Inhibition of Human UDP-Glucuronosyltransferase Enzymes by Canagliflozin and Dapagliflozin: Implications for Drug-Drug Interactions

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ABSTRACT

Canagliflozin (CNF) and dapagliflozin (DPF) are the first sodium-glucose cotransporter 2 inhibitors to be approved for clinical use. Although available evidence excludes clinically significant inhibition of cytochromes P450, the effects of CNF and DPF on human UDP-glucuronosyltransferase (UGT) enzymes are unknown. Here, we report the inhibition of human recombinant UGTs by CNF and DPF, along with the \( K_i \) values for selected recombinant and human liver microsomal UGTs. CNF inhibited all UGT1A subfamily enzymes, but the greatest inhibition was observed with UGT1A1, UGT1A9, and UGT1A10 (IC\(_50\) values \(< 10 \mu M\)). DPF similarly inhibited UGT1A1, UGT1A9, and UGT1A10, with IC\(_50\) values ranging from 39 to 66 \( \mu M\). In subsequent kinetic studies, CNF inhibited recombinant and human liver microsomal UGT1A9; \( K_i \) values ranged from 1.4 to 3.0 \( \mu M\), depending on the substrate (propofol/4-methylumbelliferone) enzyme combination. \( K_i \) values for CNF inhibition of UGT1A1 were approximately 3-fold higher. Consistent with the activity screening data, DPF was a less potent inhibitor of UGT1A1 and UGT1A9. The \( K_i \) for DPF inhibition of UGT1A1 was 81 \( \mu M\), whereas the \( K_i \) values for inhibition of UGT1A9 ranged from 12 to 15 \( \mu M\). Based on the in vitro \( K_i \) values and plasma concentrations reported in the literature, DPF may be excluded as a perpetrator of DDIs arising from inhibition of UGT enzymes, but CNF inhibition of UGT1A1 and UGT1A9 in vivo cannot be discounted. Since the sodium-glucose cotransporter 2 inhibitors share common structural features, notably a glycoside moiety, investigation of drugs in this class for effects on UGT to identify (or exclude) potential drug-drug interactions is warranted.

Introduction

Type 2 diabetes, which accounts for more than 90% of all cases of diabetes, is a chronic disease characterized by hyperglycemia due to a progressive insulin secretory defect on the background of insulin resistance (American Diabetes Association, 2012). The microvascular changes that occur in patients with diabetes may cause retinopathy, neuropathy, and chronic kidney disease. Further, type 2 diabetes associates with comorbidities, such as hyperlipidemia, hypertension, and stroke. Consequently, type 2 diabetes results in significant morbidity and mortality, which in turn have important social and economic consequences. Indeed, it has been estimated that diabetes accounts for more than 10% of total worldwide healthcare costs for adults, and this is likely to increase into the future given that the number of people with diabetes is increasing in all countries and has been projected to total almost 600 million by 2035 (International Diabetes Foundation, http://www.idf.org/diabetesatlas/update-2014).

Although metformin is the first-line drug for the treatment of type 2 diabetes, combination therapy with another agent is generally required to achieve and maintain recommended levels of glycemic control (American Diabetes Association, 2012; Australian Medicines Handbook 2014). Although most antidiabetic drugs target insulin secretion or insulin action, modulation of glucose homeostasis provides an alternative approach to glycemic control. The kidney plays a critical role in glucose homeostasis through the absorption of filtered glucose. Sodium-glucose cotransporters (SGLT) 1 and 2, located in the proximal convoluted tubule, are together responsible for almost all glucose reabsorption. Of these, the low-affinity high-capacity SGLT2 accounts for approximately 90% of glucose reabsorption under normal circumstances. Based on the early observation that phlorizin, a \( \beta \)-glucoside, inhibits SGLT1 and 2 (Kinne and Castaneda, 2011), a number of \( \beta \)-glucoside inhibitors that specifically inhibit SGLT2 and hence enhance urinary glucose excretion have been developed as antidiabetic agents.

Cangliflozin (CNF) and dapagliflozin (DPF) are the first SGLT2 inhibitors to be approved for clinical use. Both have been demonstrated to improve short-term outcomes in adults with type 2 diabetes (Plosker, 2012; Stenlof et al., 2013; Vasilakou et al., 2013). CNF and DPF are C-glucosides (Fig. 1), and available evidence indicates that glucuronidation of the glucoside moiety is the major metabolic pathway of both compounds in humans. CNF is glucuronidated at the 2- and 3-hydroxyl groups of the glucoside rings; the respective glucuronides are referred to as M5 and M7 (Mamidi et al., 2014). The urinary excretion of M5 and M7 in patients with type 2 diabetes ranges from 7 to 10% and 21 to 32% of the administered dose, respectively (Devini et al., 2013). It has been reported that CNF glucuronidation is catalyzed by
Several glucuronides were observed following incubation of DPF with hepatocytes from various species (Obermeier et al., 2010). One of these, termed M15, was the major metabolite from human hepatocytes. Following administration of radiolabeled DNF to healthy volunteers, DNF plus M15 accounted for >72% of total plasma radioactivity (Obermeier et al., 2010). A later report confirmed that M15, now identified as DPF 3-O-glucuronide, was formed by incubations of human liver, kidney, and intestinal microsomes with UDP-glucuronic acid (Kasichayanula et al., 2013b). Rates of DPF 2-O-glucuronidation by human liver microsomes (HLMs) and kidney microsomes were <5% of DPF 3-O-glucuronide. Peak plasma concentrations of DPF 3-O-glucuronide measured following a single oral 50-mg dose of DPF ranged from approximately 1 μg/l in healthy subjects to 2 μg/l in type 2 diabetes patients with moderate to severe renal impairment (Kasichayanula et al., 2013b). It has been reported that DPF 3-O-glucuronidation is catalyzed by UGT1A9 (Plosker, 2012; Kasichayanula et al., 2013b, 2014; Scheen, 2013b). It has been reported that DPF 3-O-glucuronidation is catalyzed by UGT1A9 (Plosker, 2012; Kasichayanula et al., 2013b, 2014; Scheen, 2014). As with CNF, however, actual data relating to the involvement of UGT1A9 in DPF glucuronidation appear not to have been published.

Materials and Methods

Materials

Alamethicin (from Trichoderma viride), bovine serum albumin (BSA) (essentially fatty acid free), codeine, diclofenac, β-estradiol (β-EST), β-estradiol-3-β-D-glucuronide, hecogenin, 4-methylumbelliferone (4MU), 4-methylumbellifereone β-D-glucuronide, phenylbutazone, propofol (PRO), and UDP-glucuronic acid (sodium salt) were purchased from Sigma-Aldrich (Sydney, Australia); CNF and DPF were purchased from Selleck Chemicals (Houston, TX); and codeine 6-O-β-D-glucuronide was purchased from Toronto Research Chemicals (North York, ON, Canada). Fluconazole, lamotrigine, and lamotrigine N2-β-D-glucuronide were gifts from Pfizer Australia (Sydney, Australia) and Wellcome Research Laboratories (Beckenham, UK), respectively. Supersomes expressing UGT2B4, UGT2B7, UGT2B15, and UGT2B17 and pooled HLM (150 donor pool; equal number of male and female donors) were purchased from Corning Gentest (Tewksbury, MA). Solvents and other reagents used were of analytical reagent grade.

Methods

HLM and Recombinant Human UGTs. Approval for the use of human liver tissue for in vitro drug metabolism studies was obtained from the Southern Adelaide Clinical Research Ethics Committee. HLMs were activated by preincubation with the pore-forming agent alamethicin (50 μg/mg microsomal protein) prior to use in incubations, as described by Boase and Miners (2002).

Human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 cDNAs were stably expressed in a human embryonic kidney (HEK) cell line (HEK293T) according to Uchaipichat et al. (2004). After growth to at least 80% confluence, cells were harvested and washed in phosphate-buffered saline. Cells were subsequently lysed by sonication using a Vibra Cell VCX 130 Ultrasonics Processor (Sonics and Materials, Newtown, CT). Lysates were centrifuged at 12,000g for 1 minute at 4°C, and the supernatant fraction was separated and stored in phosphate buffer (0.1 M, pH 7.4) at −80°C until use. Given the lower expression of UGT2B4, UGT2B7, UGT2B15, and UGT2B17 in HEK293 cells, Supersomes expressing these proteins were used for activity and inhibition studies.

CNF and DPF Inhibition of Recombinant UGT Enzyme Activities. Recombinant human UGT enzyme activities were determined in the absence and presence of CNF or DPF (1, 10, and 100 μM). CNF and DPF stock solutions were prepared in dimethylsulfoxide (DMSO), such that the final concentration of the solvent in the incubations was 0.5% (v/v), which has a negligible or minor effect on most UGT activities (Uchaipichat et al., 2004). An equivalent volume of DMSO was included in control incubations. The effects of CNF and DPF on UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15, and UGT2B17 activities were measured using the nonselective UGT substrate 4MU as the probe. Incubations were performed at a 4MU concentration corresponding to the apparent Km or Skm of each enzyme (Uchaipichat et al., 2004) according to the method described by Lewis et al. (2007). Protein concentrations and incubation time varied for each enzyme (Uchaipichat et al., 2004). Inhibition of UGT1A4 by CNF and DPF was assessed with lamotrigine as the probe substrate according to the method of Rowland et al. (2006), whereas the effects on UGT2B4 activity were determined with codeine as the substrate as described by Raungurat et al. (2010). Concentrations of lamotrigine and codeine used in the UGT1A4 and UGT2B4 inhibition screening studies corresponded to the respective Km or Skm values for each substrate/pair: 1.5 mM for lamotrigine/UGT1A4 and 2.0 mM for codeine/UGT2B4. Positive control inhibitors were used in all inhibition screening experiments: hecogenin (UGT1A4: 10 μM), niflumic acid (UGT1A9: 2.5 μM; UGT1A10: 100 μM), phenylbutazone (UGT1A3, UGT1A6, UGT1A7, UGT1A8, and UGT1A10: 500 μM), fluconazole (UGT2B4 and UGT2B7: 2.5 mM), and diclofenac (UGT2B15 and UGT2B17: 500 μM). The magnitude of inhibition of each positive control inhibitor (data not shown) was as expected from previous studies in this laboratory (Uchaipichat et al., 2004, 2006a,b; Raungurat et al., 2010; Miners et al., 2011). Within- and between-day coefficients for all activity assays, including with HLM as the enzyme source (see below), were <5% and 10%, respectively.

Kinetic Characterization of CNF and DPF Inhibition of Recombinant Human Liver Microsomal UGT1A1 and UGT1A9 Activities. The kinetics and mechanisms of CNF and DPF inhibition of recombinant and human liver microsomal UGT1A1 and UGT1A9 were determined using β-EST and PRO/4MU as the respective probe substrates (Uchaipichat et al., 2004; Miners et al., 2010a). The incubation conditions and analytical procedures used to quantify β-EST, 4MU, and PRO glucuronidation were as described by Miners et al. (2011). As with the inhibition screening studies, CNF and DPF were added to incubations in DMSO, such that the final concentration was 0.5% v/v.

UGT1A1. Experiments to determine the inhibitor constants with UGT1A1 (0.25 mg/ml HEK293 cell lysate) as the enzyme source included four added...
CNF (3, 6, 9, and 12 μM) or DPF (30, 60, 90, and 120 μM) concentrations at each of the three added β-EST (3, 6, and 15 μM) concentrations. Similarly, studies with pooled HLM (0.25 mg/ml) employed four added concentrations of CNF (15, 30, 45, and 90 μM) or DPF (30, 60, 90, and 120 μM) at each of the three added β-EST concentrations specified above.

UGT1A9. Experiments to characterize CNF and DPF inhibition of recombinant UGT1A9 followed the approach described for UGT1A1, except that 4MU and PRO were used as the substrates. The HEK293 cell lysate content of incubations was 0.025 mg/ml (4MU as the substrate) or 0.25 mg/ml (PRO as the substrate). The effects of four added CNF (30, 60, 90, and 120 μM) or DPF (45, 90, 135, and 180 μM) concentrations were investigated at each of the three 4MU concentrations (8, 16, and 32 μM). Similarly, the effects of four added CNF (15, 30, 45, and 60 μM) or DPF (45, 90, 135, and 180 μM) concentrations were investigated at each of the three PRO concentrations: 2, 4, and 8 μM with CNF as the inhibitor, and 10, 15, and 20 μM with DPF as the inhibitor. Inhibition experiments with HLM (0.5 mg/ml) employed only PRO as the probe substrate since 4MU is glucuronidated by multiple UGT enzymes. Experiments employed four added CNF (30, 60, 90, and 120 μM) or DPF concentrations (80, 150, 220, and 300 μM) at each of the three added PRO concentrations (10, 25, and 50 μM). In addition, incubations contained BSA, either 0.5% (CNF) or 1% (DPF) w/v, since measurement of optimal UGT1A9 activity (recombinant and human liver microsomal) requires the presence of BSA to sequester inhibitory long-chain unsaturated fatty acids (see the Results and Discussion sections). Binding of CNF, DPF, β-EST, 4MU, and PRO to enzyme sources, and where relevant, to BSA was corrected for in the presence of BSA to sequester inhibitory long-chain unsaturated fatty acids. Optimal UGT1A9 activity (recombinant and human liver microsomal) requires the presence of BSA to sequester inhibitory long-chain unsaturated fatty acids.

Measurement of the Binding of CNF, DPF, β-EST, 4MU, and PRO to HEK293 Cell Lysate, HLM, and BSA. The binding of DPF to the HEK293 cell lysate (0.025 and 0.25 mg/ml) and HLM (0.25 and 0.5 mg/ml), in the absence and presence of BSA, where indicated, was performed over the concentration range shown in Supplemental Table 1 using a commercial rapid equilibrium dialysis (RED) device (Thermo Scientific, Rockford, IL). The sample chamber contained the enzyme source (± BSA, 0.5 or 1% w/v) and DPF (in DMSO, final concentration 0.5% v/v) in phosphate buffer (0.1 M, pH 7.4), whereas the buffer chamber side contained only phosphate buffer. The respective volumes of the sample and buffer chambers were 400 and 600 μl. Dialysis experiments were performed for 8 hours. Attainment of equilibrium was demonstrated using enzyme-enzyme (HLM or HEK293 cell lysate) and buffer-buffer controls at the lowest and highest DPF concentrations investigated in each experiment.

In contrast to DPF, dialysis was not achieved with CNF in enzyme-enzyme and buffer-buffer controls over 8 hours using the commercial RED device. However, equilibrium was achieved over this time using conventional equilibrium dialysis (employing dialysis cells). Thus, the binding of CNF to the HEK293 cell lysate (0.025 and 0.25 mg/ml) and HLM (0.25 and 0.5 mg/ml) in the absence and presence of BSA, where indicated, was performed over the concentration range shown in Supplemental Table 2 according to the procedure of McLure et al. (2000) using Dianorm equilibrium dialysis cells (Munich, Germany) of 1.2-ml capacity per side, separated by a Spectrapor number 4 dialysis membrane (molecular mass cut-off: 12–14 kDa; Spectrum Medical Industries Inc., Los Angeles, CA). One cell contained the enzyme source (± BSA, 0.5 or 1% w/v) and CNF (in DMSO, final concentration 0.5% v/v) in phosphate buffer (0.1 M, pH 7.4), and the other phosphate buffer alone. The dialysis cell assembly was immersed in a water bath at 37°C and rotated at 12 rpm for 8 hours.

The potential effects of CNF and DPF on the binding of β-EST, 4MU, and PRO to enzyme sources (HEK293 cell lysate and HLM) in the absence and presence of BSA, as appropriate, was assessed over the concentration ranges shown in Supplemental Tables 3–5. β-EST and 4MU binding was measured using the commercial RED device and conditions described for DPF (above). Like CNF, equilibrium was not established in enzyme-enzyme and buffer-buffer controls over 8 hours for PRO using the RED device. However, equilibrium was attained within 8 hours using conventional equilibrium dialysis. Thus, the binding of PRO to incubation constituents was determined as described for CNF.

Following dialysis, an aliquot from each chamber of the RED device or Dianorm equilibrium dialysis cell was treated with four volumes of ice-cold methanol containing 4% glacial acetic acid and centrifuged (5000g for 10 minutes). Concentrations of CNF, DPF, β-EST, 4MU, and PRO were measured by high performance liquid chromatography using the conditions given in Supplemental Table 6. The fraction of each compound bound to incubation constituents (HEK293 cell lysate, HLM, and BSA), f_[inhibitor], was calculated as the concentration of the compound in the buffer cell divided by the concentration in the sample cell.

Data Analysis. All kinetic and inhibition experiments were performed in duplicate, and data points represent the mean of duplicate estimates (<10% variance). K_i values for CNF and DPF inhibition of recombinant and human liver microsomal UGT1A9 activities were determined by fitting the expressions given in eqs. 1–3 to experimental data using Enzfitter (version 2.0; Biosoft, Cambridge, UK), whereas K_i values for CNF and DPF inhibition of recombinant and human liver microsomal UGT1A1 activities were determined by fitting the expressions given in eqs. 4 and 5 to experimental data.

Goodness of fit of all expressions was assessed from a comparison of the parameter SE of fit, coefficient of determination (r^2), 95% confidence intervals, and F-statistic.

Competitive inhibition:

\[ v = \frac{V_{\text{max}} \times [S]}{K_m + [I]/K_i + [S]} \]  

where \( V_{\text{max}} \) is maximal velocity, [S] is substrate concentration, \( K_m \) is the Michaelis constant, [I] is the inhibitor concentration, and \( K_i \) is the inhibitor constant (for the enzyme-inhibitor [EI] complex).

Noncompetitive inhibition:

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

where \( K_i \) and \( K'_i \) are the inhibitor constants for the EI and enzyme-substrate-inhibitor [ESI] complexes.

Mixed (competitive-noncompetitive) inhibition:

\[ v = \frac{V_{\text{max}} \times [S]}{K_m(1 + [I]/K_i) + [S](1 + [I]/K'_i)} \]  

where \( K_i \) and \( K'_i \) are the inhibitor constants for the EI and ESI complexes, respectively.

Competitive inhibition of an enzyme exhibiting sigmoidal kinetics (homotropic positive cooperativity), version 1:

\[ v = \frac{V_{\text{max}} \times S_{0.5}^n}{S_{0.5}^n(1 + [I]/K_i) + [S]^{n/2}} \]  

where \( S_{0.5} \) is the concentration at half \( V_{\text{max}} \), and \( n \) is the Hill coefficient.

Competitive inhibition of an enzyme exhibiting sigmoidal kinetics (homotropic positive cooperativity), version 2:

\[ v = \frac{V_{\text{max}} \times S_{0.5}^n}{S_{0.5}^n(1 + [I]/K_i) + [S]^{n/2}} + S_0^n \]  

Ratio of the areas under the plasma concentration time curve (AUC) of the victim drug in the absence and presence of the inhibitor:

\[ \frac{\text{AUC}_{\text{inhibitor}}}{\text{AUC}_{\text{vehicle}}} = \frac{1}{1 + f_m} \]  

where \( f_m \) is the fraction of the dose metabolized by the enzyme and pathway of interest.

Hepatic inlet concentration of the inhibitor:

\[ [I]_{\text{inlet}} = [I]_{\text{max}} + k_1 \times F_a \times \text{Dose} \]  

where \( [I]_{\text{max}} \), \( k_1 \), \( F_a \), and \( Q_b \) are the maximum drug concentration in the systemic circulation associated with a given dose, absorption rate constant,
fraction absorbed from the gastrointestinal tract, and liver blood flow, respectively.

Results

CNF and DPF Inhibition of Recombinant Human UGT Enzymes. CNF inhibited all UGT1A subfamily enzymes, with IC_{50} values < 50 μM (Fig. 2). The greatest inhibition was observed with UGT1A1, UGT1A9, and UGT1A10, with respective IC_{50} values of 10, 6, and 7 μM. Inhibition of UGT2B7 and UGT2B15 was moderate (IC_{50} values of approximately 50 μM), whereas CNF had a negligible effect on UGT2B4 and UGT2B17 activities. Like CNF, DPF most potently inhibited UGT1A1, UGT1A9, and UGT1A10. However, IC_{50} values (39–66 μM) were approximately 6-fold higher than those for CNF inhibition of these enzymes. Estimated IC_{50} values for DPF inhibition of all other UGT enzymes ranged from 75 to 3500 μM.

It is known that recombinant and human liver microsomal UGT1A9, but not UGT1A1, activities are underestimated in the absence of BSA (see Discussion). Thus, experiments undertaken to characterize the inhibition kinetics of recombinant and human liver microsomal UGT1A9 included BSA in the incubation medium. The binding of CNF to albumin is extensive, and, due to limitations of assay sensitivity, the concentration of BSA added to incubations containing CNF was 0.5% w/v. The somewhat less extensive binding of DPF to albumin permitted kinetic experiments to be performed in the presence of 1% w/v BSA. Experience in this laboratory indicates that the optimal effect of BSA on UGT activities occurs for concentrations in the range of 0.5–2% w/v (for example, Rowland et al., 2007, 2008).

Binding of CNF and DPF to HEK293 Cell Lysate and HLM with and without BSA. The binding of CNF and DPF to HEK293 cell lysate and HLM was determined in the absence and presence of BSA. Data are shown in Supplemental Tables 1 and 2. The non-specific binding of CNF and DPF to each enzyme source (± BSA) was concentration independent over the range studies. The mean f_{unc} values for DPF binding to HLM and HEK293 cell lysate (both 0.25 mg/ml) in the absence of BSA (UGT1A1 inhibition studies) were
0.86 and 0.94, respectively (Supplemental Table 1). The mean \( f_{uyc} \) values for DPF binding to HEK293 cell lysate and HLM in the presence of BSA (1% w/v) ranged from 0.27 to 0.30. The addition of concentrations of \( \beta \)-EST, 4MU, and PRO at the upper end of the ranges used in inhibition experiments did not affect DPF binding.

Similar trends were observed with CNF binding, although binding of CNF to enzyme sources and BSA (0.5% w/v) was higher than for DPF (Supplemental Table 2). The mean \( f_{uyc} \) values for CNF binding to HLM and the HEK293 cell lysate (both 0.25 mg/ml) in the absence of BSA (UGT1A1 inhibition studies) were 0.38 and 0.96, respectively (Supplemental Table 2). When BSA (0.5% w/v) was added to suspensions of HEK293 cell lysate (0.025 and 0.25 mg/ml) and HLM (0.5 mg/ml) (UGT1A9 inhibition studies), CNF binding increased substantially; mean \( f_{uyc} \) values were 0.08–0.12. As with DPF, the addition of concentrations of \( \beta \)-EST, 4MU, and PRO at the upper end of the ranges used in inhibition experiments did not affect CNF binding.

It was further demonstrated that concentrations of CNF and DPF at the upper end of the ranges used in inhibition experiments did not affect 4MU binding to HEK293 cell lysate plus BSA, and PRO

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**Fig. 3.** Dixon plots for canagliflozin inhibition of the enzyme/substrate pairs: (A) UGT1A1/\( \beta \)-EST; (B) HLM/\( \beta \)-EST; (C) UGT1A9+BSA/4MU; (D) UGT1A9+BSA/PRO; and (E) HLM+BSA/PRO. Concentrations of canagliflozin and substrates are corrected for binding to the respective enzyme sources and BSA (0.5% w/v). Points are experimentally derived values (mean of duplicate estimates; < 5% variance), whereas lines are from fitting with eq. 1 (UGT1A9) or eq. 4 (UGT1A1).
binding to HEK293 cell lysate and HLM plus BSA (UGT1A9 inhibition experiments) (Supplemental Tables 3 and 4). Similarly, concentrations of CNF and DPF at the upper end of the ranges used in inhibition experiments did not affect the binding of β-EST binding to the HEK293 cell lysate and HLM (UGT1A1 inhibition experiments) (Supplemental Table 5).

Binding of CNF, DPF, β-EST, 4MU, and PRO to enzyme sources and, where relevant, to BSA was corrected for in the calculation of $K_{i,u}$ values for the inhibition of UGT1A1 and UGT1A9 (below).

**Kinetics of CNF and DPF Inhibition of Recombinant and Human Liver Microsomal UGT1A1 and UGT1A9.** β-EST was used as the UGT1A1 probe substrate, both for the recombinant and human liver microsomal enzymes. β-EST 3-glucuronidation by recombinant UGT1A1 exhibits sigmoidal kinetics, with an $S_{50}$ of 10 μM (Udomuksorn et al., 2007). It was confirmed here that β-EST 3-glucuronidation by HLM also exhibits sigmoidal kinetics, with an $S_{50}$ of 14 μM (data not shown). Experimental data for inhibition of β-EST 3-glucuronidation by CNF and DPF were poorly fit by the expressions for competitive, noncompetitive, and mixed inhibition (eqs. 1–3, Data Analysis), presumably because the concentrations of β-EST employed in the inhibition experiments (3, 6, and 15 μM) spanned the $S_{50}$ and therefore included the early curved and pseudo-linear sections of the
sigmoidal substrate concentration versus velocity plots. Thus, we modified the Hill equation, which describes sigmoidicity, to include an inhibition term analogous to the equation for competitive inhibition of an enzyme exhibiting Michaelis-Menten kinetics (eq. 1, Data Analysis). Both derived equations (eqs. 4 and 5, Data Analysis) described CNF and DPF inhibition of UGT1A1 well, both visually and statistically. However, eq. 5 gave marginally improved fits statistically (Figs. 3 and 4): F-statistic > 4980, r² = 0.999, and standard error of parameter fits < 5%. By contrast, experimental data for CNF and DPF inhibition of recombinant UGT1A9 (4MU and PRO as the substrates) and human liver microsomal UGT1A9 (PRO as the substrate) were well described by the equation for competitive inhibition of an enzyme exhibiting Michaelis-Menten kinetics (eq. 1).

Kinetic plots for CNF and DPF inhibition are shown in Figs. 3 and 4, respectively, and derived $K_{i,u}$ values are given in Table 1. CNF was a potent inhibitor of recombinant and human liver microsomal UGT1A9, with $K_{i,u}$ values in the range of 1.4–3.0 $\mu$M, depending on the substrate/enzyme combination. $K_{i,u}$ values for CNF inhibition of UGT1A1 were approximately 3-fold higher. Consistent with the activity screening data, DPF was a less potent inhibitor of UGT1A1 and UGT1A9. The $K_{i,u}$ for the inhibition of UGT1A1 by both compounds was 81 $\mu$M, whereas $K_{i,u}$ values for the inhibition of UGT1A9 ranged from 12 to 15 $\mu$M.

**Discussion**

CNF and DPF are the first SGLT2 inhibitors to be approved for clinical use. Since many patients with type 2 diabetes present with multiple comorbidities, polypharmacy is common highlighting the need to carefully evaluate potential DDIs. U.S. Food and Drug Administration guidelines now recommend that new drugs are evaluated for their potential to inhibit the major human drug metabolizing cytochrome P450 and UGT enzymes in vitro to assess their potential role as perpetrators of inhibitory DDIs (http://www.fda.gov/downloads/drugs/guidancocomplianceregulatoryinformation/guidances/ucm292362.pdf). As noted in the Introduction, it has been reported that IC₅₀ values for DP inhibition of the major drug metabolizing human liver microsomal P450 enzymes exceed 45 $\mu$M (Obermeier et al., 2010), and the product information for CNF indicates a low propensity for the inhibition of P450 enzyme activities (Invokana Product Information, 2013). However, no systematic investigations of the effects of CNF and DPF on human UGT enzymes have been reported. Here, we demonstrate that CNF is a potent inhibitor of UGT1A1 and UGT1A9 in vitro, whereas DPF inhibition of these enzymes is moderate. UGT1A1 and UGT1A9 are both expressed in liver, whereas UGT1A9 is additionally expressed in the kidney. Hence, inhibition of UGT1A1 and UGT1A9 may potentially result in the reduced clearance of drugs, nondrug xenobiotics, and endogenous compounds that are eliminated by these enzymes.

Long-chain unsaturated fatty acids released from the microsomal membrane during the course of an incubation are known to inhibit UGT1A9, but not UGT1A1, activity, resulting in over-estimation of the $K_{a}$ and $K_{i}$ values of substrates and inhibitors of this enzyme, respectively (Rowland et al., 2008; Manevski et al., 2011; Gill et al., 2012; Walsky et al., 2012). Thus, experiments to determine the $K_{a}$ values for CNF and DPF glucuronidation of recombinant and human liver microsomal UGT1A9 were undertaken in the presence of BSA, which sequesters the inhibitory fatty acids, thereby providing a more accurate value of the inhibitor constant. $K_{i,u}$ values for CNF and DPF inhibition of human liver microsomal and recombinant UGT1A9 were determined with PRO, which is a selective substrate for this enzyme (Miners et al., 2010a). Inhibition of recombinant UGT1A9 was also investigated with 4MU as the probe substrate. Although 4MU is a nonselective UGT substrate, the intrinsic clearance for 4MU glucuronidation by UGT1A9 is very high (Uchaipichat et al., 2004), and it is therefore a valuable substrate for measuring the activity of the recombinant enzyme. $K_{i,u}$ values for CNF inhibition of recombinant and human liver microsomal UGT1A9 with the three enzyme/substrate combinations were close in value (1.4–3.0 $\mu$M), as were $K_{i,u}$ values for DPF inhibition of each of the enzyme/substrate combinations (12–15 $\mu$M) (Table 1). There was also close agreement in the $K_{a}$ values for CNF (7.2–9.1 $\mu$M) and DPF (81 $\mu$M) inhibition of recombinant and human liver microsomal UGT1A1, which were determined using the selective substrate β-EST (Miners et al., 2010a).

The propensity of a compound to act as a perpetrator of inhibitory DDIs may be assessed using eq. 6 (Data Analysis) (Ito et al., 1998; Miners et al., 2010b), where the key term is the $[I]/K_{i}$ ratio. For a victim drug completely metabolized along a single metabolic pathway by a single enzyme, $f_m = 1$ and eq. 6 simplifies to the expression $AUC$ ratio = $(1 + [I]/K_{i})$. In the absence of data that allow calculation of the hepatic input concentration (i.e., $k_{a}$ and/or $F_{a}$), the inhibitor concentration is generally taken as the maximum plasma unbound concentration of the putative perpetrator (for example, Raungrut et al., 2010).

The reported mean maximum plasma concentrations of CNF at steady state for doses of 100 and 300 mg/day (the highest recommended dose) are 1227 $\mu$g/l (2.8 $\mu$M) and 4678 $\mu$g/l (107 $\mu$M), respectively (Devineni et al., 2013). These concentrations are similar to or exceed the $K_{i,u}$ values observed for CNF inhibition of UGT1A1 (1.4–3.0 $\mu$M) and UGT1A1 (7.2–9.1 $\mu$M). Based on these CNF plasma concentrations and $K_{i,u}$ values, approximately 30–40% and 215–245% increases in the AUC ratio for exclusive UGT1A1 substrates are predicted for the 100 and 300 mg/day doses, respectively, using the simplified equation given above. For exclusive UGT1A9 substrates, predicted AUC ratio increases range from 215 to 245% and 450 to 740% for the 100 and 300 mg/day CNF doses, respectively. Assuming a fraction unbound in plasma of 0.01 (Devineni et al., 2013), the corresponding maximum unbound concentrations of CNF in plasma are 0.03 and 0.11 $\mu$M, respectively, both of which are lower than the range of $K_{i,u}$ values observed for CNF inhibition of UGT1A1 and UGT1A9. Although it is expected that the unbound concentration of the inhibitor in blood reflects the drug concentration in hepatocytes, we and others have previously observed that optimal prediction of DDI potential arising from the inhibition of UGT and cytochrome P450 enzymes is obtained using the total perpetrator drug concentration (Ito et al., 2004; Brown et al., 2005; Rowland et al., 2006; Raungrut et al., 2010). Thus, inhibition of UGT1A1 and UGT1A9 by CNF in vivo cannot be discounted. However, it is likely that the magnitude of the predicted increases in

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**TABLE 1**

Derived $K_{i,u}$ values for canagliflozin and dapagliflozin inhibition of recombinant and human liver microsomal UGT1A1 and UGT1A9

<table>
<thead>
<tr>
<th>Enzyme Source/Substrate</th>
<th>$K_{i,u}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNF</td>
<td>DPF</td>
</tr>
<tr>
<td>UGT1A1/β-estradiol</td>
<td>7.2 ± 1.4</td>
</tr>
<tr>
<td>HLM/β-estradiol</td>
<td>9.1 ± 0.2</td>
</tr>
<tr>
<td>UGT1A9/4MU</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>UGT1A9/propranol</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>HLM/propranol</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

*: Standard error of parameter fit.
The mean maximum plasma concentration of DPF (10 mg/day) at steady state is reported as 169 μg/l (0.41 μM), and the unbound fraction in plasma is reported as 0.09 (Plosker 2012; Kasichayanula et al., 2014), providing a maximum unbound concentration of 0.04 μM. Thus, both the mean total and unbound DPF concentrations are low compared with the \( \frac{K_{\text{in}}}{\text{i,u}} \) ratios for inhibition of UGT1A1 and UGT1A9. Interestingly, however, coadministration of the known UGT1A9 inhibitor mefenamic acid (Gagani et al., 2007) increased the area under the plasma concentration time curve of DPF in healthy subjects by 51% (Kasichayanula et al., 2013a).


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