22ND NORTH AMERICAN ISSX MEETING MONTRÉAL

P82

Development of UGT1A7 and UGT1A8 Inhibition Assays Using Recombinant Enzymes as a Test System

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Abstract

It is well established that the liver has the greatest abundance of UDP-glucuronosyltransferase (UGT) enzymes, while the kidney and gastro-intestinal tract are considered important extra-hepatic sites for drug metabolism. Although hepatic UGT enzymes play a key role in the metabolism of drugs, the significance of intestinal UGTs in pharmacokinetics and drug-drug interactions has recently gained prominence[1]. UGT1A7, UGT1A8, and UGT1A10 enzymes are expressed mainly in the small intestine and colon and are absent in the liver. Orally administered drugs that are extensively metabolized by intestinal UGT enzymes exhibit poor bioavailability. Drugs that inhibit these isoforms may greatly alter the bioavailability of orally co-administered drugs that are substrates of intestinal UGTs. Guidelines provided by the 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 an investigational drug[2]. Methods to determine inhibition of hepatic UGT enzymes are well established; however, there is a need to develop methods to assess inhibition potential of intestinal enzymes. The purpose of this study was to develop an assay to assess inhibition of UGT1A7 and UGT1A8 isoforms using recombinant enzymes. Propofol served as the probe substrate for both enzymes. Linear metabolic conditions and kinetic parameters were determined in recombinant UGT1A7 and UGT1A8 enzymes. Sigmoidal enzyme kinetics was observed for propofol glucuronidation using recombinant UGT1A7 and UGT1A8 enzymes, with S_{50} values of 45 µM and 81 µM, respectively. Inhibition of propofol glucuronidation was achieved using magnolol (UGT1A7) and troglitazone (UGT1A8) with IC_{50} values of 0.93 and 0.82 µM, respectively. This newly developed *in vitro* method for UGT1A7 and UGT1A8 enzymes can be utilized to assess the inhibition potential of new chemical entities.

Introduction

In addition to the liver and kidney, UGTs are highly expressed in the gastrointestinal tract, where they have the potential to influence the pharmacokinetics and biological effects of ingested drugs and xenobiotic[3]. UGT1A7, 1A8, and 1A10 are enzymes present exclusively in the small intestine and can significantly influence the pre-systemic 'first-pass' metabolism and drug bioavailability, the extent of enterohepatic cycling and the clearance of drugs from plasma[3]. The significance of intestinal UGTs is exemplified by raloxifene, a selective estrogen receptor modulator for the treatment of osteoporosis. Orally administered raloxifene is extensively metabolized in the intestine by UGT1A8, UGT1A10, and UGT1A1, which leads to poor bioavailability (2%)[4].This raises the possibility that drugs that inhibit UGTs expressed in the intestine may greatly alter the bioavailability of co-administered oral drugs, such as raloxifene, which are substrates of these UGTs.

Objective

The goal of this study was to develop *in vitro* methods to assess inhibition of UGT1A7, and UGT1A8 by new chemical entities in drug discovery and development programs using recombinant enzyme test system and provide a tool to improve confidence in the evaluation of potential inhibition *in vivo*.

Materials and Methods

Materials: cDNA-expressing UGT Corning® Supersomes™, 0.5M Tris-HCl buffer, pH 7.5, 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. Propofol, magnolol, troglitazone, and labetalol were obtained from Sigma-Aldrich. Propofol β-D-glucuronide and propofol d-17 β-D-glucuronide were obtained from Toronto Research Chemicals.

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 recombinant UGT1A7/1A8 (0.05 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, propofol (2.8-500 µM) was added and assay initiated with addition of UDPGA (5 mM) and incubated at 37˚C for 30 minutes.

Inhibition Assay (IC_{50} Determination): Incubation mixture containing propofol (30 µM for UGT1A7 and 80 µM for UGT1A8), 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 recombinant UGT1A7/1A8 (0.05 mg/mL) and transferred to pre-warmed 96-well microplates. Following a 5-minute pre-incubation period, magnolol, troglitazone, or control solvent (DMSO) was added at a final concentration of 1% (v/v). The reaction was initiated with addition of UDPGA (5 mM) then incubated at 37˚C for 30 minutes.

Reaction Phenotyping Assay: Incubation mixture containing 8 mM $MgCl_2$ and 0.025 mg/mL alamethicin in 50 mM Tris-HCl buffer pH 7.5, was placed in a water-bath at 37˚C for 10 minutes, followed by addition of recombinant UGT (0.5 mg/mL) and transferred to pre-warmed 96-well microplates. Following a 5-minute pre-incubation period, propofol (10 µM) was added and assay initiated with addition of UDPGA (5 mM) then incubated at 37˚C for 30 minutes. All incubations were quenched by addition of stable-labeled internal standards in acetonitrile, centrifuged, and samples transferred to fresh 96-well microplates for LC-MS/MS analysis.

LC-MS/MS Conditions: Propofol β-D-glucuronide and propofol –d17 β-D-glucuronide was quantified by LC-MS/MS using 4000 QTRAP® or API 4000 LC-MS/MS systems (Sciex) coupled with CTC/LEAP or Shimadzu auto-samplers. Samples were separated on a ZORBAX SB-C_{18} 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 at a flow rate of 0.75 mL/min. Standard curves were prepared in UGT control matrix, containing protein, cofactors and buffer at concentrations equal to that of assay samples. LC-MS/MS conditions for propofol β-D-glucuronide and propofol-d17 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 sigmoidal model.

Table 1. LC-MS/MS Conditions for the Analysis of Propofol β-D-glucuronide and Propofol-d17 β-D-glucuronide

UGT Enzyme	Metabolite and IS	Detection Mode	MRM Transition	Declustering Potential	Collision Energy	Standard Curve Range (nM)
UGT1A7 and UGT1A8	Propofol β-D-glucuronide	Negative	353.3 → 177.1	-64	-37	9.77 - 5000
	Propofol-d17-β-D-glucuronide		370.4 → 194.4	-64	-37	-

Table 2. Kinetic Parameters in Recombinant UGT1A7 and UGT1A8

UGT Enzyme	Substrate	Model	S_{50} (µM)		Vmax (pmol/mg/min.)		Hill Slope	
			Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
UGT1A7	Propofol	Sigmoidal	42	47	3899	5939	1.8	2.1
UGT1A8	Propofol	Sigmoidal	80	81	1627	1497	1.9	2.2

Table 3. Incubation Conditions for the Inhibition Assay and IC_{50} values for UGT1A7 and UGT1A8

UGT Enzyme	Protein Concentration (mg/mL)	Substrate	Substrate Concentration (µM)	Incubation Time (min.)	Inhibitor	IC_{50} (µM)	
						Assay 1	Assay 2
UGT1A7	0.05	Propofol	30	30	Magnolol	0.93	0.88
					Troglitazone	1.6	-
UGT1A8	0.05	Propofol	80	30	Magnolol	34	-
					Troglitazone	0.82	1.1

Results and Discussion

Experiments were conducted in recombinant UGT1A7 and recombinant UGT1A8 Corning Supersomes to determine protein concentration and incubation times within the linear range of metabolite formation. Propofol β-D-glucuronide and stable-labeled isotope were analyzed using the conditions in **Table 1**. Propofol has previously been used as a probe substrate for UGT1A8 isoform and atypical enzyme kinetics have been reported[5]. In the current study, we tested propofol as a substrate for UGT1A7 isoform and compared kinetics with UGT1A8. Sigmoidal kinetics for propofol glucuronidation were observed following incubation with both the isoforms. S_{50} and V_{max} values, as well as Hill slope, were determined for each reaction (**Table 2, Figure 1**). The S_{50} value for UGT1A8 was approximately 2-fold higher than the S_{50} value for UGT1A7. The V_{max} value for UGT1A7 was approximately 3-fold higher than the V_{max} value for UGT1A8. These data suggest that propofol binding affinity is relatively stronger for UGT1A7 than UGT1A8.

Magnolol, a known inhibitor of UGT1A7[6], was used to evaluate inhibition of propofol glucuronidation with UGT1A7 and UGT1A8 (0.05 mg/mL) following an incubation period of 30 minutes. Propofol concentration at or below the average apparent S_{50} value for each isoform was used. Magnolol demonstrated potent inhibition of UGT1A7 with a mean IC_{50} value of 0.91 µM (n=2) (**Table 3**, **Figure 2**), whereas inhibition of UGT1A8 was relatively lower as compared to UGT1A7 with an IC_{50} value of 34 µM. Additionally, troglitazone, which is a known inhibitor of UGT1A8[5], was used to evaluate inhibition of propofol glucuronidation with UGT1A7 and UGT1A8 (0.05 mg/mL) following an incubation period of 30 minutes. Troglitazone was a strong inhibitor of both UGT1A7 and UGT1A8 with IC_{50} values of 1.6 µM and 0.96 µM (n=2), respectively (**Table 3**, **Figure 2**).

Propofol glucuronidation was evaluated in a panel of cDNA-expressing UGTs (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17). As expected, UGT1A9 was the main enzyme responsible for the glucuronidation of propofol. Among the UGT enzymes present exclusively in the intestine (UGT1A7, UGT1A8, and UGT1A10), UGT1A7 and 1A8 are the only enzymes involved in the metabolism of propofol. Although UGT1A10 shares as high as 93% to 94% amino acid sequence similarity with UGT1A7, UGT1A8, and UGT1A9[7], it was interesting to note that it was not involved in the glucuronidation of propofol (**Figure 3**).

References

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Figure 1. Enzyme Kinetics for Propofol in Recombinant UGT1A7 and UGT1A8 (insets show Eadie Hofstee plots)

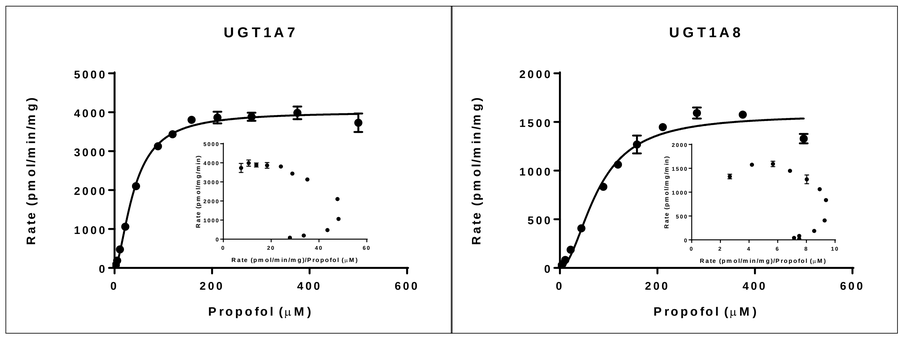


Figure 2. Representative IC_{50} Curve for Inhibition of UGT1A7 and UGT1A8 in Recombinant Enzymes using Magnolol and Troglitazone as Inhibitors

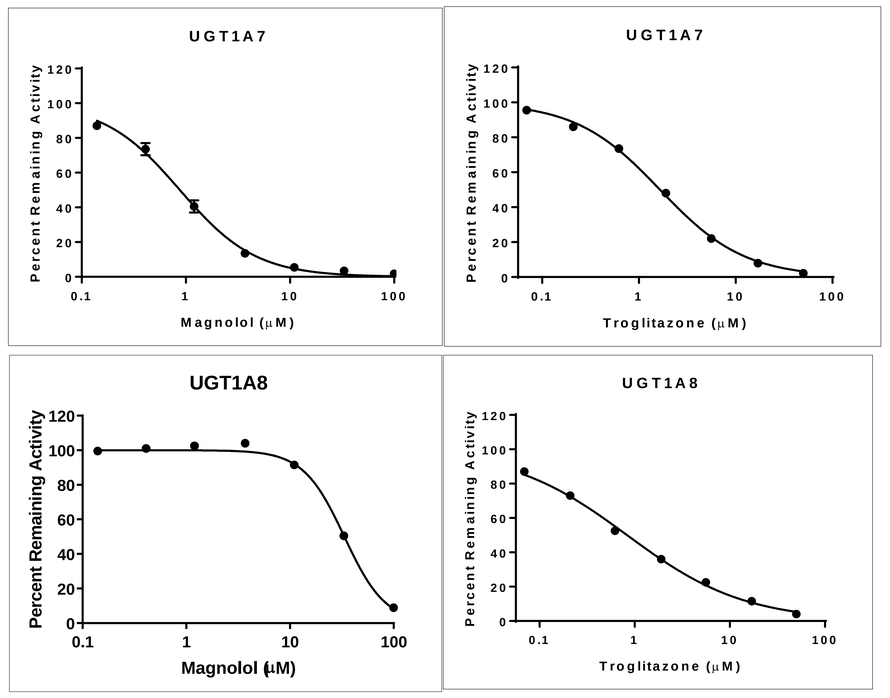
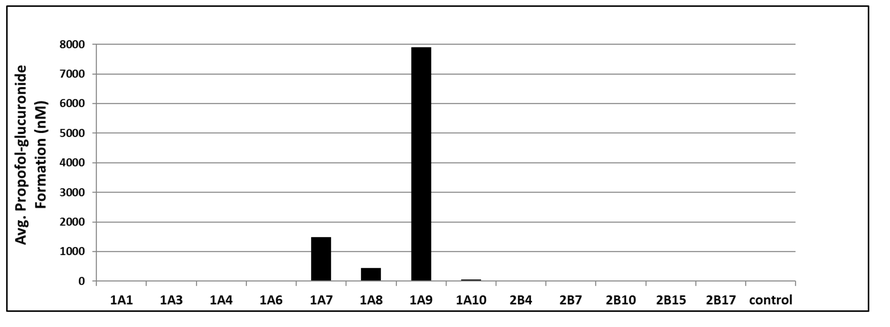


Figure 3. Glucuronidation of Propofol in a Panel of Recombinant UGT Isoforms



Summary and Conclusions

- *In vitro* assays were developed to evaluate inhibition of UGT1A7 and UGT1A8 isoforms using propofol as the probe substrate. These assays can aid in the prediction of *in vivo* DDI potential of orally co-administered drugs that are substrates of intestinal UGT enzymes.
- Magnolol showed potent inhibition of UGT1A7 mediated propofol glucuronidation with an IC_{50} value of 0.91 µM. Inhibition of UGT1A8 mediated propofol glucuronidation by magnolol was relatively lower with an IC_{50} value of 34 µM. The selectivity of magnolol could be evaluated further to use as a tool for UGT reaction phenotyping of enzymes in human intestinal microsomes.
- Troglitazone was a strong inhibitor of UGT1A7 and UGT1A8 mediated propofol glucuronidation with similar IC_{50} values, 1.6 µM and 0.96 µM, respectively.
- Besides UGT1A9, glucuronidation of propofol was found to be mediated by UGT1A7 and UGT1A8, that are enzymes present exclusively in the intestine. UGT1A10 was not involved in the glucuronidation of propofol.
- Further work to determine *in vitro* conditions to test the inhibition of UGT1A10, known to be a pharmacologically important intestinal UGT isoform, is warranted.