Post-transcriptional Regulation of UGT2B10 Hepatic Expression and Activity by Alternative Splicing

Adrien Labriet, Eric P. Allain, Michèle Rouleau, Yannick Audet-Delage, Lyne Villeneuve, and Chantal Guillemette

Pharmacogenomics Laboratory, Centre Hospitalier Universitaire de Québec Research Center and Faculty of Pharmacy, Québec, Canada Research Chair in Pharmacogenomics, Université Laval, Québec, Canada

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ABSTRACT
The detoxification enzyme UDP-glucuronosyltransferase UGT2B10 is specialized in the N-linked glucuronidation of many drugs and xenobiotics. Preferred substrates possess tertiary aliphatic amines and heterocyclic amines, such as tobacco carcinogens and several antidepressants and antipsychotics. We hypothesized that alternative splicing (AS) constitutes a means to regulate steady-state levels of UGT2B10 and enzyme activity. We established the transcriptome of UGT2B10 in normal and tumoral tissues of multiple individuals. The highest expression was in the liver, where 10 AS transcripts represented 50% of the UGT2B10 transcriptome in 50 normal livers and 44 hepatocellular carcinomas. One abundant class of transcripts involves a novel exonic sequence and leads to two alternative (alt.) variants with novel in-frame C termini of 10 or 65 amino acids. Their hepatic expression was highly variable among individuals, correlated with canonical transcript levels, and was 3.5-fold higher in tumors. Evidence for their translation in liver tissues was acquired by mass spectrometry. In cell models, they colocalized with the enzyme and influenced the conjugation of amitriptyline and levomedetomidine by repressing or activating the enzyme (40%–70%; \(P < 0.01\)) in a cell context-specific manner. A high turnover rate for the alt. proteins, regulated by the proteasome, was observed in contrast to the more stable UGT2B10 enzyme. Moreover, a drug-induced remodeling of UGT2B10 splicing was demonstrated in the HepaRG hepatic cell model, which favored alt. variants expression over the canonical transcript. Our findings support a significant contribution of AS in the regulation of UGT2B10 expression in the liver with an impact on enzyme activity.

Introduction

N-linked glucuronidation is an important inactivation route for amine-containing drugs and xenobiotics (Kaivosaaari et al., 2011; Kato et al., 2013). Two of the 19 UDP-glucuronosyltransferases (UGTs), UGT2B10 and UGT1A4, are the main drivers of N-linked glucuronidation (Chen et al., 2008a; Kerdpin et al., 2009; Kato et al., 2013). Long described as an orphan UGT, the discovery that UGT2B10 is crucial for the detoxification of tobacco carcinogens has raised much attention and rationalized the importance of characterizing this unique UGT (Chen et al., 2007, 2008b; Kaivosaaari et al., 2007; Berg et al., 2010; Murphy et al., 2014). UGT2B10 is one of the main liver UGT enzymes based on mRNA [quantitative polymerase chain reaction (PCR) and deep RNA-sequencing data] and protein levels (quantitative mass spectrometry–based proteomics data) (Court et al., 2012; Fallon et al., 2013; Margaillan et al., 2015; Tourancheau et al., 2017). Expression has also been reported in the breast, testis, gallbladder, tongue, and tonsils, although at much lower levels than hepatic expression (Haakensen et al., 2010; Jones and Lazarus, 2014).

UGT2B10 displays a preference for tertiary aliphatic amines and heterocyclic amines. These structures are found in several clinically used drugs, such as antihistamines, antipsychotics, and antidepressants, including several of the tricyclic class such as imipramine and amitriptyline (Kaivosaaari et al., 2008, 2011; Kato et al., 2013; Kazmi et al., 2015; Pattanawongsa et al., 2016), whereas endogenous substrates have yet to be identified. Although UGT1A4 substrate preference significantly overlaps with that of UGT2B10, the latter presents a greater affinity and clearance for many tertiary cyclic amines at a therapeutic concentration (Kato et al., 2013). One structural determinant of the specificity toward amine substrates may be the residues Pro40 of UGT1A4 and Leu34 of UGT2B10, located in their substrate binding domain, a position that is otherwise a strictly conserved histidine residue (His40, coordinates of UGT1A1) in all human UGTs (Kerdpin et al., 2009).

Acknowledgments
The Cancer Genome Atlas; UGT, UDP-glucuronosyltransferase.

ABBREVIATIONS: alt., alternative; AS, alternative splicing; BLAST, Basic Local Alignment Search Tool; CAR, constitutive androstane receptor; Enzo, endoglycosidase; ER, endoplasmic reticulum; FXR, farnesoyl X receptor; G, glucuronide; GTEx, The Genotype-Tissue Expression; GW4064, 2-[2,6-Dichlorophenyl]-4-(3'-carboxy-2-chlorostilbene-4-yl)oxy methyl-5-isopropylisoxazole; i4, isoform 4; i5, isoform 5; MG132, N-Benzoyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal; MRM, multiple reaction monitoring; MS, mass spectrometry; PCR, polymerase chain reaction; PXR, pregnane X receptor; RNA-seq, RNA sequencing; ROS263397, (S)-4-(3-fluoro-2-methyl-phenyl)-4,5-dihydro-oxazol-2-ylamine; SNP, single nucleotide polymorphism; TCGA, The Cancer Genome Atlas; UGT, UDP-glucuronosyltransferase.
Materials and Methods

Analysis of UGT2B10 mRNA Expression

To carefully analyze UGT2B10 expression and AS patterns, raw RNA sequencing (RNA-seq) data were downloaded from public databases and realigned to the recently established UGT transcriptome [Tourancheau et al., 2016; The Genotype-Tissue Expression (GTEX) (http://www.gtexportal.org/home/) and The Cancer Genome Atlas (TCGA) (https://gdc.cancer.gov)]. RNA-seq data sets were obtained from dbGaP at http://www.ncbi.nlm.nih.gov/gap through the database of Genotypes and Phenotypes (dbGaP) accession number phs000424.v6.p1, project ID 13346. The GTEx normal liver data was assessed using the edgeR package for R. Normalized counts were then converted to counts per million or transcripts per million using transcript length. Reverse-transcription PCR analysis of UGT2B10 transcripts was performed as previously described (Tourancheau et al., 2016), using primer sequences provided in Supplemental Table 1. The Basic Local Alignment Search Tool (BLAST) of the NCBI (https://blast.ncbi.nlm.nih.gov/) served to search sequence similarity between the novel UGT2B10 sequences and other genes in humans and other species. The Protein BLAST (blastp) suite was used to search “nonredundant protein sequences” and “reference proteins” whereas the translated nucleotide (blastn) suite was used to search the “nucleotide collection” and “reference RNA sequences” with the unique amino acid sequences of alt. proteins.

Expression Vectors and Human Cell Models (HEK293 and HepG2)

To study alt. transcripts and proteins, expression vectors were produced from the UGT2B10_v1 pcDNA6 construct (Beaulieu et al., 1998) using the Q5 Site-Directed Mutagenesis kit (New England Biolabs Ltd., Whitby, ON, Canada).

Antibodies.
Mass Spectrometry–Multiple Reaction Monitoring. Detection of peptides unique to alt. UGT2B10 was as described (Rouleau et al., 2016) with minor modifications. In brief, human liver S9 fraction (8 mg of proteins Xenotech LLC, Lenexa, KS) was lysed for 45 minutes on ice in a total volume of 4 ml of Lysis Buffer containing 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% (w/v) Igepal CA-630 (Sigma-Aldrich, St. Louis, MO), 1 mM dithiothreitol, and complete protease inhibitor cocktail (Roche, Laval, QC, Canada). Lysates were centrifuged for 15 minutes at 13,000g, and UGT2B10 was immunoprecipitated with 10 μg of the monoclonal anti-UGT2B10 (ab57685; Abcam) for 1 hour at 4°C on an orbital shaker. Protein complexes were captured by an overnight incubation at 4°C with protein G-coated magnetic beads (200 μl of Dynabeads; Thermo Fisher Scientific). Beads were washed in lysis buffer and with 50 mM ammonium bicarbonate and stored at −20°C until analysis. Tryptic digests of UGT2B10 were prepared and analyzed by mass spectrometry (MS)–coupled multiple reaction monitoring (MRM) on a 6500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Sciex, Concord, ON, Canada) as previously described (Rouleau et al., 2016).

In brief, MS analyses were conducted with an ionspray voltage of 2500 V in positive ion mode. Peptides were desalted on a 200-μM × 6-mm chip trap ChromXP C18 column, 3 μM (Eksigent; Scieix), at 2 μM/min solvent A (0.1% formic acid). Peptides were then eluted at a flow rate of 1 μM/min with a 30-minute linear gradient from 5% to 40% solvent B (acetonitrile with 0.1% formic acid) and a 10-minute linear gradient from 40% to 95% solvent B. MRM analyses were performed using the four most intense transitions for each of the target peptides for the light and heavy forms. The UGT2B10 signature peptides were detected in tryptic digests of the immunoprecipitated UGT2B10 samples, and peptide identity was confirmed by coinjection of isotopically labeled [15C6,18N2]lys and [13C6,15N4]Arg synthetic peptides (Pierce Protein Biotechnology, Thermo Fisher Scientific).

Glucuronidation Assays. For enzymatic assays in intact cells (in situ assays), two cell models were used (HEK293 and HepG2 cells). Cells were seeded in 24-well plates (HEK293: 8 × 10^5 cells/well; HepG2: 2.25 × 10^5 cells/well). Assays were initiated 48 hours after seeding by replacing the culture medium with fresh medium (1 ml/well) containing a UGT2B10 substrate (amitriptyline, 7.5 and 75 μM). Cells were incubated for 4 hours, and media were then collected and stored at −20°C until glucuronide (G) quantification by high-performance liquid chromatography–tandem mass spectrometry. Assays were replicated at least two independent experiments in triplicates. Separation of amitriptyline and levomedetomidine was performed on an ACE Phenyl column 3 mm × 4.6 mm (Canadian Life Science, ON, Canada). Isocratic condition with 70% methanol/30% water/3 mM ammonium formate with a flow rate of 0.9 ml/min was used to elute amitriptyline G. A linear gradient was used to elute levomedetomidine G, with 5% methanol/95% water/1 mM ammonium formate as initial conditions followed by a 10-minute linear gradient to 90% methanol/10% water/1 mM ammonium formate. The glucuronides were quantified by tandem mass spectrometry (API 6500; Biosystems-Sciex, Concord, ON, Canada). The following mass ion transitions (m/z) were used: 377.1 for UGT2B10_v1 and 201.1 for UGT2B10_v2.

For a quantitative assessment of UGT2B10 gene expression, we performed a realignment of public GTEX and TCGA RNA-seq data from human tissue samples to the fully annotated UGT variant sequence database. This database was created based on RNA-seq experiments previously conducted with several human tissues (Tourancheau et al., 2016). Data indicated that the UGT2B10 transcriptome is composed of one canonical and 10 alt. transcripts arising from the single UGT2B10 gene. The UGT2B10 alt. transcripts are created by partial intronization of exons 1–2, exon skipping of exons 4–6, and inclusion of novel terminal exons 6b or 6c. UGT2B10 transcripts and encoded isoforms are depicted in Fig. 1. Data revealed UGT2B10 as one of the highest expressed UGTs in normal liver samples, whereas variants arising from alternative splicing represented nearly 50% of the UGT2B10 hepatic transcriptome (Fig. 2A). Some variant classes were remarkably abundant, with levels comparable to those of the canonical UGT2B10 v1 transcript encoding the UGT2B10 enzyme—namely, those with N-terminal truncations and with novel C-terminal sequences (Fig. 2A). Total UGT2B10 expression was 2.15-fold (P = 0.013) higher in hepatocellular carcinoma (n = 44) relative to normal livers (n = 50), and was highly variable among individuals (coefficients of variation of 93%–117%) (Fig. 2B). In contrast to the high hepatic levels, expression of UGT2B10 in other normal tissues surveyed was much lower, with values below 1 transcript per million in the bladder, breast, colon, kidney, lung, prostate, and skin (data not shown).

Alternative Splicing Creates UGT2B10 Variants with Novel In-Frame C-Terminal Sequences Detected in Human Liver at the Protein Level. Splicing events generating UGT2B10 transcripts (n9 and n10) harboring novel C-terminal sequences were of particular interest for this study. These alt. variants are produced by intronization of parts of the canonical exon 6 and inclusion of a novel exon 6c (Fig. 1). These AS events appeared specific to humans and were not noted in other species based on BLAST searches in the nonredundant nucleotide collection and
RNA reference sequence databases. Transcripts were abundant in human livers and were 3.5-fold higher in hepatic tumors relative to normal tissues. They represented, on average, 15% of total UGT2B10 expression in normal liver tissues and 25% in hepatocellular carcinoma tissues (Fig. 2A and B).

The considerable interindividual variability for these alt. transcripts was higher than for the total and canonical UGT2B10 hepatic expression. We also noted a significant positive correlation between the expression of the canonical and alt. transcripts ($r = 0.854 – 0.915$, $P < 0.001$) (Fig. 2C).

The putative UGT2B10 proteins encoded by these alt. transcripts are referred to as UGT2B10 isoforms 4 and 5, named i4 and i5, respectively. They are predicted to retain the substrate and the cosubstrate (UDP-glucuronic acid) binding domains coupled to a novel C-terminal sequence (Fig. 1). Isoform 4 lacks the 43 C-terminal amino acids including the transmembrane domain and the positively charged C-terminal tail of the UGT2B10 enzyme that are replaced by 10 novel amino acids encoded by exon 6c (Fig. 3A). As for isoform 5, because a smaller portion of exon 6 is intronized, the encoded protein is predicted to retain 18 of the 24 transmembrane domain residues and to be extended by 65 novel amino acids, half of which are encoded by a frame shift in exon 6, and half by exon 6c (Fig. 1 and Fig. 3A).

Validation of endogenous protein expression was possible for UGT2B10_i5 through the identification of a unique peptide sequence by targeted MS-MRM of liver UGT2B10 immunoprecipitated from multiple donors (Fig. 3B and C). This result is in line with the detection of the corresponding transcripts by PCR in human livers (Supplemental Fig. 2A). The endogenous expression of UGT2B10_i4 could not be addressed by this approach given the short and hydrophobic nature of the C-terminal unique sequence.

Alternative Isoforms with Novel C-Terminal Sequences Colocalized with the UGT2B10 Enzyme and Modified Its Activity In Vitro. The alt. UGT2B10 isoforms were stably expressed in the embryonic kidney cell line HEK293, devoid of endogenous UGT expression, alone or with the canonical UGT2B10 enzyme. In addition, their expression was examined in the liver cell model HepG2 that endogenously expresses the UGT2B10 enzyme and conjugates substrates of the enzyme, such as amitriptyline and levomedetomidine. We initially confirmed protein expression using immunoblot and immunofluorescence experiments using an anti-UGT2B10 antibody (1845) that targets amino acids encoded by exon 1 and therefore recognizes the three UGT2B10

![Fig. 1. Schematic overview of UGT2B10 mRNA transcripts and encoded proteins.](https://www.ncbi.nlm.nih.gov/gene/7365)


*Nomenclature from Tourancheau et al. (2017)*

Focus of this study

Regulation of UGT2B10 by Alternative Splicing 517 at ASPET Journals on December 4, 2021 dmd.aspetjournals.org Downloaded from
isoforms (Fig. 4A). Indeed, we detected UGT2B10_i4 and UGT2B10_i5 near their predicted molecular masses of 57 and 66 kDa in cell models (Fig. 4A).

The subcellular distribution of each protein was examined in the HEK293 models, where the canonical and alt. proteins were detected and largely restricted to an ER localization. This was confirmed by the colocalization with the ER marker protein disulfide isomerase (Fig. 4B). Each isoform also displayed minor perinuclear and Golgi localization (Supplemental Fig. 3). The glycosylation status of each protein was studied by subjecting microsomes from HEK293 cell models to Endo H glycosidase, which cleaves N-linked sugars on asparagine acquired by ER-resident enzymes, and to O-glycosidase, which removes serine and threonine O-linked complex sugars acquired in the Golgi. Each UGT2B10 protein was sensitive to Endo H treatment, revealed by the shift to a higher mobility protein band upon treatment (Fig. 4C). In contrast, their mobility was not affected by a treatment with O-glycosidase.

Enzymatic assays in intact cells were subsequently conducted in the hepatic cell model HepG2 that expresses the endogenous UGT2B10 enzyme. Compared with the reference cell line (stably transfected with pcDNA6), expression of alt. UGT2B10_i4 isofom enhanced the glucuronidation activity of the UGT2B10 enzyme with two drug substrates, amitriptyline and levomedetomidine, by 1.5- to 2-fold (Fig. 5, A and B). In turn, the presence of alt. UGT2B10_i5 significantly impaired the glucuronidation of amitriptyline and tended to reduce that of levomedetomidine as well by 20%–25%. Glucuronidation assays conducted with the UGT-negative HEK293 cells stably expressing either alt. UGT2B10 isoforms revealed no transferase activity for amitriptyline and levomedetomidine. When coexpressed with the UGT2B10 enzyme, we observed a significant 23%–65% inhibition of glucuronidation activity by HEK293 cells in the presence of the alt. i4 or i5 proteins (Fig. 5, A and B). Since we observed a colocalization of alt. isoforms and the UGT2B10 enzyme in the ER, we addressed their potential interaction as a possible regulatory mechanism. Immunoprecipitations were conducted with an anti-V5 epitope antibody using cell models stably expressing the UGT2B10 enzyme and transiently expressing either alt. isofom tagged with the V5 epitope. The UGT2B10 enzyme. Compared with the reference cell line (stably transfected with pcDNA6), expression of alt. UGT2B10_i4 isofom enhanced the glucuronidation activity of the UGT2B10 enzyme with two drug substrates, amitriptyline and levomedetomidine, by 1.5- to 2-fold (Fig. 5, A and B). In turn, the presence of alt. UGT2B10_i5 significantly impaired the glucuronidation of amitriptyline and tended to reduce that of levomedetomidine as well by 20%–25%. Glucuronidation assays conducted with the UGT-negative HEK293 cells stably expressing either alt. UGT2B10 isoforms revealed no transferase activity for amitriptyline and levomedetomidine. When coexpressed with the UGT2B10 enzyme, we observed a significant 23%–65% inhibition of glucuronidation activity by HEK293 cells in the presence of the alt. i4 or i5 proteins (Fig. 5, A and B). Since we observed a colocalization of alt. isoforms and the UGT2B10 enzyme in the ER, we addressed their potential interaction as a possible regulatory mechanism. Immunoprecipitations were conducted with an anti-V5 epitope antibody using cell models stably expressing the UGT2B10 enzyme and transiently expressing either alt. isofom tagged with the V5 epitope. The UGT2B10 Fig. 2. Alternative splicing diversifies the hepatic UGT2B10 transcriptome. (A) Relative levels of canonical (v1) and alt. transcript classes in the normal human liver and in hepatocellular carcinoma. The ΔC-term+new sequence class is predominantly composed of n9 and n10 transcripts, whereas transcript n8 represents less than 2% in normal and tumor tissues. (B) Interindividual variability in UGT2B10 expression for all transcripts (total), canonical (v1), and alt. transcripts (n9n10). Boxes represent 25–75 percentiles, whiskers 10–90 percentiles. Median is indicated by the horizontal line and mean by a “+.” (C) Correlation between UGT2B10_v1 and n9n10 expression in normal livers and hepatocellular carcinoma. All expression data were derived by a realignment of RNA-seq data from GTEx (n = 50) and TCGA (n = 44) to the fully annotated UGT variant sequence (Tourancheau et al., 2016). CV, coefficient of variation; FC, fold change; N, normal tissues; T, tumor tissues; TPM, transcripts per million.
enzyme was immunoprecipitated with each alt. isofrom, indicating their ability to form complexes (Fig. 3C).

Alternative Isoforms Have Shorter Half-Lives than the UGT2B10 Enzyme and Are Targeted for Degradation by the Proteasome. Protein stability was evaluated in both cell models. The UGT2B10 enzyme displayed a half-life over 16 hours in both HEK293 (exogenous expression) and HepG2 (endogenous expression) cell models, whereas the alt. isoforms displayed superior turnover rates. The alt. UGT2B10_i5 was the least stable, with short half-lives of 1.9 and 0.7 hours in HEK293 and HepG2, respectively (Fig. 6A). The turnover rate of isoform i4 differed between the two cell models, and was 11.5 hours in HEK293 but much shorter in HepG2 (1.5 hours). In HepG2 cells, we noted a significant recovery of alt. protein expression, even rising above those of nontreated cells by 16 hours after initiation of the cycloheximide treatment. This was not observed for the UGT2B10 enzyme, nor for any of the UGT2B10 proteins in HEK293 cells. This observation implied a possible inactivation in HepG2 cells, which was confirmed by the detection of two glucuronides of cycloheximide (G1 and G2) with HepG2 microsomes (Supplemental Fig. 5). This was further validated using microsomal fractions of pooled human livers and UGT supersomes. In these experiments, UGT1A3 and UGT1A4 (G1) as well as UGT1A1 and UGT2B4 (G1 and G2) also conjugated some cycloheximide (Supplemental Fig. 5), with some of them detected in HepG2 cells and not in the HEK293 cell model. As a consequence, this may lead to an inaccurate assessment of UGT protein half-lives using this approach in the HepG2 model.

Accordingly, the difference in protein stability between the UGT2B10 enzyme and alt. proteins was further addressed by proteasomal inhibition. Whereas the enzyme levels were nearly unperturbed by inhibition of the ubiquitin-proteasome system, alt. UGT2B10 and, more particularly, isoform i5 were stabilized, indicating that they were degraded via the ubiquitin-proteasome system. Proteasomal inhibition for 16 hours increased the ratio of alt. isoform/UGT2B10 enzyme in HepG2 liver cell models, whereas stabilization was more modest in HEK293 (Fig. 6B). Increased levels of alt. isoforms were not derived from enhanced transcription, verified at the mRNA level (Supplemental Fig. 2B).

Differential Induction of UGT2B10 Alternative Transcripts in Liver Cells by Phenobarbital and a Constitutive Androstane Receptor Agonist. HepaRG cells constitute a good surrogate system to study hepatic functions and response to drug treatments. An analysis of public HepaRG RNA-seq data (Li et al., 2015) with the exhaustive UGT transcriptome revealed an expression of canonical and alt. UGT2B10 in

Fig. 3. Alternate UGT2B10 protein is expressed in the human liver. (A) C-terminal amino acid sequences of the UGT2B10 enzyme (i1) and alt. isoforms i4 and i5. Sequences unique to each alt. proteins are italicized. (B) Experimental approach for the detection of UGT2B10_i5 by immunoprecipitation and MRM. (C) The common UGT2B10 peptide NSWNFK (left) and the alt. specific peptide LLGSSNPPILASQR (right) were detected in tryptic digests of UGT2B10 immunoprecipitated from human liver samples (upper chromatograms). The chromatograms of control peptides (lower chromatograms) labeled with stable isotopes mixed with the immunopurified UGT2B10 confirmed the identity of i1 and i5 peptides. Representative chromatograms are shown (n = 2). K, ER retention signal; TMD, transmembrane domain.
The recent expansion of the pharmacogene transcriptome by AS has shed light on a novel mechanism regulating drug metabolism and clearance (Bellemare et al., 2010a;b; Guillaumette et al., 2010, 2014; Rouleau et al., 2014, 2016; Chhibber et al., 2016; Tourancheau et al., 2016, 2017). Our study of the UGT2B10 transcriptome, encoding a key detoxification enzyme specialized in N-glucuronidation of multiple harmful xenobiotics (Kaivosaa et al., 2007, 2011; Kato et al., 2013), demonstrated that AS accounts for a large proportion of UGT2B10 gene expression, especially in the liver. This observation held true in liver tumors, where UGT2B10 expression was enhanced 2-fold in hepatocellular carcinoma. Indeed, our analysis of next-generation sequencing data revealed that UGT2B10 expression prevails in the liver, whereas in all other tissues surveyed, including the lung, its expression was low to undetected. This is consistent with the expression determined at the RNA level in several human tissues, including those of the aerodigestive tract (Ohno and Nakajin, 2011; Court et al., 2012; Jones and Lazarus, 2014). This supports that a main detoxification site of UGT2B10-dependent N-glucuronidation is the liver, where UGT2B10 is one of the most abundant UGT enzyme based on proteomics data (Fallon et al., 2013; Sato et al., 2014; Marguillan et al., 2015). In addition, AS also provides an explanation for the multiple observations reporting a lack of correlation between mRNA and protein expression, such as in hepatocellular carcinoma, where the RNA expression remained equivalent between tumor tissues and adjacent normal tissues, whereas glucuronidation activity was drastically decreased (Lu et al., 2015).

With a focus on one abundant class of hepatic alt. UGT2B10 variants containing a novel 3′ terminal exon that were confirmed at the protein level in human liver samples and in heterologous expression models, we exposed their regulated expression and influence on UGT2B10 enzyme activity in vitro. In fact, the transcriptional regulation of UGT2B10 has been poorly studied. A response element for the bile acid-sensing farnesoyl X receptor (FXR) was recently uncovered in the UGT2B10 promoter region and participated in the induction of UGT2B10 by the FXR agonists GW4064 (3-(2,6-Dichlorophenyl)-4-(3′-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole) and chenodeoxycholic acid (Lu et al., 2017a). In the cell model HepaRG, a surrogate to human primary hepatocytes in drug-metabolism studies (Antherieu et al., 2012), RNA-seq data revealed drug-induced regulation of the UGT2B10 transcriptome by both the CAR and pregnane X receptor (PXR). The superior induction of alt. UGT2B10 by CAR and PXR agonists observed herein, especially in HepaRG cells devoid of CAR expression, further raises the possibility of a PXR-dependent remodeling of splicing events at the UGT2B10 locus that may significantly influence UGT2B10 detoxification activity. Whether other receptors such as FXR, previously reported to regulate UGT2B10 transcription (Lu et al., 2017a), also influence splicing remains to be addressed. When expressed in human cells, alt. UGT2B10 acted as a regulator of the glucuronidation activity of the UGT2B10 enzyme, possibly conveyed by heterologous complexes formed between the enzyme and alternative proteins, a regulatory mechanism documented for other human UGTs (Bushey and Lazarus, 2012; Menard et al., 2013; Rouleau et al., 2014, 2016). Our findings further suggest a cell-specific influence given that, in HepaRG cells, an increased N-glucuronidation of the UGT2B10 substrates aminopyrine and levomeodetomidine by the endogenous enzyme was observed, in contrast with a repression of enzyme activity in HEK293 cells. The endogenous expression of additional UGTs other than UGT2B10 in HepG2 cells and different protein turnover rates could be among factors influencing their functions. Likewise, the impact of AS on UGT2B10 activity has been documented previously by findings of a common polymorphism (rs116294140) that disrupts a splice site in exon 3 and introduces a premature stop codon possibly triggering non-sense mRNA decay (Fowler et al., 2015). This polymorphism, particularly more frequent among African Americans, significantly reduced N-glucuronidation of drugs such as ROS263397 as well as nicotine and cotinine (Murphy et al., 2014; Fowler et al., 2015). In fact, occurrences of this splice site variant as well as the coding variant Asp67Tyr (rs61750900) were estimated to collectively explain over 24% of interindividual variability in cotinine glucuronidation (Patel et al., 2015). Our results support that alternative splicing at the UGT2B10 locus may be a major factor contributing to this variability in the constitutive expression of the gene, with a potential impact on responses to substrates of the UGT2B10 pathway.

The alternate UGT2B10 proteins with novel in-frame C-terminal sequences are predicted to include the entire putative catalytic domain (Radominska-Pandya et al., 2010), although their enzyme activity could...
not be confirmed in standard in vitro assay conditions when expressed in HEK293 human cells. This could be due to an inadequate topology of the alt. proteins essential for enzyme function. In silico analysis of the novel exonic sequence with NCBI BLAST tools did not reveal a match with other nucleotide or amino acid sequences of any organism. The splicing event at the UGT2B10 locus appears specific to humans. This is consistent with the low to undetectable activity toward preferred UGT2B10 substrates such as N-heterocyclic amines and aliphatic tertiary amines in most primates and other mammals, suggesting that UGT2B10 expression occurs preferentially in humans (Kaivosaari et al., 2007, 2008; Zhou et al., 2010; Lu et al., 2017b). The novel appended amino acid sequence encoded in the alternative frame by exon 6 is, however, related to that of putative alternative variants of several other human proteins, including CLCN3 (chloride exchange transporter 3), ALG9 (alpha-1, 2-mannosyltransferase), and CHFR (E3-ubiquitin ligase), supporting that the new sequence may encode a conserved domain (Supplemental Fig. 6).

As for subcellular localization, our immunofluorescence data indicated that the alt. isoforms, partially or completely lacking the transmembrane domain and devoid of the positively charged lysine tail (Fig. 3), are nonetheless ER-resident proteins when expressed in HEK293 human cells. Although studied in a limited set of UGTs, the high sequence similarity of ER retention elements among UGTs supports a common ER retention mechanism. ER residency of UGT enzymes is mediated by at least four protein regions: the N-terminal signal peptide, a short hydrophobic patch in the N-terminal substrate binding domain, the C-terminal transmembrane region, and the cytoplasmic dilysine motif containing the ER retention signal “KXXKK” (K, lysines; X, any amino acids) (Jackson et al., 1993; Ciotti et al., 1998; Ouzzine et al., 1999; Barre et al., 2005). In line with our observations, these structural features may be partially redundant, as none appears strictly essential to ER retention. The Endo H sensitivity and lack of sensitivity toward O-glycosidase for the UGT2B10 enzyme and alt. UGT2B10 also support ER residency. Consistent with their colocalization, the potential of alt. UGT2B10 to interact with the UGT2B10 enzyme suggests that this may be a mechanism underlying their regulatory function.

The preferential stabilization of alt. isoforms by proteasomal inhibition and their shorter half-lives relative to the UGT2B10 enzyme suggest that distinct pathways may govern the turnover of alt. isoforms and the enzyme. This also suggests that a small variation in RNA levels has the potential to affect AS protein expression level. The significant abundance of alt. transcripts encoding these proteins may indicate that hepatic cells are poised to adapt levels of regulatory isoforms in response to various endogenous or exogenous stimuli. This is supported by the preferential mRNA expression of alternates in HepaRG cells treated with nuclear receptor agonists. Although the structural determinants of UGT protein stability are scarcely known, mechanisms modulating UGT turnover may significantly contribute to the regulation of their detoxifying functions, a well documented aspect for some drug-metabolizing cytochrome P450s (Zhukov and Ingelman-Sundberg, 1999; Kim et al., 2016 and references therein). When expressed in the HEK293 model, half-lives of UGTs were more than 12–16 hours for...
UGT2B4, UGT2B7, UGT2B10, and UGT2B15, whereas UGT1A1 and UGT2B17 were more labile with half-lives less than 3 hours (Turgeon et al., 2001; Rouleau et al., 2016; this study). However, given that cycloheximide is glucuronidated by several UGT enzymes as demonstrated herein, alt. UGT protein half-lives established by translational inhibition with this compound in cells expressing UGTs likely constitute an inaccurate estimate that will be influenced by the enzymes expressed.

A limitation of our study is the lack of detection of alt. UGT2B10 by immunoblotting in pooled liver microsomes from 50 individuals using the polyclonal anti-UGT2B10 antibody #1845. Their expression varied widely among individuals (coefficient of variation of 155%), with some having no or barely detectable hepatic alt. UGT2B10 variants. Levels of alt. proteins in our human liver pool may be too low for detection by immunoblotting. In contrast, the detection of alt. UGT2B10 by MS-MRM was performed following an immunoprecipitation step that enriched UGT2B10, thus improving the sensitivity of detection. The high turnover rate of the alt. proteins, as observed in the two cell models, may also influence our ability to detect them in human livers by immunoblotting. Additional experiments are required to ascertain the expression of alt. UGT2B10 proteins in individual human liver samples and their expression ratio relative to the UGT2B10 enzyme.

In conclusion, our study reveals that AS creates a diversified UGT2B10 transcriptome and represents half of the UGT2B10 expression in the human liver, with a wide interindividual variability. Alternate UGT2B10 proteins may significantly influence the UGT2B10-dependent detoxification of amine-containing drugs, such as antipsychotic and tobacco metabolites, and are expected to modulate endogenous substrates of the UGT2B10 enzyme, which are currently unknown. Our study further highlights the long-term stability of the UGT2B10 enzyme that contrasts with the lability of alt. proteins, the latter being regulated by proteasomal degradation. Most interestingly, we further exposed a preferential induction by PXR and CAR inducers of alt. UGT2B10 with novel in-frame C-terminal sequences in hepatic cells, implying a fine regulation of the AS process by xenosensing transcription factors. Our study highlights an important regulatory role of AS in UGT2B10 expression and detoxification functions that may explain part of the significant variability in N-glucuronidation, largely mediated by the UGT2B10 pathway in the liver. We thus believe that interindividual
Fig. 7. Differential induction of alt. UGT2B10 by phenobarbital and a CAR agonist in HepaRG cells. Expression data for UGT2B10 canonical and alt. transcripts n/0 and n/10 (encoding i and j) in HepaRG cells, wild type (WT) or with a CAR knockout (KO), were obtained from the public RNA-seq data GSE71446. Cells were treated either with vehicle [dimethylsulfoxide (DMSO)], phenobarbital (PB; 1 μM), or the CAR agonist CTCG (1 μM) for 24 hours as previously described (Li et al., 2015). **P < 0.01; ***P < 0.001.

differences in the clinical response to UGT2B10 substrates are likely to be understood through the AS process affecting both the constitutive and inducible expression of UGT2B10.

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Authorship Contributions

Participated in research design: Labriet, Allain, Rouleau,Audet-Delage,Guillemette.

Conducted experiments: Labriet, Allain,Audet-Delage, Villeneuve.

Performed data analysis: Labriet,Allain,Rouleau,Audet-Delage,Villeneuve,Guillemette.

Wrote or contributed to the writing of the manuscript: Labriet,Rouleau,Guillemette.

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**Address correspondence to:** Dr. Chantal Guillemette, Pharmacogenomics Laboratory, Centre Hospitalier Universitaire de Québec Research Center, Université Laval, 2705 Boul. Laurier, R4701.5, Québec, Canada, G1V 4G2. E-mail: Chantal.Guillemette@crchudequebec.ulaval.ca
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Fig. S1. Specificity of anti-UGT2B10 #1845 for human UGT enzymes. The microsomal fraction prepared from HEK293 cells (UGT negative) stably expressing each of the seven UGT2B enzymes or transfected with the empty vector pcDNA6 (-) was probed with the anti-UGT2B10 to demonstrate specificity. HL, human liver microsomes. Short and long exposures of the same immunoblot is provided to ascertain specificity.
Fig. S2. RT-PCR amplification of UGT2B10 transcripts. A. Validation of UGT2B10_n9 and UGT2B10_n10 expression in human liver. Primers (arrows; 4203F and 4211R) were located in exon 1 and in exon 6c common to both variants. PCR products corresponding to n9 and n10 could not be distinguished because they differed by only 71 base pairs (bp). The identity of PCR products as n9 and n10 was verified by Sanger sequencing. B. RT-PCR detection of transcripts in cell models exposed to MG132. PCR strategies specified on the right either amplified both v1 and n10 or only n10. GAPDH served as a positive and loading control. L: molecular weight ladder.
Fig. S3. Subcellular localization of UGT2B10 and alternative isoforms expressed in HEK293. UGT2B10 proteins were detected with #1845, and cellular compartments with specific markers as described in Materials and Methods. In merged images, nuclei are stained with DRAQ5. Bar represents 10 µm. Control HEK293 cells were transfected with the empty vector pcDNA6.
Fig. S4. Stability of UGT2B10 proteins - Replicate assay. The half-life of UGT2B10 proteins were determined by translational inhibition with cycloheximide (CHX) in HEK293 and HepG2 cell models. Protein levels were measured by densitometry scanning of immunoblots from two independent assays. Half-lives were averaged from the two biological replicates. Related to Fig.6A.
Fig. S5. Analysis of cycloheximide glucuronidation by high-performance liquid chromatography tandem mass spectrometry. A. Human liver microsomes produced two cycloheximide glucuronides (G1 and G2). Representative chromatograms of cycloheximide glucuronides (top) and their fragmentation pattern (bottom). Putative glucuronidation sites are indicated by blue arrows in the chemical structure of cycloheximide. B. Glucuronidation activity of microsomes from human liver, HepG2 pcDNA6, HEK-UGT2B11 and of commercial UGT supersomes. Two independent assays were conducted in triplicate.
Fig. S6. Sequence similarity of the UGT2B10_i5 unique sequence with other alt. human proteins. A BLASTp search with the unique C-terminal UGT2B10_i5 amino acid sequence revealed a conserved region encoded by the frame shift in exon 6 (boxed). asterisk: conserved amino acid; colon: conservative substitution. Alignment was produced with the Clustal Omega tool available at www.Uniprot.org.