94.9 ± 1.2</td>
<td>2.28</td>
<td>2.15</td>
<td>0.06</td>
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<tr>
<td>M5a</td>
<td>98.3 ± 0.1</td>
<td>10.8</td>
<td>375 ± 7</td>
<td>34.7</td>
<td>32.8</td>
<td>0.94</td>
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<tr>
<td>Total</td>
<td>37.0</td>
<td>35.0</td>
<td>1.00</td>
<td></td>
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<tr>
<td>HLMs without BSA</td>
<td>3130 ± 1620</td>
<td>1690</td>
<td>734 ± 363</td>
<td>86.8 ± 46.8</td>
<td>0.434</td>
<td>0.410</td>
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</tr>
<tr>
<td>M5c</td>
<td>77.5 ± 3.1</td>
<td>41.9</td>
<td>384 ± 4</td>
<td>9.16</td>
<td>8.66</td>
<td>0.95</td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>9.59</td>
<td>9.07</td>
<td>1.00</td>
<td></td>
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</tr>
</tbody>
</table>

*HLM incubations were conducted in the presence and absence of 2% BSA as described under Materials and Methods.
*Ertugliflozin unbound fraction (f_{u,ert}) in HLMs (0.25 mg/ml) without BSA and with 2% BSA was 0.54 and 0.11, respectively.
*Apparent K_m values exceeded the highest ertugliflozin concentration in incubation (1000 µM) due to substrate inhibition kinetics.

| (rhUGTs) (1A1, 1A3, 1A4) | 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17, and nontransfected control | were obtained from Corning Life Sciences (Woburn, MA). Pooled human kidney microsomes (HKMs) prepared from eight mixed-gender donors were obtained from Xenotech (Kansas City, KS). HLM 3A5*1*1, 3A5*3*3 microsomes pools were prepared as previously described (Tseng et al., 2014). Recombinantly expressed human CYPs (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) were obtained from either Invitrogen (Life Technologies, Waltham, MA) or BD BioSciences (Franklin Lakes, NJ).

**General UGT Incubation Conditions**

UGT incubations were performed in a buffer mixture containing 100 mM Tris HCl buffer (pH 7.5 at 37°C) with or without 2% BSA, 5 mM MgCl_2, and microsomal or recombinant protein. The buffer mixture was preincubated with alamethicin (0.1 µg/ml) on ice for 15 minutes to allow for alamethicin pore formation. The incubation mixture was warmed to 37°C prior to the addition of ertugliflozin. Reactions were initiated by the addition of 5 nM UDPGA and terminated by protein precipitation with acetonitrile containing internal standard (IS).

**UGT Substrate Saturation in HLMs and rhUGTs**

Using the general UGT incubation conditions described above, reactions with 0.25 mg/ml protein (i.e., HLM, rhUGT1A9, and rhUGT2B7) and ertugliflozin (1–1000 µM) for HLMs, 0.3–1000 µM for rhUGTs) were initiated by the addition of UDPGA and terminated at 30 or 40 minutes by protein precipitation with acetonitrile containing IS. All samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), where M5c and M5a formation were quantified against standard curves. The experimental conditions selected for enzyme kinetics were chosen after conduct of preliminary reaction rate experiments to ensure linear reaction velocity with respect to protein concentration and incubation time. HLM and rhUGT incubations for M5a formation were performed in triplicate. Incubations for M5c formation were conducted on several days (n = 6) in both HLMs and rhUGTs.

**UGT Phenotyping**

Ertugliflozin was incubated in the presence of 3 rhUGTs (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17) and nontransfected control to assess which isoforms contribute to glucuronide formation. The general incubation mixture without 2% BSA contained 0.5 mg/ml protein, either rhUGT or vector control, and ertugliflozin (7, 77, and 770 µM) representing approximately 0.1x, 1x, and 10x the HLM apparent substrate concentration at half-maximal velocity (K_m) for the primary glucuronidation (M5c) pathway (Table 1). The incubation mixtures were prewarmed to 37°C prior to the addition of UDPGA and terminated over a time course by protein precipitation with acetonitrile containing IS. Samples were analyzed by LC-MS/MS, where ertugliflozin depletion and both M5c and M5a formation were measured by analyte/IS area ratios. All incubations were performed in duplicate.

Ertugliflozin (5 µM) was incubated with HLMs in the presence of 2% BSA with and without UGT chemical inhibitors for a quantitative assessment of UGT isomerase contribution. General incubation conditions and sample preparation were similar to those described for UGT substrate saturation experiments. Reversible inhibitors were incubated at the following concentrations: 10 µM tramadol (UGT1A9), 10 µM digoxin (UGT1A9), and 3 µM 16β-phenyllongifolol (UGTs 2B4/2B7). Control incubations were performed in the absence of chemical inhibitors (HLM control). The incubation mixture was warmed to 37°C for 5 minutes prior to the addition of ertugliflozin. Reactions were initiated by the addition of UDPGA and terminated over a time course by protein precipitation with acetonitrile containing IS. Samples were analyzed using LC-MS/MS, where M5a and M5c formation were quantified against standard curves. Selective chemical inhibition experiments were conducted in duplicate.

**CYP Substrate Saturation in HLMs and Recombinantly Expressed Human CYP Enzymes**

HLM incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4), 3 mM MgCl_2, 0.1 mg/ml HLMs, and ertugliflozin (0.01–1000 µM). Incubations were warmed to 37°C prior to the addition NADPH (1.2 mM). Reactions were terminated after 10 minutes by transferring aliquots of the incubation mixture to acetonitrile containing IS. Incubations were conducted in duplicate. For substrate saturation experiments in recombinantly expressed human CYP enzymes (rhP450s) (2C8, 3A4, and 3A5), incubation conditions were similar to those described previously in HLMs. The rhP450 concentration was 10 pmol/ml (0.11 mg/ml) protein, the incubation time was 10 minutes, and ertugliflozin concentrations ranged from 0.01 to 500 µM. All samples were analyzed by LC-MS/MS and quantified against standard curves of M1, M2, and M3. The experimental conditions selected for enzyme kinetics were chosen after conduct of preliminary reaction rate experiments in HLMs to ensure linear reaction velocity with respect to protein concentration and incubation time.

**CYP Phenotyping**

Ertugliflozin was incubated in the presence of eight rhP450s (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) at a concentration of 10 pmol/ml. Potassium phosphate buffer (100 mM), rhP450s or nontransfected control, and ertugliflozin (10 µM for M1 and M2 formation, or 50 µM for M3 formation) were prewarmed to 37°C. Incubations were initiated with 1.2 mM NADPH followed by addition of substrate. Reactions were terminated at 10 minutes by protein precipitation with acetonitrile containing IS. The recombinant CYP panel was incubated in triplicate. All samples were analyzed by LC-MS/MS and quantified against standard curves of M1, M2, and M3. Ertugliflozin (10 or 50 µM) was incubated with HLMs in the presence of CYP selective chemical inhibitors for the determination of CYP isomerase contribution. General incubation conditions and sample preparation were similar to those described for CYP substrate saturation experiments. Reversible inhibitors were incubated at the following concentrations: 0.64 µM montelukast (CYP2C8), 10 µM sulfaphenazole (CYP2C9), 3 µM benzylcinnamate (CYP2C19), 1 µM quinidine (CYP3A4), and 1 µM ketoconazole (CYP3A4/5). Inactivators were preincubated at the following concentrations: 10 µM furafylline (CYP1A2), and 3 µM PPAR (CYP2B6). For incubations containing reversible inhibitors, microsomes were preincubated with inhibitors for 5 minutes prior to initiating the
Ertugliflozin Phenotyping

Ertugliflozin Clearance in HLMs and HKMs by Substrate Depletion

The clearance of ertugliflozin was investigated in HLMs and HKMs in the presence of the individual and combined cofactors (e.g., UDPGA and/or NADPH) to estimate the in vitro fraction of metabolic clearance (fCL) by UGT or CYP enzyme families. Incubations were performed using the general UGT incubation conditions described above with and without 2% BSA containing 2 mg/ml HLMs or HKMs. The incubation mixture was warmed to 37°C prior to the addition of UDPGA (5 mM) or NADPH (1.6 mM), either as a mixture or individually. Reactions were initiated by the addition of ertugliflozin (1 μM final) and terminated over a time course from 0 to 180 minutes by protein precipitation with acetonitrile containing IS. All incubations were performed in duplicate. Samples were analyzed by LC-MS/MS and quantified against standard curves of ertugliflozin, M5c, and M5a.

Determination of Unbound Fraction of Ertugliflozin in HLMs

Using the equilibrium dialysis method previously described (Di et al., 2012), the fraction unbound (fu) in HLMs was determined using 1 μM ertugliflozin, M5c, and M5a. To determine the relative contributions of CYPs 3A4 and 3A5 to the metabolism of ertugliflozin, additional experiments were conducted in a pooled lot of HLMs prepared from individual HLM lots selected for either the *1/*1 genotype (HLM 3A5 *1/*1) associated with CYP3A5 extensive metabolizers or *3/*3 genotype (HLM 3A5 *3/*3) associated with CYP3A5 poor metabolizers (Tseng et al., 2014). Microsomal incubations with ertugliflozin (10 and 50 μM) were conducted as described above for HLMs (0.1 mg/ml protein, 10-minute incubation) and included 1 μM ketoconazole (CYP3A4/5 inhibitor) or 1 μM CYP3Cide (CYP3A4 inhibitor). Pretreatment of the incubates varied based on the presence of the competitive inhibitor ketoconazole (no preincubation with NADPH) or the inactivator CYP3Cide (10-minute preincubation with NADPH). The control incubation for these experiments was conducted with no preincubcation. These experiments were conducted in duplicate. All samples were analyzed by LC-MS/MS and quantified against standard curves of M1, M2, and M3.

Data Analysis

Clearance by Substrate Depletion. The unbound in vitro intrinsic clearance (CLint,u) and scaled in vivo whole organ intrinsic clearance (CLint,tot,u) was determined at a single ertugliflozin concentration and determined by depletion using the following equations:

\[
\text{CL}_{\text{int,u}} = \frac{0.693}{k_i} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{\text{mg microsomes}}{\text{mg tissue}} \times \frac{\text{Ygoftissue}}{\text{kg bodyweight}} / f_u
\]

where the elimination rate constant (k_i) is negative slope of natural log of the average peak area ratio versus time, and f_u is the fraction unbound in the incubation (fu,inc). For HLMs, X and Y were 45 and 21, respectively. The CLint,u was also determined by metabolite formation at a single ertugliflozin concentration using the equation

\[
f_{\text{CL, renal}} = \frac{\text{CL}_{\text{int,u, renal}}}{\text{CL}_{\text{int,u, renal}} + \text{CL}_{\text{int,u, hepatic}}}
\]

Clearance in HLMs Using Enzyme Kinetic Parameters. In general, substrate concentration [S] and velocity (v) data were fit to the appropriate enzyme kinetic model by nonlinear least-squares regression analysis to derive the apparent enzyme kinetic parameters, maximal rate of metabolism (Vmax), and Km or S50. The Michaelis-Menten model (eq. 6), the substrate activation inhibition model (eq. 7) which incorporates the Hill coefficient (n), and the substrate inhibition model (eq. 8) were evaluated for best fit of the data. Best fit was determined using R² goodness of fit, Eadie-Hofstee fit, and Akaike information criterion.

\[
v = \frac{V_{\text{max}} \times S}{(K_m + S)}
\]

\[
v = \frac{V_{\text{max}} \times S}{(S_0 + S^n)}
\]

\[
v = \frac{V_{\text{max}} \times S}{K_m + S \times (1 + \frac{1}{n})}
\]

The exception was formation of M1 in rhCYP2C8. An unweighted linear fit was used to calculate the formation slope [i.e., apparent intrinsic clearance (CLint)] since M1 formation was not saturable (Supplemental Fig. 3) with the highest concentration of ertugliflozin in incubation (500 μM). The CLint was calculated using either eq. 9 for typical kinetics or for M1 formation in rhCYP2C8, CLint was equivalent to the linear slope. Equation 10 was used to calculate CLint atypical kinetics incorporating the Hill coefficient.

\[
CL_{\text{int,tot,u}} = \frac{V_{\text{max}}}{K_m}
\]

\[
CL_{\text{int,u}} = \frac{V_{\text{max}}}{S_0 \times (n-1) \times (n-1)}
\]

The contribution of each metabolic pathway (as represented by each metabolite) to the total clearance of ertugliflozin (fCL) was calculated using eq. 11:

\[
f_{\text{CL}} = \frac{\text{CL}_{\text{int,u, x}}}{\text{CL}_{\text{int,u, x}} + \text{CL}_{\text{int,u, renal}} + \text{CL}_{\text{int,u, hepatic}}}
\]

where CLint,u, x is the unbound apparent intrinsic clearance for metabolite x, and CLint,u, renal is the sum of unbound scaled intrinsic apparent clearance values for all metabolites measured.

Fraction of Metabolism Using Selective Chemical Inhibitors in HLMs. The effect of selective chemical inhibitors on the formation of each metabolite was calculated using eq. 12:
To determine the isoform specific contribution to total clearance, metabolite specific contributions were adjusted for fractional clearance:

\[
\text{fm} = \frac{\text{Inhibition}_x \times \text{fm}_{CL_{tot},u}}{100}
\]

where Inhibition$_x$ is the contribution of a specific isoform to the formation of metabolite $x$, and fm$_{CL_{tot},u}$ is the fraction of total clearance represented by metabolite $x$. Since two inhibitors were used for UGT1A9, the mean % inhibition was used for the final fm estimate.

Using Relative Activity Factor or Intersystem Extrapolation Factor to Scale Recombinantly Expressed Enzyme Clearance to HLM Clearance. The rhUGT 1A9 and 2B7 CL$_{int}$,u was calculated using eq. 9, which was then scaled to HLM CL$_{int}$,u using eq. 14:

\[
\text{HLM} \text{ CL}_{int, u} = \text{RAF} \times \text{rhCL}_{int, u}
\]

where RAF is the relative activity factor for an individual enzyme based on selective probe substrate CL$_{int}$ values (eq. 15), rhCL$_{int}$,u is the unbound intrinsic apparent clearance in rhUGTs, and CL$_{int}$,u is the unbound intrinsic apparent clearance scaled to liver. The RAF value for UGT1A9 (0.8) was generated using mycophenolic acid as the substrate. The UGT2B7 RAF value of 2.4 was generated using zidovudine.

\[
\text{RAF} = \frac{\text{HLMCL}_{int, u}}{\text{rhCL}_{int, u}}
\]

For rhCYP 3A4, 3A5, and 2C8 incubations, the rhCL$_{int, u}$ was calculated (eq. 9). An intersystem extrapolation factor (ISEF) was applied in conjunction with the abundance of the CYP in the liver (Supplemental Table 2) to scale to a hepatic CL$_{int, u}$.


**HLM CL\textsubscript{int,a} = rhCL\textsubscript{int,a} \times ISEF \times tissue abundance\textsuperscript{pmol mg\textsuperscript{-1}}** \quad (16)

**Delineation of the Fraction Metabolized via CYP3A4 versus CYP3A5.** The linear portion of the mean regression line \((n = 2)\) was used to determine a single slope of each incubation condition \((k\text{el})\). The slope of the line was used to determine the half-life \((t\text{\textsubscript{1/2}; minutes})\) at each incubation condition \((eq. 1)\), which was then extrapolated to the \(CL\text{int,a}\) like \(eq. 2\). The contribution by CYP3A5 was calculated using the following equations \((Tseng et al., 2014)\):

\[
\text{Calculated } \% \text{ CYP3A4 Contribution} = \frac{CL\text{int,a} - CL\text{int,CYP3A4}}{CL\text{int,a}} \times 100 \quad (17)
\]

\[
\text{Calculated } \% \text{ CYP3A5 Contribution} = \% \text{ CYP3A} - \% \text{ CYP3A4} \quad (18)
\]

where \(CL\text{int,a}\) is the clearance in HLMs without inhibitor.

**Results**

**UGT and CYP Substrate Saturation in HLMs.** The metabolite formation kinetics for ertugliflozin glucuronides M5c (major) and M5a (minor) were determined after incubation of ertugliflozin in HLMs in the absence and presence of 2% BSA \((Fig. 2; Table 1)\). In the absence of BSA, M5a exhibited substrate inhibition kinetics, and the estimated apparent \(K\text{m,u}\) value \((3130 \text{ m}\text{M})\) exceeded the highest ertugliflozin concentration in incubation \((1000 \mu\text{M})\). All other profiles displayed typical Michaelis-Menten kinetics. In general, the \(K\text{m,u}\) values were lower in the presence of 2% BSA for M5c \((4\text{-fold})\) and M5a \((41\text{-fold})\) resulting in \(K\text{m,u}\) values of 10.8 and 41.7 \(\mu\text{M}\), respectively. Inclusion of BSA had minimal effect on M5c \(V\text{max}\), whereas M5a \(V\text{max}\) decreased in the presence of BSA from \(734 \pm 363\) to \(94.9 \pm 1.2\) pmol/min per milligram. Overall, the formation \(CL\text{int,a}\) for M5c and M5a increased \(>3.8\text{-fold}\) in the presence of BSA primarily driven by a lower \(K\text{m,u}\). The highest total \(CL\text{int,a}\) for glucuronidation \((37 \mu\text{L/min per milligram})\) was observed in the presence of BSA with M5c \((34.7 \mu\text{L/min per milligram})\) the primary and M5a \((2.28 \mu\text{L/min per milligram})\) the minor metabolic route.

CYP enzyme kinetic and intrinsic clearance values were determined for the formation of M1, M2, and M3 in HLMs \((Fig. 3; Table 2)\). Visual inspection of the Eadie-Hofstee plots for the M1, M2, and M3 reaction rates demonstrated nonlinear profiles consistent with activation kinetics. For all three metabolites, a Michaelis-Menten equation including a Hill coefficient best described the formation kinetics and was selected for determination of enzyme kinetic parameters. \(S\text{50,u}, V\text{max},\) and Hill coefficient values were \(73.0 \text{ m}\text{M}, 166 \text{ pmol min}\text{^{-1} mg\textsuperscript{-1}},\) and \(1.54\) for M1, \(51.6 \text{ m}\text{M}, 31.4 \text{ pmol min}\text{^{-1} mg\textsuperscript{-1}},\) and \(1.59\) for M2, and \(93.0 \text{ m}\text{M}, 24.3 \text{ pmol min}\text{^{-1} mg\textsuperscript{-1}},\) and \(1.56\) for M3, respectively.

**Fig. 3.** Substrate saturation profile of ertugliflozin oxidative metabolites M1, M2, and M3 derived from pooled HLMs. The kinetic profiles exhibited a sigmoidal behavior, as illustrated by the Eadie-Hofstee graphs. Data are expressed as the means from duplicate experiments.

**Table 2**

<table>
<thead>
<tr>
<th>HLM metabolite</th>
<th>(S\text{50}) (\mu\text{M})</th>
<th>(S\text{50,u}^a) (\mu\text{M})</th>
<th>(V\text{max}) pmol min\textsuperscript{-1} mg\textsuperscript{-1}</th>
<th>Hill coefficient</th>
<th>(CL\text{max}) (\mu\text{L min}\text{^{-1} mg}\textsuperscript{-1})</th>
<th>(CL\text{int,a}) (\mu\text{L min}\text{^{-1} kg}\textsuperscript{-1})</th>
<th>(f\text{CL,CYP})</th>
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<tbody>
<tr>
<td>M1</td>
<td>88.0</td>
<td>73.0</td>
<td>166</td>
<td>1.54</td>
<td>1.19</td>
<td>1.12</td>
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<td>M2</td>
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<tr>
<td>M3</td>
<td>112</td>
<td>93.0</td>
<td>24.3</td>
<td>1.56</td>
<td>0.136</td>
<td>0.129</td>
<td>0.083 (0.844)^b</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>—</td>
<td>24.3</td>
<td>1.56</td>
<td>1.64</td>
<td>1.55</td>
<td>0.99 (1.0)^b</td>
</tr>
</tbody>
</table>

\(^a\)Ertugliflozin unbound fraction \((f\text{u,inc})\) was calculated as 0.83 for 0.1 mg/ml protein in incubation based on the measured value of 0.397 at 0.76 mg/ml HLMs.

\(^b\)Values in parenthesis are the fractional clearances normalized to a value of 1.0.
Based on these kinetic parameters, the respective M1, M2, and M3 concentrations of 7, 77, and 770 μmol/min per milligram with 13 rhUGT enzymes in the absence of 2% BSA. Ertugliflozin was incubated at 77 μmol/min per milligram, and 22.4 μmol/min per milligram, respectively. Zidovudine was used to generate a RAf for UGT1A9 and UGT2B4/2B7 of 0.86 and 0.14, respectively. The rhUGT CLint prior to RAf scaling yields fm values of 0.20 and 0.80, respectively. The combined inhibition of overall in vitro glucuronidation in HLMs resulted in a fm for UGT1A9 and UGT2B4/2B7 of 0.81 and 0.19, respectively.

Enzyme kinetic characterization describing formation of M5c and M5a in rhUGT 1A9 and 2B7 were performed in the presence of 2% BSA (Fig. 2; Table 4). All profiles exhibited typical Michaelis-Menten kinetics. For M5c, the Km,u and Vmax in rhUGTs 1A9 and 2B7 was 14.7 μM and 375 μmol/min per milligram (rhCLint,u = 25.4 μl/min per milligram), and 22.4 μM and 2.63 ± 0.18 pmol/min per milligram (rhCLint,u = 0.177 μl/min per milligram), respectively. For M5a, the Km,u and Vmax in rhUGTs 1A9 and 2B7 were 14.9 μM and 5.09 ± 0.15 pmol/min per milligram (rhCLint,u = 0.343 μl/min per milligram), respectively. The rhCLint,u was scaled to hepatic Clint,u using the appropriate RAf (Table 4). For UGT1A9, a mycophenolic acid generated RAf of 0.8 (Lin et al., 2015) was used to scale M5c and M5a formation to a hepatic microsomal Clint,u of 20.4 and 0.274 μl/min per milligram, respectively. Zidovudine was used to generate a UGT2B7 RAf of 2.4 (Lin et al., 2014). UGT2B7 mediated hepatic Clint,u for M5c and M5a formation was 2.81 and 0.613 μl/min per milligram, respectively. Overall, addition (M5c and M5a) of the RAf-adjusted scaled HLM Clint for UGT1A9 (20.7 μl/min per milligram) and UGT2B7 (3.42 μl/min per milligram) results in the combined UGT-mediated Clint,u of 24.1 μl/min per milligram (CLint,cu = 22.7 μl/min per kilogram) or RAf-adjusted fm for UGT1A9 and UGT2B7 of 0.86 and 0.14, respectively. (The rhUGT Clint prior to RAf scaling yields fm for UGT1A9 and UGT2B7 of 0.986 and 0.014, respectively.

### CYP Phenotyping of Ertugliflozin

CYP reaction phenotyping or enzyme mixing was conducted examining ertugliflozin metabolite formation in eight rhP450s (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5), which revealed M1 formation was quantifiable in rhCYPs 1A2, 3A4, and 3A5, M2 was quantifiable in rhCYPs 1A2, 2C8, 2C19, 2D6, 3A4, and 3A5, and M3 was quantifiable in rhCYPs 2D6, 3A4, and 3A5 (Supplemental Fig. 2).
Inhibition of ertugliflozin (5 μM) glucuronide formation in pooled HLMs using UGT-selective chemical inhibitors

Data are expressed as the means from duplicate experiments after HLM incubations as described under Materials and Methods. The relative contribution values (fm) were calculated from data scaled to 100% inhibition.

<table>
<thead>
<tr>
<th>UGT enzyme</th>
<th>Inhibitor</th>
<th>Total (M5a + M5c)</th>
<th>M5a</th>
<th>M5c</th>
<th>Total (scaled to 100%)</th>
<th>Relative contribution CLint,u</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM Control incubation</td>
<td>4.92</td>
<td>—</td>
<td>—</td>
<td>4.92</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1A9</td>
<td>3.91</td>
<td>3.91</td>
<td>20.6</td>
<td>17.2</td>
<td>81.8</td>
<td>81.8</td>
</tr>
<tr>
<td>M1</td>
<td>6.42</td>
<td>6.42</td>
<td>12.8</td>
<td>7.88</td>
<td>88.0</td>
<td>88.0</td>
</tr>
<tr>
<td>2B4/2B7</td>
<td>1.02</td>
<td>1.02</td>
<td>10.9</td>
<td>7.04</td>
<td>81.8</td>
<td>81.8</td>
</tr>
<tr>
<td>M2</td>
<td>4.08</td>
<td>4.08</td>
<td>8.91</td>
<td>5.09</td>
<td>88.0</td>
<td>88.0</td>
</tr>
<tr>
<td>2C8</td>
<td>3.99</td>
<td>3.99</td>
<td>13.9</td>
<td>8.94</td>
<td>81.8</td>
<td>81.8</td>
</tr>
<tr>
<td>M3</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.996</td>
<td>0.996</td>
<td>0.996</td>
</tr>
</tbody>
</table>

The effect of CYP selective chemical inhibitors on the metabolism of ertugliflozin in HLMs was also investigated. The metabolic formation rates of M1, M2, and M3 in HLMs were most significantly inhibited in the presence of ketoconazole resulting in percent inhibition values of 91.9%, 86.8%, and >81.8%, respectively (Table 5). After scaling inhibition responses to 100%, fm for CYP3A4/5 was predicted to be 0.72 for M1, 0.15 for M2, and >0.080 for M3. M2 was also significantly inhibited in the presence of montelukast resulting in a percent inhibition value of 24.7% and a predicted fm CYP2C8 of 0.042. Minor contributions by the remaining CYP isoform inhibitors were observed for M1 (2C8, 2C9, 2D6), M2 (1A2, 2B6, 2C9, 2D6), and M3 (2B6, 2C8, 2C9), although inhibition did not achieve statistical significance (P > 0.01) and was considered negligible.

To further discern the contribution of CYP isoforms 3A4 and 3A5 to ertugliflozin metabolism, incubations with the CYP3A4 selective inactivator CYP3cide and dual CYP3A4/5 inhibitor ketoconazole were conducted in CYP3A5 high (HLM 3A5 *1/*1) and low expressing (HLM 3A5 *3/*3) microsomes (Fig. 5; Supplemental Table 4). There were statistically significant differences (P < 0.01) in the percent inhibition of M1 and M2 resulting from incubation with CYP3cide (75% and 68%) when compared with ketoconazole (84% and 86%) indicating a contribution by CYP3A5 in metabolism of ertugliflozin, albeit small. The calculated in vitro contribution of CYP3A5 to overall CYP3A metabolism of M1 and M2 was 9.7% and 18%, respectively (Supplemental Table 4). In HLM 3A5 *1/*1, the percent inhibition of M3 by CYP3cide (81%) was not statistically different when compared with ketoconazole (88%). When incubated in microsomes devoid of CYP3A5 expression (HLM 3A5 *3/*3), there was no statistically meaningful difference between the effects of CYP3cide and ketoconazole on any of the metabolites.

Ertugliflozin kinetic parameters were generated for all three metabolites in rhCYP 3A4, 3A5, and 2C8 (Supplemental Fig. 3; Table 4). rhCYP3A4 kinetics was best described by the substrate inhibition model with K i,u, V max, and inhibition constant for substrates exhibiting substrate inhibition kinetics (K i,u) values of 120 μM, 37.0 pmol/min per picomole and 1090 μM for M1, 135 μM, 11.0 pmol/min per picomole and 587 μM for M2, and 213 μM, 15.6 pmol/min per picomole and 397 μM for M3, respectively. Based on these kinetic parameters, the respective M1, M2, and M3 CLint,sc,u values were 4.78, 1.27, and 1.13 ml/min per kilogram (after ISEF and abundance scaling).

Cholesteryl 4α-hydroxylase activity (CHOL) in HepaRECs expressing rhCYP3A5 was not statistically different when compared with rhCYP2C8 (84% and 86%) (Supplemental Table 4). In HLM 3A5 *1/*1, the percent inhibition of M3 by CYP3cide (81%) was not statistically different when compared with ketoconazole (88%). When incubated in microsomes devoid of CYP3A5 expression (HLM 3A5 *3/*3), there was no statistically meaningful difference between the effects of CYP3cide and ketoconazole on any of the metabolites.

Ertugliflozin Phenotyping

The Km,u, V max, a n d Ki values for M1 and M2 formation were not calculated due to insufficient solubility to achieve saturation rates. M3 formation with rhCYP2C8 was detectable but did not have a meaningful difference from the effects of CYP3cide and ketoconazole on any of the metabolites.

The total scaled, ISEF-adjusted, CLint,sc,u for 2C8 contribution to ertugliflozin metabolism was 0.083 ml/min per kilogram. In rhCYP3A5, M1 and M2 reaction rates were fit to a substrate inhibition model, and M3 was fit to a Michaelis-Menten model for determination of enzyme kinetic parameters. K m,u, V max, a n d K i values for M1 and M2 formation were 32.0 μM, 0.0361 pmol/min per picomole, and 568 μM. The resulting hepatic CLint,sc,u value for M2 was 0.0654 ml/min per kilogram. Kinetic parameters for M1 with rhCYP2C8 were not calculated due to insufficient solubility to achieve saturation V max. A CLint,sc,u Value for M1 of 0.0174 ml/min per kilogram was calculated from the slope of substrate concentration versus formation rate data. M3 formation with rhCYP2C8 was detectable but did not have sufficient measurable concentration data to generate a kinetic curve. The total scaled, ISEF-adjusted, CLint,sc,u for CYP2C8 contribution to ertugliflozin metabolism was 0.083 ml/min per kilogram. In rhCYP3A5, M1 and M2 reaction rates were fit to a substrate inhibition model, and M3 was fit to a Michaelis-Menten model for determination of enzyme kinetic parameters. K m,u, V max, a n d K i values for M1 and M2 formation were 175 μM, 5.47 pmol/min per picomole, and 1660 μM and 163 μM, 4.93 pmol/min per picomole, and 771 μM, respectively. The K m,u and V max values for M3 formation are 495 μM and 1.66 pmol/min per picomole. Based on these kinetic parameters, the respective M1, M2, and M3 rhP450 CLint,u Values were 0.0313, 0.0302, and 0.0044 μl/ min per picomole, respectively. HLM CLint,u values were not calculated due to lack of CYP3A5 ISEF. The total CLint,sc,u for CYPs was 7.26 ml/min.
Enzyme kinetic parameters for ertugliflozin metabolite formation in recombinantly expressed CYP and UGT enzymes

Data are expressed as the means ± S.D. from triplicate incubations or means from duplicate experiments. Recombinant enzyme kinetics were scaled to HLM CLint using the HLM Scaling Factors (RAF for UGTs and ISEF for CYPs) as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Enzyme</th>
<th>Vmax</th>
<th>Km</th>
<th>Km,HLM</th>
<th>Km, HKM</th>
<th>rhCLint,sc,a</th>
<th>HLM scaling factor</th>
<th>CLint,sc,a</th>
<th>CLint,sc,a</th>
<th>Combined CLint,sc,a</th>
<th>fm by enzyme family</th>
</tr>
</thead>
</table>
| M5a        | UGT1A9 | 5.09 | 0.15 | 135 ± 13 | 14.9    | 0.343        | 0.80              | 0.274      | 0.259      | 22.7  | UGT: 0.76 (1A9:2B7)
|            | UGT2B7 | 6.55 | 0.28 | 233 ± 28 | 25.6    | 0.256        | 2.40              | 0.613      | 0.580      | 0.86 (0.14) |
| M5c        | UGT1A1 | 375  | —    | 134 ± 6  | 14.7    | 0.117        | 2.40              | 2.81       | 2.66       | —         | —                 |
|            | CYP2A4 | 37.0 | —    | 148 ± 10 | 1090    | 0.308        | 16.4              | 5.05       | 4.78       | 7.26    | CYP: 0.24 |
|            | CYP3A5 | 5.47 | —    | 230 ± 17 | 1660    | 0.0313       | —                 | —          | —          | —         | —                 |
| M2         | CYP3A4 | 11.0 | —    | 167 ± 13 | 587     | 0.0815       | 16.4              | 1.34       | 1.27       | —         | —                 |
|            | CYP3A5 | 4.93 | —    | 215 ± 16 | 771     | 0.0302       | —                 | —          | —          | —         | —                 |
|            | CYP2C8 | 0.0361 | — | 32.0 ± 28 | 568    | 0.00125      | 55.2              | 0.092       | 0.0654     | —         | —                 |
| M3         | CYP3A4 | 15.6 | —    | 263 ± 21 | 397     | 0.0732       | 16.4              | 1.20       | 1.13       | —         | —                 |
|            | CYP3A5 | 1.66 | —    | 495 ± 37 | 367     | 0.0044       | —                 | —          | —          | —         | —                 |
|            | CYP2C8 | —    | —    | —       | —       | —            | —                 | —          | —          | —         | —                 |

<sup>a</sup> Vmax for UGT metabolism is reported as picomole per minute per milligram protein and for CYP metabolism is picomole per minute per milligram protein.

<sup>b</sup> Ertugliflozin unbound fraction (fu,inc) in rhCYP 3A4 was 0.81 for 0.11 mg/ml protein concentration, 0.11 in HLMs with 2% BSA, 0.11 in HLMs with 2% BSA and 2% CYP, 0.76 for 0.16 mg/ml rhCYP 3A5, 0.90 for 0.06 mg/ml protein rhCYP 2C8.

<sup>c</sup> UGT1A9 and UGT2B7 RAfs were generated using myophenolic acid and zidovudine as probe substrates, respectively.

<sup>d</sup> CYP2A4 CLint, ISEF was 2.3 and CYP and UGT abundance was 24 pmol/mg. An ISEF is not available for CYP3A5.

<sup>e</sup> The fm by enzyme family is based on combined CYP and UGT CLint,sc,a.

<sup>f</sup> M1 CYP2C8 CLint was determined by slope of formation since solubility prevented determination of individual kinetic parameters.

**Clearance of Ertugliflozin in HLMs and HKMs at 1 μM**

Ertugliflozin clearance was investigated in HLMs and HKMs obtained from incubations in cofactors. As measured by substrate depletion, the scaled unbound human intrinsic clearance (CLint,sc,a) obtained from incubations in HLMs and HKMs was 27.1 and 2.93 ml/min per kilogram, respectively (Table 6), resulting in a combined CLint,sc,a of 30.0 ml/min per kilogram.

Clearance of Ertugliflozin in HLMs and HKMs was also conducted to measure CLint,u with individual UGT and CYP cofactors. The HLM UGT-mediated CLint,sc,a was 33.9 ml/min per kilogram, and the CYP mediated CLint,sc,a was 1.55 ml/min per kilogram. The combined HLM CLint,sc,a was 35.5 ml/min per kilogram. The UGT-mediated CLint,sc,a, for HKMs was 3.66 ml/min per kilogram. The combined HLM and HKM CLint,sc,a was 39.2 ml/min per kilogram corresponding to a fm of 0.91 and 0.09 for liver and kidney, respectively. Using the well stirred model, the systemic clearance (CLsys) was 1.55 ml/min per kilogram, where the CLsys for liver and kidney was 0.90 and 0.10, respectively.

**Non-specific Binding of Ertugliflozin in HLMs.** Under UGT experimental conditions, the fm measured in an equilibrium dialysis experiment was 0.54 and 0.11 in HLMs (0.25 mg/ml protein) in the absence and presence of 2% BSA, respectively. The fm in HLM at 0.76 mg/ml was 0.397. The fraction unbound for CYP HLM (0.1 mg/ml protein), rhCYP3A4 (0.11 mg/ml protein), rhCYP3A5 (0.16 mg/ml), and rhCYP2C8

<table>
<thead>
<tr>
<th>CYP</th>
<th>Inhibitor</th>
<th>Rate (pmol·min⁻¹·mg⁻¹)</th>
<th>Inhibition (scaled to 100%)</th>
<th>fm&lt;sub&gt;CYP&lt;/sub&gt;</th>
<th>Rate (pmol·min⁻¹·mg⁻¹)</th>
<th>Inhibition (scaled to 100%)</th>
<th>fm&lt;sub&gt;CYP&lt;/sub&gt;</th>
<th>Rate (pmol·min⁻¹·mg⁻¹)</th>
<th>Inhibition (scaled to 100%)</th>
<th>fm&lt;sub&gt;CYP&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>Control (no preincubation)</td>
<td>7.02</td>
<td>—</td>
<td>—</td>
<td>2.05</td>
<td>—</td>
<td>—</td>
<td>5.51</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C2C8</td>
<td>0.64 μM montelukast</td>
<td>6.04</td>
<td>13.9</td>
<td>—</td>
<td>1.54</td>
<td>24.7 (22.1)</td>
<td>0.042</td>
<td>4.51</td>
<td>18.2</td>
<td>—</td>
</tr>
<tr>
<td>C2C9</td>
<td>10 μM sulfaphenazole</td>
<td>6.28</td>
<td>10.4</td>
<td>—</td>
<td>1.82</td>
<td>11.1</td>
<td>—</td>
<td>4.81</td>
<td>12.7</td>
<td>—</td>
</tr>
<tr>
<td>C2C19</td>
<td>3 μM benzylnirvanol</td>
<td>7.58</td>
<td>—</td>
<td>8.01</td>
<td>2.34</td>
<td>—14.4</td>
<td>—</td>
<td>7.14</td>
<td>—29.7</td>
<td>—</td>
</tr>
<tr>
<td>C2D6</td>
<td>1 μM quinidine</td>
<td>6.75</td>
<td>3.83</td>
<td>—</td>
<td>1.99</td>
<td>3.01</td>
<td>—</td>
<td>5.74</td>
<td>—4.18</td>
<td>—</td>
</tr>
<tr>
<td>3A4A/ 5</td>
<td>1 μM ketoconazole</td>
<td>0.569</td>
<td>91.9 (100)</td>
<td>0.72</td>
<td>0.271</td>
<td>86.8 (77.9)</td>
<td>0.15</td>
<td>&lt;1.00</td>
<td>&gt;81.8 (100)</td>
<td>&gt;0.080</td>
</tr>
<tr>
<td>HLM</td>
<td>Control (with preincubation)</td>
<td>5.79</td>
<td>—</td>
<td>—</td>
<td>1.66</td>
<td>—</td>
<td>—</td>
<td>4.52</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1A2</td>
<td>10 μM furafylline</td>
<td>6.17</td>
<td>—</td>
<td>5.67</td>
<td>1.66</td>
<td>0.200</td>
<td>—</td>
<td>4.96</td>
<td>—</td>
<td>9.66</td>
</tr>
<tr>
<td>2B6</td>
<td>3 μM PPP</td>
<td>5.97</td>
<td>—</td>
<td>3.17</td>
<td>1.66</td>
<td>0.200</td>
<td>—</td>
<td>4.34</td>
<td>4.06</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> M1 and M2 were generated from 10 μM ertugliflozin and M3 was generated from 50 μM ertugliflozin.

<sup>b</sup> Values reported only for those inhibitors that resulted in a statistically significant change in rate compared with solvent control (P < 0.01).

Data are expressed as the means from duplicate experiments. The percent inhibition data were scaled to 100% as described under Materials and Methods. The relative contribution values (fm) were calculated from data scaled to 100% inhibition.
2017), allowing consistent approaches to conduct core in vitro UGT and CYP phenotyping experiments (Zhang et al., 2007; Miners et al., 2010; Zientek and Youdim, 2015; Di, 2017). Core studies involved identification of human relevant metabolites from elimination routes, defining fCL, from enzyme kinetics, qualitative rhUGT mapping, and quantitative detailing with isoform-selective chemical inhibitors to define in vitro fm. Orthogonal phenotyping approaches informed core studies, which involved scaling recombinant enzyme kinetics (quantitative assessment) using RAF or ISEF, and genotyped tissue fraction studies (i.e., CYP3A5). Based on consistent and robust results defining glucuronidation and UGT1A9 as the primary metabolic enzyme, UGT correlation analyses (Achour et al., 2017) were not pursued.

Human relevant metabolites (Fig. 1) initially identified in human hepatocyte incubations (Kalugtukar et al., 2011) and quantified from human mass-balance studies included three primary regiosomer glucuronides (M5a, M5b, M5c) with M5c representing >80% of UGT-mediated CL (Kalugtukar et al., 2011; Miao et al., 2013). The minor glucuronide (M5b) accounted for <5% of human glucuronidation and was not formed in appreciable amounts in rhUGTs or HLMs to conduct phenotyping. UGT reaction phenotyping of the two primary glucuronides (M5c and M5a), representing >95% of human glucuronidation, was conducted. Oxidative metabolism resulted in elimination of three primary metabolites (M1, M2, M3) and secondary glucuronides (M6a, M6b), assumed secondary to oxidative metabolism, which collectively accounts for a minor (12%) human CL route.

Enzyme kinetic assays defining fCL, were conducted in HLMs since UGT-selective chemical inhibitors are not qualified for use in human hepatocytes. HLM formation kinetics for M5c (major) and M5a (minor) were performed with 2% BSA to sequester fatty acids released during microsomal incubations since fatty acids could inhibit activity for some UGT isoforms (e.g., UGT1A9 and UGT2B7), lowering the unbound Km and hence increasing CLint,u (Rowland et al., 2007; Manevski et al., 2011; Walsky et al., 2012; Miners et al., 2017). Ertugliflozin UGT-mediated CLint,u (9.59 ml/min per milligram) increased 3.9-fold (37.0 ml/min per milligram) in the presence of 2% BSA, primarily driven by a lower unbound Km with BSA (Table 1), similar to UGT1A9 substrates propofol and mycophenolic acid (Rowland et al., 2008; Gill et al., 2012) or UGT2B7 substrates zidovudine (Manevski et al., 2011; Walsky et al., 2012) and naloxone (Kilford et al., 2009; Gill et al., 2012). In vitro formation of M5c (34.7 µl/min per milligram) was approximately 15-fold greater than M5a (2.8 µl/min per milligram), consistent with human disposition studies indicating M5c as the major human glucuronide (Miao et al., 2013). In vitro formation of oxidative metabolites (1.64 µl/min per milligram) was significantly lower than

### Table 6

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>Substrate depletion ( ^{\text{a,b}} )</th>
<th>Metabolite formation ( ^{\text{b,c,d}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{1/2} )</td>
<td>( \text{CL}_{\text{tot},u} )</td>
</tr>
<tr>
<td>HLM</td>
<td>110</td>
<td>28.6</td>
</tr>
<tr>
<td>HKM</td>
<td>62.0</td>
<td>50.8</td>
</tr>
<tr>
<td>Combined</td>
<td>——</td>
<td>30.0</td>
</tr>
</tbody>
</table>

\( f_{\text{CL}} \): half-life.

\( ^{\text{a}} \) Clearance by substrate depletion was determined in the presence of combined cofactors for UGT and CYP metabolism.

\( ^{\text{b}} \) Substrate depletion and UGT metabolite formation experiments were conducted in the presence of 2% BSA.

\( ^{\text{c}} \) Clearance estimates by metabolite formation were determined in the presence of individual cofactors.

\( ^{\text{d}} \) CLint,u scaled with well-stirred liver or kidney model using blood flow of 20 and 18 ml/min per kilogram, respectively, \( f_{\text{CL}} \) of 0.064, and \( C_{\text{ox}}/C_{\text{CL}} \) of 0.66.

Value represents formation of M5a scaled from ertugliflozin concentration of 1 µM in HLMs and HKMs. The HLM value is comparable to full enzyme kinetic clearance (32.8 ml/min per kilogram) as presented in Table 1.

Value represents formation of M5c scaled from ertugliflozin concentration of 1 µM in HLMs and HKMs. The HLM value is comparable to full enzyme kinetic clearance (2.15 ml/min per kilogram) as presented in Table 1.

### Discussion

Ertugliflozin ADME characterized in a human \( ^{14}\text{C} \) mass-balance study demonstrated the primary clearance mechanism was UGT-mediated metabolism (86%) with minor contributions from CYP-mediated metabolism (12%) and renal clearance (2%) (Miao et al., 2013). Reaction phenotyping studies reported herein defined UGT1A9 as the primary enzyme responsible for ertugliflozin metabolic CL with minor contributions from UGT2B7, UGT2B4, CYP3A4, CYP3A5, and CYP2C8. Quantitative UGT reaction phenotyping has advanced significantly (Miners et al., 2010; Zientek and Youdim, 2015; Di, 2017), allowing consistent approaches to conduct core in vitro UGT and CYP reaction phenotyping.}

![Percent inhibition of CYP metabolites M1, M2, and M3 by ketoconazole (CYP3A4/5) and CYP3cide (CYP3A4) in pooled HLM 3A4 ×11 microsomes.](image)

**Fig. 5.** Percent inhibition of CYP metabolites M1, M2, and M3 by ketoconazole (CYP3A4/5) and CYP3cide (CYP3A4) in pooled HLM 3A4 ×11 microsomes. The percent inhibition was used to calculate CYP3A4 and CYP3A5 contributions to M1, M2, and M3 formation (Fig. 6). Mean data represent duplicate incubations.

(0.06 mg/ml protein), were calculated as 0.83, 0.81, 0.76, and 0.90, respectively, using the differential protein binding equation (Austin et al., 2002).

Data are presented as the means of two replicate experiments.
glucuronidation, resulting in \( f_{\text{CL}} \) estimates of hepatic glucuronidation versus oxidative metabolism of 96% and 4%, respectively. These \( f_{\text{CL}} \) estimates predict major human conjugative CL mechanisms well (88%) when not accounting for renal CL, and somewhat underestimate observed human oxidative CL (12%). A possible explanation for the underestimation of observed human oxidative CL is the potential reabsorption of ertugliflozin, which is generated from hydrolysis of biliary secreted ertugliflozin glucuronides in the gastrointestinal tract. The potential reabsorption and subsequent metabolism by CYP3A4 in the intestine may account for the increased contribution of oxidative metabolism to ertugliflozin metabolism in vivo, resulting in an underestimation of CYP-mediated metabolism using in vitro studies. However, a physiologically based pharmacokinetic model for ertugliflozin, which does not contain this reabsorption component, adequately characterizes ertugliflozin pharmacokinetics across a dose range of 0.5–300 mg, indicating that any potential reabsorption does not significantly contribute to ertugliflozin disposition and the role of oxidative metabolism in ertugliflozin metabolism remains small (Callégari et al., 2020).

Qualitative recombinant UGT enzyme mapping identified UGT1A9 as the apparent major enzyme responsible for M5c glucuronide formation, whereas extrahepatic UGTs 1A7 and 1A8 also demonstrated catalytic turnover (Fig. 4). At higher concentrations, formation of M5c was also catalyzed by UGT1A3 and UGT2B7, albeit at apparently low turnover rates. In addition to UGT1A9, subsequent kinetic experiment with rhUGTs (Table 4) and chemical inhibition studies (Table 3) indicated a minor but more substantial role for UGT2B7 in M5c glucuronidation (Fig. 2). This increase in \( C_{\text{LINT}} \) is likely due to the inclusion of 2% BSA in rhUGT kinetic assays and HLM chemical inhibitor studies, which have demonstrated significant increases in rhUGT CL\( _{\text{LINT}} \) (9-fold) for UGT2B7 substrates such as zidovudine (Walsky et al., 2012). In turn, M5a glucuronidation was apparently primarily catalyzed by UGTs 1A9, 2B7, and 2B4 (Fig. 4) with low turnover rates from other UGT1A enzymes. Subsequent phenotyping studies demonstrated a more significant contribution of UGT2B7 relative to UGT1A9 than apparent from rhUGT mapping.

Quantitative chemical inhibition studies conducted in the presence of digoxin and tranilast (Lapham et al., 2012) demonstrated that UGT1A9 (\( f_{\text{m}} = 0.81 \)) is the primary enzyme responsible for ertugliflozin glucuronidation, whereas the minor route (\( f_{\text{m}} = 0.19 \)) was assigned to UGT2B7 and UGT2B4 after incubation with 16\( b \)-phenyllongifolol (Bichlmaier et al., 2007; Lapham et al., 2012). Although 16\( b \)-phenyllongifolol was previously considered a selective inhibitor of UGT2B7, recent development of a selective UGT2B4 substrate assay in HLMs in our laboratory demonstrated that 16\( b \)-phenyllongifolol (3 \( \mu \)M) is a non-selective inhibitor against UGT2B7 and UGT2B4 substrates resulting in >90% inhibition (unpublished data). Quantitative proteomics could provide insight into the potential contribution of UGTs 2B4 and 2B7 toward ertugliflozin minor glucuronidation route (\( f_{\text{m}} = 0.19 \)). Expression of UGT2B4 (picomole per milligram protein) in pooled HLMs is approximately 50% lower than UGT2B7 (Fallon et al., 2013b; Achour et al., 2017). Since 16\( b \)-phenyllongifolol completely inhibited UGTs 2B4 and 2B7 activity in HLMs and assuming that UGT2B4 and UGT2B7 HLM enzyme kinetic parameters for ertugliflozin glucuronidation are similar, one-third of UGT2B2 ertugliflozin glucuronidation could be assigned to UGT2B4 (\( f_{\text{m}} = 0.06 \)) and two-thirds to UGT2B7 (\( f_{\text{m}} = 0.13 \)).

Orthogonal assays were also employed to substantiate or inform UGT chemical inhibition phenotyping based on increasing evidence of successful RAF scaling of rhUGT CL\( _{\text{LINT}} \) to HLMs with utility in UGT prototyping (Roqueuge et al., 2010; Zhu et al., 2012). Scaling of rhUGT CL\( _{\text{LINT}} \) for UGTs 1A9 and 2B7 provided similar estimates of relative in vitro \( f_{\text{m}} \) (0.86 vs. 0.14) compared with chemical inhibition (0.81 vs. 0.19). Although not the focus of this study, it is of interest that the RAF-adjusted in vivo CL\( _{\text{LINT}} \) for glucuronidation (22.7 ml/min per kilogram) was within 2-fold or approximately 0.65-fold the HLM glucuronidation CL\( _{\text{LINT}} \) (34.9 ml/min per kilogram).

Since UGT1A9 and UGT2B7 are the primary and secondary respective UGTs expressed in human kidney (Fallon et al., 2013a; Zientek and Youdim, 2015), it is important to consider extrahepatic metabolic CL for ertugliflozin. Because ertugliflozin is not subject to significant intestinal first-pass metabolic extraction (\( F_{\text{gi}} = 0.98 \)) (Callégari et al., 2020) and UGT1A9 expression level in the intestine is very low or undetectable (Fallon et al., 2013a; Zientek and Youdim, 2015), it is of interest that the RAF-adjusted in vivo CL\( _{\text{LINT}} \) for glucuronidation (22.7 ml/min per kilogram) is approximately 2- to 3-fold higher (Mukai et al., 2014; Zientek and Youdim, 2015). However, CL\( _{\text{LINT}} \) scaled for tissue CL is approximately 10-fold lower for human kidney relative to liver, primarily due to the differences in organ...
Fig. 7. Integration of “bottom-up” core in vitro phenotyping and orthogonal studies to establish in vitro $f_m$ and “middle-out” in vivo $f_m$ for ertugliflozin based on “top-down” human mass balance disposition. [Adapted from Figure 7 (Minors et al., 2020)]
weight. Only the UGT1A9 metabolite (M5c) was quantifiable in HKMs, in line with relative fm of UGT for ertugliflozin and expression levels of UGTs 1A9 and 2B7 (Fallon et al., 2013a; Margaillan et al., 2015), whereas oxidative metabolism was not observed (Table 6). Similar results for UGT1A9 substrate propofol demonstrated Vmax and CLint were 2.1- and 3.7-fold higher in HKMs relative to HLMs (Mukai et al., 2014). It is therefore anticipated that kidney metabolic CL will account for approximately 10% of total systemic ertugliflozin CL, primarily mediated by UGT1A9.

Minor oxidative CL routes (<20%) could prove important during physiologically based pharmacokinetic model development, particularly when assessing CYP3A4-mediated enzyme induction for victim drugs (Gandelman et al., 2011). Chemical inhibition studies demonstrated most of the ertugliflozin oxidative metabolism was mediated by CYP3A (Gandelman et al., 2011). Chemical inhibition studies demonstrated when assessing CYP3A4-mediated enzyme induction for victim drugs pharmacokinetic model development, particularly

When assessing CYP3A4-mediated enzyme induction for victim drugs, the liver microsomes: comparison of two proteomic methods and correlation with catalytic activity. Drug Metab Dispos 45:1102-1112.


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