Assays to evaluate UGT glucuronidase inhibition by new chemical entities have become increasingly important tools in drug discovery to evaluate drug-drug interaction (DDI) liabilities. Glucuronidation accounts for approximately 35% of Phase II drug metabolism and represents the primary clearance pathway for ~10% of the most prescribed drugs.

Guidelines provided by European Medicines Agency (EMA) recommend studying inhibition of UGTs if direct glucuronidation is one of the major elimination pathways of the investigational drug. We have recently developed improved and robust methods of assessing inhibition of UGT1A1, UGT1A4, UGT1A6, and UGT2B7 enzymes using pooled human liver microsomes, drug-like specific probe substrates, LC-MS/MS analysis with stable-labeled isotope internal standards. These newly established methods utilize an ultra-low concentration (0.01 mM) of enzyme protein designed to reduce impact of free fatty acids (e.g., arachidonic, linoleic, and oleic acid), which can be potent inhibitors of certain UGTs (UGT1A9 and UGT2B7) and may confound results. Additionally, lower enzyme protein can reduce non-specific binding of the test article. Unlike cytochrome P450 enzymes, UGT enzymes are stable over longer incubation times and, in some cases, we incorporated incubation periods up to 60 minutes to allow for increased metabolite production resulting in improved sensitivity of detection, while retaining initial-rate conditions. The substrate concentration selected for each assay was determined by first characterizing the enzyme kinetics of each reaction. 17β-Estradiol, trifluoperazine, deferiprone, and propranolol (AZT) were the substrates selected for UGT1A1, UGT1A4, UGT1A6, and UGT2B7, respectively. Nicardipine was used as a non-selective control inhibitor for UGT1A1, UGT1A4, UGT1A6, and UGT2B7, which provided IC50 values of 0.20 µM, 2.9 µM, 12 µM, 0.64 µM, and 2.9 µM, respectively.

In conclusion, UGT inhibition assays incorporating ultra-low HLM protein concentrations have become more feasible as the assays were designed to reduce artifacts, they should provide an excellent tool to more confidently evaluate in vivo DDI potential of new chemical entities.

### Results and Discussion

Experiments were conducted for each UGT isoform to select protein concentration and incubation times within the linear range of metabolite formation. Km, and Vmax values were determined for each reaction using non-linear regression (Figure 1). A substrate concentration of 100 nM was used to determine the mean IC50 values for each incubation time. For UGT1A1, UGT1A4, UGT1A6, and UGT2B7, we selected a 10-minute incubation and a final enzyme concentration of 0.5 µM HLM for protein inhibition experiments. Although addition of BSA to an incubation can sequester fatty acids and reverse the inhibitory effects24, this introduces complexity to inhibition assays (e.g., comparison across enzymes unaffected by fatty acids or estimating free fraction). Use of ultra-low concentrations of micromolar protein in vivo assays is expected to reduce assay artifacts and allow more confident evaluation in vivo DDI potential of new chemical entities.

### Materials and Methods

**Materials:** Human liver microsomes (Corning Cat. No. 451171), UGT Reaction Mix Solution A (Corning Cat. No. 451190), 0.5 M Tris HCl, pH 7.5 were obtained from Corning Life Sciences. Substrates, metabolite standards, stable-labeled internal standards, and inhibitors were purchased from SLABV (SLABV Co., Ltd., Tokyo, Japan). 17β-Estradiol, trifluoperazine, nicardipine, propranolol, deoxy-α-D-glucuronic acid, deoxy-β-D-glucuronic acid, deoxy-D-glucuronic acid, deoxypropranolol, deoxypropranolol d5-Glucuronic acid, trifluoperazine d16, d17, propranolol d17, and zidovudine (AZT) were used as stable-labeled internal standards.

**Inhibition Assay:** Incubation mixtures contained probe substrate and UGT Reaction Mix Solution A, probe substrate and UGT Reaction Mix Solution B, and probe substrate and UGT Reaction Mix Solution C in a 96-well plate. The samples were placed in a 37°C water bath for 15 minutes, followed by addition of HLM (0.01 mg/ml) and transferred to a 37°C incubation for 5 minutes, followed by addition of final enzyme concentration of 0.01 mM. Incubation was terminated by addition of equal volume of cold internal standard in acetonitrile with 0.1% formic acid and transferred to a 96-well plate for LC-MS/MS analysis.

**LC-MS/MS Conditions:** The isotope-labeled internal standards were analyzed using an Ultimate 3000 HPLC system coupled with Q-TRAP 4000 QTRAP™ and 4000 LC/MS systems (Applied Biosystems/MDS Sciex) coupled with C18 column (2.1 x 50 mm, 5 µm) with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phases (0.15/0.85 methanol:water in water was used for quantification of deoxypropranolol glucuronide) at a flow rate of 0.5 mL/min. Standard curves were prepared in 0.1 M Tris buffer containing 0.01 mM, pooled HLM and UGT Reaction Mix A and B. LC-MS/MS analysis for each metabolite and stable-labeled internal standard were determined by non-linear regression using XLS (DOS). The IC50 values were determined by using the SigmaPlot four-parameter equation.

**Data Analysis:** Kinetic parameters were determined by non-linear regression using XLS (DOS). For the inhibition assays, IC50 values were determined by using SigmaPlot four-parameter equation. The results were used to evaluate DDI risk and meet regulatory agency requirements. The use of low concentrations of HLM protein (0.01 mM) is expected to reduce assay artifacts associated with inhibitory free fatty acids, membrane partitioning, and inhibitor depletion. Nicardipine was found to be a universal inhibitor of the five UGT enzymes with IC50 values ranging from 0.22 to 12 µM.