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Development of an *In Vitro* Method as a Tool to Assess UDP-Glucuronosyltransferase (UGT) 2B10 Inhibition

Timothy P. Creegan, Lisa Jacob, Ritu Singh, and J. George Zhang
Corning Incorporated, Life Sciences, Corning Gentest℠ Contract Research Services, 6 Henshaw St., Woburn, MA USA

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Abstract

UGT2B10, once thought of as an orphan enzyme, has gained increased relevance in drug development for the ability to catalyze N-glucuronidation of a number of known xenobiotics containing aliphatic tertiary amines and aromatic N-heterocyclic groups[1]. This has prompted the need to screen compounds for their ability to inhibit UGT2B10 metabolism and evaluate the potential for drug-drug interactions (DDIs) when co-administrated with known UGT2B10 substrates. Guidelines provided by European Medicines Agency (EMA) on the investigation of drug interactions recommend studying inhibition of UGTs if direct glucuronidation is one of the major elimination pathways of the investigational drug[2]. We have recently developed a robust assay to assess inhibition of UGT2B10 using cotinine, as a specific probe substrate and amitriptyline as an inhibitor. Following the determination of linear metabolic conditions, the kinetic parameters, K_m and V_{max}, were established in human liver microsomes (HLM) and recombinant UGT2B10. The inhibition assay was developed using low HLM concentration (0.05 mg/mL) to limit the potential of free-fatty acid inhibition and avoid the need for fatty-acid sequestration using BSA. The average IC_{50} with amitriptyline as the inhibitor from two separate experiments was 9.6 µM. Further, the use of cotinine as a reaction phenotyping marker was assessed by testing the metabolism of cotinine with a panel of recombinant UGTs. The data confirmed cotinine metabolism by UGT2B10 with minor contribution from UGT1A4 which is consistent with the known overlapping substrate specificity of the two enzymes from the published results. The kinetic parameters for cotinine in recombinant UGT1A4 were also determined. Selected known UGT2B10 chemical inhibitors were tested at multiple concentrations with a panel of recombinant UGT enzymes to determine the extent of inhibition overlap. In conclusion, an *in vitro* method was developed as a tool to assess the potential of new chemical entities to inhibit UGT2B10 enzyme in accordance with the regulatory requirements.

Introduction

UGT2B10, a member of the UDP-glucuronosyltransferase superfamily, has gained importance in drug discovery screening for its ability to catalyze, like UGT1A4, the N-glucuronidation of many clinically relevant compounds. Robust assays are needed to assess new chemical entities containing aliphatic tertiary amines or aromatic N-heterocyclic groups for their potential to inhibit UGT2B10.

Further, an ultra-low protein concentration used in human liver microsomes (HLMs) assays is preferred to avoid artifacts due to inhibitory effects of long-chain unsaturated fatty acids and allow more confident evaluation of *in vivo* DDI potential.

The goal of this study was to develop an *in vitro* method to assess inhibition of UGT2B10 in HLM by new chemical entities in drug discovery and development programs and provide a tool to evaluate potential inhibition *in vivo*.

Materials and Methods

Materials: HLM (Corning Cat. No. 452117), cDNA-expressing UGT Corning® Supersomes™, UGT Reaction Mix: Solution A containing UDPGA (Corning Cat. No. 451300), UGT Reaction Mix: Solution B containing Tris-HCl (pH 7.5), $MgCl_2$ and alamethicin (Corning Cat. No. 451320) were obtained from Corning Life Sciences. Amitriptyline, cotinine, doxepin hydrochloride, ß-estradiol, β-estradiol 3-(β-D-glucuronide) sodium salt, 7-hydroxy-4-(trifluoromethyl)coumarin (7-HFC), labetalol, mianserin hydrochloride and trifluoperazine (TFP) dihydrochloride were obtained from Sigma. (-)Cotinine N-β-D-glucuronide, (-)cotinine-d3 N-β-D-glucuronide and 4-trifluoromethylumbelliferyl β-D-glucuronide potassium salt were obtained from Toronto Research Chemicals. Trifluoperazine N-Glucuronide and trifluoperazine-d3 N-D-glucuronide were purchased from TLC Pharmaceutical Standards. Sodium 17β-estradiol-16,16,17-d3 3-glucuronide was purchased from CDN Isotopes.

Enzyme Kinetics Assay: Incubation mixture containing 8 mM $MgCl_2$ and 0.025 mg/mL alamethicin in 100 mM Tris-HCl buffer pH 7.5 was placed in a water-bath at 37˚C for 10 minutes, followed by addition of HLM (0.5 mg/mL) or recombinant UGT2B10/1A4 (0.5 mg/mL) and transferred to pre-warmed 96-well microplates (maintained at 37˚C on a heating block). Following a 5-minute pre-incubation period, cotinine (0.28-50 mM or 1.1-200 mM (UGT1A4)) was added and assay initiated with addition of UDPGA (5 mM) and incubated at 37˚C for 120 minutes (**Table 2, Figure 1**).
Inhibition Assay (IC_{50} Determination): Incubation mixture containing 1 mM cotinine, 8 mM $MgCl_2$ and 0.025 mg/mL alamethicin in 100 mM Tris-HCl buffer pH 7.5, was placed in a water-bath at 37˚C for 10 minutes, followed by addition of HLM (0.05 mg/mL) and transferred to pre-warmed 96-well microplates. Following a 5-minute pre-incubation period, amitriptyline or control solvent (DMSO) was added at a final concentration of 1% (v/v). The reaction was initiated with addition of UDPGA (5 mM) and incubated at 37˚C for 60 minutes (**Table 3, Figure 2**).
Reaction Phenotyping Assay: Incubation mixture containing 8 mM $MgCl_2$ and 0.025 mg/mL alamethicin in 100 mM Tris-HCl buffer pH 7.5, was placed in a water-bath at 37˚C for 10 minutes, followed by addition of rUGT (0.5 mg/mL) and transferred to pre-warmed 96-well microplates. Following a 5-minute pre-incubation period, cotinine (5 mM or 20 mM) was added and assay initiated with addition of UDPGA (5 mM) and incubated at 37˚C for 120 minutes (**Figure 3**).
Inhibition Assay (rUGTs with enzyme-specific substrate): Incubation mixture containing rUGTs, 8 mM $MgCl_2$ and 0.025 mg/mL alamethicin in 100 mM Tris-HCl buffer pH 7.5, was transferred to a 96-well microplate. Following a 5-minute pre-incubation period, substrate was added (150 µM estradiol for UGT1A1 and 1A3; 50 µM 7HFC for UGT1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17; 200 µM TFP for UGT1A4; 20 mM cotinine for UGT2B10) followed by amitriptyline, doxepin, mianserin or control solvent. Assay initiated with addition of UDPGA (5 mM) and incubated at 37˚C for 20 minutes (120 minutes for UGT2B10) (**Figure 4**).

All incubations were quenched by addition of stable-labeled internal standards (or labetalol) in acetonitrile, centrifuged and samples transferred to fresh 96-well microplates for LC-MS/MS analysis.

LC-MS/MS Conditions: Cotinine N-β-D-glucuronide and cotinine-d_3 N-β-D-glucuronide quantified by LC-MS/MS using 4000 QTRAP® and API 4000 LC-MS/MS systems (Sciex) coupled with CTC/LEAP or Shimadzu auto-samplers. Samples separated on a HILIC column (4.6 x 100 mm, 5 µm) with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phases at a flow rate of 0.8 mL/min. Standard curves prepared in HLM or UGT control matrix, containing protein, cofactors and buffer at concentrations equal to that of assay samples. LC-MS/MS conditions for cotinine N-β-D-glucuronide and cotinine-d_3 N-β-D-glucuronide are listed in **Table 1**.

Data Analysis: Kinetic parameters determined by non-linear regression using GraphPad Prism. For the inhibition assay, IC_{50} values determined by using sigmoidal Hill four-parameter equation. For K_m/V_{max}, kinetic parameters determined using substrate inhibition model**.**

Figure 1. Enzyme Kinetics for Cotinine in HLM, rUGT2B10, and rUGT1A4 (Inlets Show Eadie Hofstee Plots)

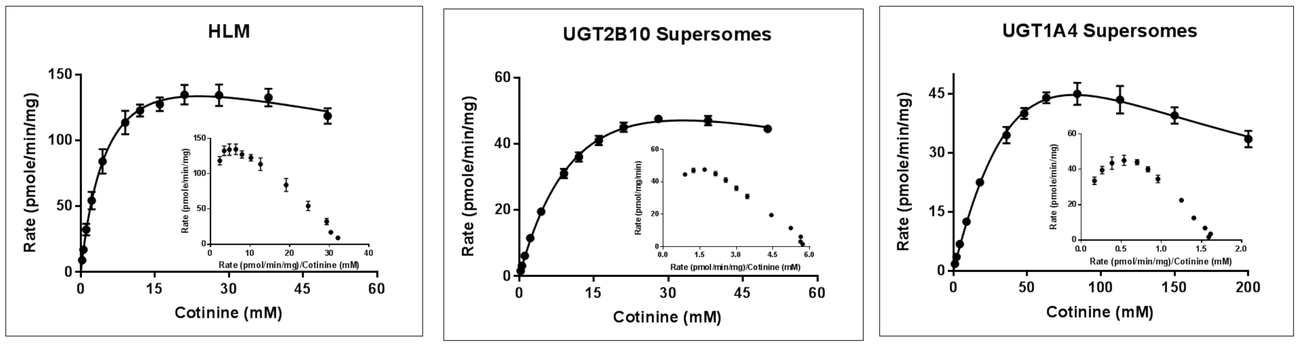


Figure 2. Representative IC_{50} Curve for Inhibition of UGT2B10 in HLM

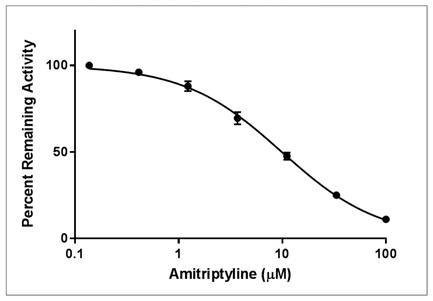


Figure 3. N-Glucuronidation of Cotinine in a Panel of Recombinant UGT Isoforms

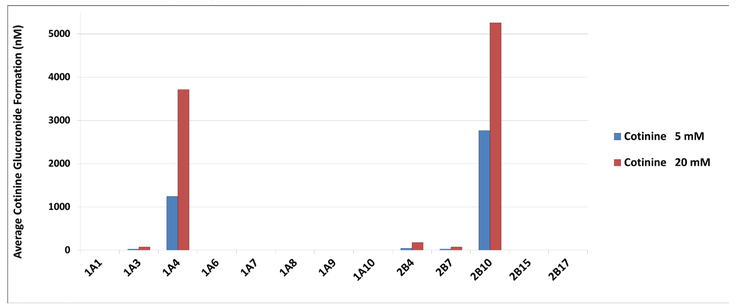


Table 1. LC-MS/MS Conditions for the Analysis of Cotinine N-β-D-glucuronide and Cotinine-d_3 N-β-D-glucuronide

UGT Enzyme	Metabolite and IS	Polarity	MRM transition	Standard Curve Range (nM)
UGT2B10	Cotinine N-ß-D-glucuronide	+	353.2-177.1	9.77-5000
	Cotinine-d3 N-ß-D-glucuronide		356.3-180.0	-

Table 2. Kinetic Parameters in HLM and Recombinant UGT2B10 and UGT1A4

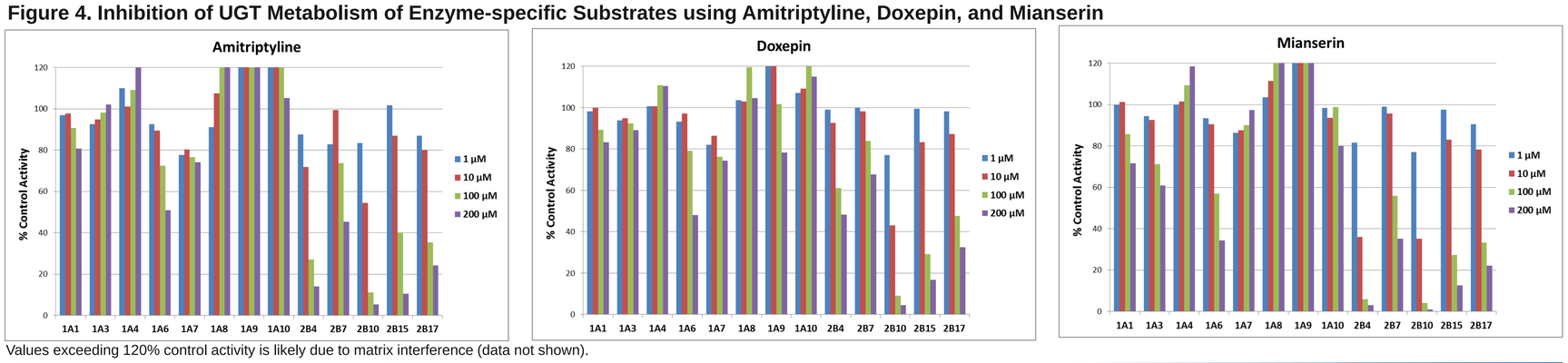
Enzyme Source	Substrate	K_m (mM)	V_{max} (pmol/mg/min)	K_m (V_{max}) Literature Value[1]	Lit. Ref.
HLM	Cotinine	3.2	146	3.3 (297)	1
rUGT2B10		17	97	2.8 (44)	1
rUGT1A4		114	170	-	-

[1] Km values determined using Michaelis-Menton model

Table 3. Inhibition of Cotinine Metabolism in HLM using Amitriptyline

UGT2B10 Enzyme Source	Substrate	Inhibitor	IC50 (µM)			Average IC_{50} (µM)
			Assay 1	Assay 2	Assay 3	
HLM	Cotinine	Amitriptyline	10	9.1	9.8	9.7

Figure 4. Inhibition of UGT Metabolism of Enzyme-specific Substrates using Amitriptyline, Doxepin, and Mianserin



Values exceeding 120% control activity is likely due to matrix interference (data not shown).

Results and Discussion

Experiments were conducted in HLM, and recombinant UGT2B10 (rUGT2B10) Corning Supersomes to determine protein concentration and incubation times within the linear range of metabolite formation. K_m and V_{max} values were determined for each reaction using non-linear regression (**Table 2, Figure 1**). Kinetic parameters were also determined for recombinant UGT1A4 (rUGT1A4) using UGT2B10 assay conditions (**Table 2, Figure 1**). Cotinine N-β-D-glucuronide and stable-labeled isotope were analyzed using the conditions in **Table 1**. Enzyme kinetic parameters for HLM, rUGT2B10, and rUGT1A4 were determined using a substrate inhibition model (listed in **Table 2**). The rUGT1A4 K_m value was approximately 7-fold higher than rUGT2B10 apparent K_m. These data suggest stronger UGT2B10/cotinine affinity and a larger contribution of UGT2B10 to N-glucuronidation of cotinine than UGT1A4 in HLM. These results are in good agreement with previously published data[1].

Amitriptyline, shown to be an inhibitor of UGT2B10[1], was used to evaluate inhibition of cotinine metabolism at 0.05 mg/mL HLM with an incubation time of 60 minutes. Cotinine concentration below the average apparent K_m value was used. Amitriptyline demonstrated potent inhibition with a mean IC_{50} value of 9.7 µM (**Figure 2**).

A panel of cDNA-expressing UGTs (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17) incubated in the presence of 5 mM or 20 mM cotinine for 120 minutes showed glucuronidation by UGT2B10 and, to a relatively lesser extent, by UGT1A4. This observation was expected due to known overlapping substrate specificities[1] for these enzymes and suggests a relatively higher contribution of UGT2B10 to cotinine N-glucuronidation than UGT1A4 (**Figure 3**).

Additionally, cDNA-expressing UGTs panel (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17) were incubated in the presence of known UGT2B10-specific inhibitors, amitriptyline, doxepin, and mianserin[1], at 1 µM, 10 µM, 100 µM, and 200 µM to access UGT2B10 specificity. Amitriptyline showed good UGT2B10 inhibition at 10 µM (45% inhibition), but also showed slight UGT2B4 inhibition (28%). Doxepin showed relatively higher inhibition of UGT2B10 at 10 µM (57% inhibition) with inhibition of all other isoforms less than 15%. Mianserin showed significant inhibition of UGT2B10 at 10 µM (65% inhibition), but also showed significant UGT2B4 inhibition (64%) (**Figure 4**).

Summary and Conclusions

- *In vitro* assay was developed to evaluate inhibition of UGT2B10 enzyme in HLM. The assay may be used to evaluate DDI risk and meet regulatory agency requirements.
- Low HLM protein (0.05 mg/mL) was used to reduce the potential of assay artifacts due to inhibitory-free fatty acids, membrane partitioning, and inhibitor depletion.
- Amitriptyline was used as an inhibitor of UGT2B10 enzyme in HLM. Average IC_{50} value was 9.7 µM.
- Kinetic characterization confirmed higher affinity of UGT2B10 (K_m 17 mM) to N-glucuronidation of cotinine than UGT1A4 (K_m 114 mM).
- Metabolism of cotinine using a panel of cDNA-expressing UGTs showed glucuronidation by UGT2B10 and, to a relatively lesser extent, by UGT1A4. Data suggests a relatively higher contribution of UGT2B10 to cotinine N-glucuronidation than UGT1A4. Data further supported by apparent K_m results.
- Amitriptyline, doxepin, and mianserin demonstrated inhibition of UGT2B10 at all concentrations tested, but only doxepin (10 µM) demonstrated UGT2B10-specific inhibition. All other UGT isoforms experienced less than 15% inhibition at this concentration.
- Doxepin (10 µM) may be used as an inhibitor in reaction phenotyping studies with HLM to assess the contribution of UGT2B10 in the metabolism of new chemical entities.

References

1. Pattanawongsa A, Nair PC, Rowland A, and Miners JO. (2015) Drug Metab Dispos 44:378-388.
2. Fowler S, Kletzl H, Finel M, Manevski N, Schmid P, Tuerck D, Norcross RD, Hoener MC, Spleiss O, and Iglesias VA. (2015) J Pharmacol Exp Ther 352:358-367.
3. European Medicines Agency, “Guideline on the Investigation of Drug Interactions”. January, 2013.

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