Glucuronidation activity of individual UDP-glucuronosyltransferases

Comparison of activity and substrate specificity among recombinant human UGT enzymes and differences between dog and human subfamily 1A UGT enzymes

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ACADEMIC DISSERTATION

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Abstract

Cells express UDP-glucuronosyltransferases (UGTs) in tissues and organs along the routes of xenobiotics uptake, excretion and detoxification. UGTs are a group of important conjugation enzymes that transfer a sugar moiety from the cofactor, UDP-α-glucuronic acid, onto a nucleophilic group in substrate molecules. This glucuronidation reaction converts compounds into more water-soluble forms, and thereby enhances their excretion into bile, feces or urine.

Frequently prescribed drugs are often eliminated, directly or after oxidative or hydrolytic metabolism, through glucuronidation. Normally, several of the 19 human UGTs may participate in the glucuronidation of a certain drug, but at different rates. Occasionally, however, the formed glucuronide originates from the activity of just one particular UGT. In such cases, the drug’s glucuronidation rate depends on the enzyme’s expression level, and any polymorphism that affects either activity or regulation, could affect the conjugation rate. If the drug’s clearance is altered, it can influence drug efficacy, risks of side effects, and interactions with other drugs. Therefore, the goal of the published papers in this thesis is not only to determine the overall formation of glucuronides, but to characterize the activity of individual enzymes.

In Dr. Finel’s laboratory, we expressed the majority of UGTs used in these studies as recombinant proteins in Sf9 insect cells. Producing our own enzymes gave me an advantage of having sufficient amounts of human UGTs, including some UGTs that are not commercially available or have low activity. In addition, this enabled us to prepare variant enzymes with non-synonymous mutations, as well as to clone and express dog UGTs. The enzymatic activity was measured by detecting the product formation by HPLC and in some cases the confirmation of the glucuronide was done by LC-MS/MS. The experimental design was to express UGT enzymes and test their individual activities with several drugs or other commonly used probe substrates. In some publications, results with cloned, expressed enzymes were compared to results from glucuronidation activity in hepatic and intestinal microsomes.

The present thesis compares the glucuronidation activities at four levels: between variants or UGT preparations, between different UGTs, between UGT subfamilies and between human and dog UGT1A subfamily enzymes.

At the single enzyme level, the published results revealed that UGT1A4-P24T polymorphism affects the signal sequence cleavage. Consequently, the change in the cleavage site influences the length of the mature protein, at least with recombinant enzymes, leading to altered enzyme kinetics. The observed kinetic behavior suggests that the variant enzyme consists of active and inactive forms of UGT1A4.

Similarly, an inactive, improperly-folded form of an enzyme could explain the difference in activities between the commercial UGT1A10 and our highly active UGT1A10 (both expressed in insect cells). My 2017 published paper compared the glucuronidation activities of our UGT1A10 with several other enzymes. The results indicated that the role of UGT1A10 in the small intestine is significantly more substantial than previously thought.

Activity of UGT1A10 was also investigated in another article, which identified enzymes that glucuronidate two structurally similar environmental toxins, bisphenols S and F. The
results revealed that bisphenol S is a good substrate for UGT1A9 (expressed in the liver and kidney), whereas bisphenol F is a better substrate for the highly homologous intestinal enzyme, UGT1A10.

Catalytic differences between many UGTs were studied in the presence or absence of fatty acid-free bovine serum albumin (that binds potentially inhibitory fatty acids) and differences were found in a compound-dependent manner. The same study revealed that there might be differences between UGT subfamilies. The presence of bovine serum albumin may increase the reaction limiting velocity ($V_{max}$) more profoundly in most members of UGT1A than in members of UGT2, in addition to affecting the apparent $K_m$.

Species difference in activities of cloned, expressed dog UGT1As with human UGT1As were the subject of another paper. The results revealed that, perhaps with the exception of UGT1A6 (and UGT1A1), human and dog UGT1A counterparts do not exist, at least not directly. Consistently, in liver and intestinal microsomes, there are large differences between dog and human in glucuronidation rates for a set of test substrates. Not only rates of the glucuronidation can differ between dog and human UGT1As, but also the position where the glucuronide conjugate is attached on the target molecule could change. Furthermore, differences in tissue activity for some substrates were observed. The results indicated that, in general, dogs are not a good animal model to study glucuronidation in humans.

In summary, the substrate specificities of UGTs are currently difficult to predict. Prediction of glucuronidation activity requires carefully planned experiments and expertise in analyzing the results. The interpretation of the results should be done keeping in mind that a single enzyme activity measurement, when done alone in vitro, may only partially contribute to the overall activity in a given tissue. Furthermore, when comparing activities, it should be remembered that there are activity differences between UGT preparations, as well as the effects of additives such as albumin. To resolve the contribution that one enzyme makes in a tissue is not straightforward and requires additional experiments to overcome the limitations researchers currently face during in vitro assays. One goal of this thesis is to highlight and clarify certain questions related to the underestimated UGT1A10 activity in the literature. The cloning and characterization of the dog UGT1A family members will give other researchers in vitro tools to further study glucuronidation in dogs. The main purpose of the thesis was not only to determine and compare the glucuronidation activity of recombinant UGTs, but to investigate reasons that lead to differences in enzyme activity and to evaluate the role and function of the enzymes in drug metabolism. The driving force to improve assay conditions and data interpretation in vitro was often simply to ask the question: which UGTs catalyze glucuronidation of a substrate in a given tissue and at what rate?
Acknowledgements

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Helsinki, January 2019

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List of original publications

This thesis is based on the following publications:


The publications are referred to in the text by their roman numerals. Publication I is also included in the thesis of Nenad Manevski (Activity and Enzyme Kinetics of Human UDP-Glucuronosyltransferases: Studies of Psilocin Glucuronidation and the Effects of Albumin on the Enzyme Kinetic Mechanism, 2013).
**Abbreviations**

ACN  Acetonitrile  
ALI  Atractylenolide I  
AMC  7-Amino-4-methylcoumarin  
AUC  Area under the curve  
AZT  Zidovudine, azidothymidine  
BAV  Bavachinin  
BIL  Bilirubin  
BPA  Bisphenol A  
BPF  Bisphenol F  
BPS  Bisphenol S  
BSA  Bovine serum albumin  
CYP  Cytochrome P450  
DIF  Diclofenac  
DME  Dexmedetomidine  
E1  Estrone  
E2  Estradiol  
EE  Ethynylestradiol  
eni-E2  Epiestradiol  
eni-TS  Epitestosterone  
ER  Endoplasmic reticulum  
*E. coli*  *Escherichia coli*  
ETC  Entacapone  
FLD  Fluorescence detection  
6-HI  6-Hydroxyindole  
HE  Hill equation  
HEK293  Human embryonic kidney 293 cells  
HIM  Human intestinal microsomes  
HLM  Human liver microsomes  
HPLC  High-performance liquid chromatography  
IND  Indomethacin  
*Km*  Michaelis-Menten constant  
LC-MS/MS  Liquid chromatography-tandem mass spectrometry  
LME  Levomedetomidine  
4-MU  4-Methylumbelliferone  
MeOH  Methanol  
MM  Michaelis-Menten kinetics  
MRF  Morphine  
mRNA  Messenger RNA  
1-NP  1-Naphthol  
NCHN  N-(3-carboxypropyl)-4-hydroxy-1,8-naphthalimide  
NIL  Nilotinib  
NSAID  Nonsteroidal anti-inflammatory drugs
<table>
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<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
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<tr>
<td>R-PR</td>
<td>R-propranolol</td>
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<tr>
<td>S-PR</td>
<td>S-propranolol</td>
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<tr>
<td>qRT-PCR</td>
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<td>RED</td>
<td>Rapid equilibrium dialysis</td>
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<td>rs</td>
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<td>SI</td>
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<td>SN-38</td>
<td>7-Ethyl-10-hydroxycamptothecin</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TFP</td>
<td>Trifluoperazine</td>
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<tr>
<td>TS</td>
<td>Testosterone</td>
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<td>UDP</td>
<td>Uridine diphosphate</td>
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<td>UDPGA</td>
<td>Uridine 5’-diphosphoglucuronic acid, UDP-glucuronic acid</td>
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<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
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<tr>
<td>$V_{\text{max}}$</td>
<td>Reaction limiting velocity, maximum velocity</td>
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1 Introduction

Our cells need substances from outside the human body. To get those substances, we breathe, drink and eat, but at the same time we expose our body to compounds that can be harmful or toxic. Many of these unwanted compounds, as well as non-polar drugs and cell waste, would remain in our body unless enzymes modify the structures of compounds to more water-soluble forms. For instance, in the glucuronidation reaction, the enzyme adds a glucuronic acid moiety to a compound’s structure, which facilitates compound’s excretion into bile or urine. The enzymes catalyzing glucuronidation reactions, the uridine 5’-diphospho-glucuronosyltransferases (UGTs), are the topic of this thesis.

The human UGTs that prefer uridine 5’-diphosphogluconic acid (UDP-glucuronic acid, UDPGA) as a sugar are divided into two families, UGT1 and UGT2. In certain substrates they may add a sugar group from UDP-glucose or UDP-xylose cofactors (Mackenzie et al., 2005, Mackenzie et al., 2003). Humans have 19 UGTs with broad and partially overlapping substrate specificities. Only a few specific substrates are known for an individual enzyme. UGT substrates are mostly small and lipophilic molecules, such as common drugs and toxins, as well as a number of important endogenous substrates, such as steroids, bile acids, and bilirubin. The UGT proteins have a signal sequence at the beginning of their N-terminus that translocate them to the inside of the endoplasmic reticulum (ER) membrane. At the other end of the UGT protein, a single C-terminal transmembrane helix anchors the protein in the membrane. The N-terminal signal sequence is cleaved off following translocation of the UGT to the ER in mature UGT and active site for UDPGA and substrates faces the luminal side of the ER. Various tissues have divergent UGT expression patterns, mainly at the sites, which are active in detoxification and removal or prevention of the unwanted compounds, such as intestine, liver, and kidney.

To characterize the enzymes involved in the glucuronidation process, assays usually include commercially available single UGTs, “Supersomes®”, combined with assays done with tissue microsomes or hepatocytes from human donors. The challenge in these determinations is that UGTs often have overlapping substrate specificities, meaning that several UGTs can catalyze the same reaction. The different UGTs have their own affinities, however, and therefore the glucuronidation activity in microsomal assays represents a mixture of individual UGT activities.

UGT-selective or -specific substrates and inhibitors help to identify the enzymes that are responsible for the observed glucuronidation activity in microsomes. Unfortunately, the enzyme-specific substrates and inhibitors are not available for all UGTs. We lack the other means to determine the amount of active UGT in these reactions, so often glucuronidation rate is reported relative to the milligrams of protein in an enzyme source. Rates for an individual enzyme may be influenced experimentally by several factors, including situations when UGT preparations contain a large portion of an inactive form of the enzyme (Oda et al., 2012). In addition, only a few papers about UGT expression at protein level in tissues have been published (Sato et al., 2014, Fallon et al., 2013), which complicates the in vitro-in vivo extrapolation.

Many of the experiments involving glucuronidation in this thesis were studied at the single enzyme level. The studied UGTs were cloned and expressed in insect cells as
recombinant proteins with a C-terminal His-tag. These recombinant enzymes were then used as enzyme source in glucuronidation assays, either as insect cell homogenates or in UGT-rich membrane preparations. As for UGT preparations, our laboratory does not aim for high protein concentration or activity, but for high activity per mg of expressed protein, which means a lower proportion of inactive UGT. The C-terminal His-tag enables the determination of expression levels for each enzyme preparation with monoclonal antibodies directed against the His-tag. These individual enzymes are then tested with a number of structurally divergent compounds. Glucuronidation assay results from the liver or intestinal microsomes are combined with recombinant assays to estimate the total activity of UGTs in that tissue, and the relative contribution of each UGT. For example, this was done to identify the enzymes responsible for glucuronidation of bisphenols F and S. The human polymorphic variant UGT1A4-P24T was cloned and expressed for enzyme activity analyses compared to wild-type UGT1A4. The mutation in this variant occurs in the signal sequence, which normally is cut off from the mature protein, and thus it was anticipated that this variant would have little effect on enzyme activity. The most recent study investigated UGT1A10 activity and its role in extrahepatic glucuronidation with the aim to clarify the contradictory reports on UGT1A10 activity in the literature. The effect of addition of bovine serum albumin (BSA) examines how recombinant UGTs’ glucuronidation activity is affected by albumin addition. The paper focuses on those enzymes that had not been previously examined for the “albumin effect”. Besides individual enzymes, the differences between UGT1 and UGT2 families are examined. The dog UGT research project characterized dog UGT1A enzymes and investigated if any of them would resemble human UGTs by their substrate specificities.
2 Review of the literature

This review of the literature gives an overview of UGTs and describes how individual enzyme activities are measured in vitro by glucuronidation assays. The first part of the review introduces the UGT enzymes and their roles in drug metabolism, their substrate specificities and expression in the liver and extrahepatic tissues. The second part of the review summarizes the common recombinant UGT sources and recombinant expression systems. The third part of the literature review focuses on the in vitro glucuronidation assays.

2.1 Introduction to UGT enzymes

2.1.1 UGTs protect us from accumulation of harmful compounds

The number of drug metabolizing enzyme genes increased rapidly when animals moved from the sea onto land and started to consume terrestrial plants (Gonzalez and Nebert 1990, Nebert and Dieter 2000). Plants responded to the threat by developing secondary metabolites for their protection, whereas animals evolved such enzymes that could detoxify and excrete these plant-derived toxic compounds. Compounds that originate from outside the body are called xenobiotics, and this group include both phyto- and environmental toxins, as well as present day drugs. The drug metabolizing enzymes do not only protect the body from food derived toxins, but also prevent the accumulation of hydrophobic compounds from the environment inside the cells. These compounds could be xenobiotics and their metabolites, or endogenous compounds such as steroids and bile acids or waste metabolites like bilirubin. Our detoxification enzymes that were originally developed to protect against plant toxins, is now mainly called drug metabolizing enzymes, since besides handling endobiotics, xenobiotics and -toxins from plants, these enzymes eliminate a wide spectrum of synthetic xenobiotics, such as man-made chemicals, pollutants, and drugs.

The elimination pathways for hydrophobic xenobiotics usually consists of the activity of two types of reactions, followed by efflux transport of the hydrophilic product metabolite(s). The most common reactions are oxidations by cytochrome P450 (CYPs) or hydrolytic reactions by esterases and amidases (Phase I metabolism) and conjugation reactions by UGTs, sulfotransferases, N-acetyltransferases, and glutathione-S-transferases (Phase II metabolism). UGTs detoxify and prevent accumulation of numerous xeno- and endobiotics by catalyzing the attachment of a polar ionized glucuronic acid moiety, which greatly increases the metabolite’s hydrophilicity. The product metabolite (glucuronide) is mostly a better substrate than the original molecule was to the cell’s drug efflux transporters and hence it is easier to excrete it from the body and avoid re-entry by diffusion. In addition, in contrast to oxidative reactions that may bioactivate compounds into reactive metabolites, glucuronidation reactions generally yield less toxic products than the parent compound. Most glucuronides are, in general, biologically and pharmacologically inactive compounds.
Glucuronidation is often the most important conjugation reaction in drug metabolism and UGTs are involved in the elimination of many common drugs (Williams et al., 2004). In the drug development process, in vitro glucuronidation studies are performed with recombinant enzymes to characterize metabolism and to estimate the pharmacokinetics and pharmacodynamics of drug candidates. Glucuronidation studies play a substantial part in the understanding of absorption, distribution, metabolism and excretion of new drugs (ADME properties). Carefully performed in vitro glucuronidation assays give much information about the safety and efficacy of the drug candidate before they are further tested in animals or humans. If one UGT is primarily responsible for the glucuronidation of a drug, its potential polymorphisms must be further investigated for effects on an individual’s dose and drug safety. In addition, it is recommended to study UGT inhibition if the drug is glucuronidated by a single UGT and if glucuronidation is the major reaction in this drug’s metabolism. (See European Medicines Agency website for the guidelines on UGT inhibition studies, https://www.ema.europa.eu/documents/scientific-guideline/guideline-investigation-drug-interactions_en.pdf, accessed Nov 2018).

UGTs are approximately 530 amino acids long proteins. These enzymes have signal sequence in their N-terminus of about 25 amino acid, which directs it to the ER and is cleaved off while the nascent protein is translocated across the ER membrane. The variable N-terminal half of the enzyme binds the aglycone and thus determines the substrate specificity of the UGT (Meech and Mackenzie 1997). The conserved C-terminal part of UGT binds the co-substrate (cofactor) and consists of a 44 amino acid long donor binding motif. At the very end of the C-terminus there is a single trans-membrane helix that is followed by a short cytoplasmic segment. This transmembrane helix and certain other hydrophobic regions of protein anchor the enzyme into ER membrane (Bock 2016, Meech and Mackenzie 1997, Laakonen and Finel 2010, Ciotti et al., 1998).

### 2.1.2 The genes of human UGTs

The current nomenclature of the human UGTs is in line with the recommendations of Human Gene Nomenclature Guidelines (https://www.genenames.org/about/guidelines, accessed Aug 2018, Wain et al., 2002). The UGT gene nomenclature follows their evolution differences, the first number is the family, the letter after this indicates the subfamily and the last number identifies the individual gene. The superfamily of human UDP-glycosyltransferases consists of 22 full-length proteins that are divided into four different families based on their gene sequences. With the exception of UGT1 family, all the other human UDP-glycosyltransferases were chronologically named based on their discovery. The UGT1 and UGT2 families generally add glucuronic acid into their target molecules from the cofactor UDPGA, whereas UDP-glycosyltransferase families 3 and 8 mainly use other sugars as their cofactors (Mackenzie et al., 2005). The enzymes of 3 family utilize UDP-glucose, UDP-xylose and UDP-N-acetylgalcosamine, whereas UDP-glycosyltransferase 8 transfers the sugar from UDP-galactose (Meech and Mackenzie 2010). This thesis focuses on glucuronide conjugation catalyzed by the UGT1 and UGT2
families, and therefore after this Section 2.1.2, the members of UDP-glycosyltransferase 3 and 8 families will not be further addressed.

UGT1A is the only subfamily of UGT1 in humans. Approximately half of the human UGTs belong to this subfamily and all of them are encoded by the same single gene on chromosome 2q37. The UGT1A gene complex is 218 kb in length and each of the 13 separate first exons has its expression regulated by its own promoters (Gong et al., 2001). The different UGT1As are made by exon sharing, namely mature UGT1A mRNAs share four exons, which means that the UGT1As differ only by their first exon, which encodes the N-terminal half of the protein (encoding the substrate binding site). The first exon is then spliced onto exons 2-5 that encode for the C-terminus and UDPGA binding site. Figure 1 presents the 13 different first exons in combination with the shared exons. Only nine of UGT1As are functional, the other four (UGT1A2, UGT1A11, UGT1A12, UGT1A13) are pseudogenes in humans, because their first exons carry stop codons in the reading frame. Naming the UGT1A genes follows the first exons and starts with UGT1A1 that has its first exon closest to the common exon 2-5 in the gene (see Figure 1). This naming is in arithmetic order up to UGT1A7. After UGT1A7 the names order follows the order of gene discovery.

**Figure 1**  
Schematic presentation of the UGT1A gene that encodes all the UGT1A proteins. Gray text color and a character “P” indicates pseudogene (exon 1). The UGT1A10 is shown as an example of exon sharing (middle). The figure is not scaled, and it was drawn based on the information from HGNC Database (HUGO Gene Nomenclature Committee, HGNC, European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK; www.genenames.org, accessed Nov 2018).

The human UGT2 family is divided into two subfamilies, UGT2A and UGT2B, and their genes are located on chromosome 4q13 spanning 1200 kb. The genomic order of ten UGT2 genes and five pseudogenes is presented in Figure 2. Each gene of the UGT2 subfamily has six exons (unlike Figure 1, separate exons are not shown), UGT2A1 and UGT2A2 share five exons, similarly to UGT1As (Mackenzie et al., 2005).
UGT3A1 and UGT3A2 are the two UGT3 family enzymes. The UGT3 genes have seven exons and they are located on chromosome 5p13 (Mackenzie et al., 2005). The only member of the human UGT8 family has five exons and it is encoded on chromosome 4, similarly to the UGT2 family, but situated further away, in location 4q26 (Mackenzie et al., 2005).

Several UGTs have alternative transcripts (splice variants). In the UGT1 family, the last exon, no. 5 (see Figure 1), was shown to have two transcripts, 5a or 5b (Girard et al., 2007). The longer transcript, 5a, encodes the full-length UGT1A, whereas the 5b is shorter, lacks the part encoding the transmembrane helix and results in inactive enzyme (Girard et al., 2007). Alternative transcripts were proposed to alter the glucuronidation activity also by forming a dimer with a full-length protein and regulating the activity of UGTs in a tissue specific manner (Rouleau et al., 2016). Besides the UGT1 family, splice variants can be found also in the UGT2 family (Menard et al., 2013, Tourancheau et al., 2018).

### 2.1.3 Other animal UGTs

In addition to variability in substrate specificity, the glucuronidation sites and reaction velocities differ between species. A classic example for species differences in glucuronidation is the cat (Felis Catus) that lacks glucuronidation activity toward phenolic compounds. Cats express only few UGT1As and UGT1A6 is a pseudogene in this species. For this reason, common drugs, such as acetaminophen and salicylates, are toxic in cats (Shrestha et al., 2011, Court and Greenblatt 1997).

In opposite to cats that are obligate carnivores, herbivorous mammals such as sheep highly express UGTs that can glucuronidate phenolic compounds (Kobayashi et al., 1999). Moreover, insects that eat plants have hundreds of different UGTs in their UGT families, although many of the insect UGTs are likely involved in endobiotic regulation rather than detoxification (Ahn et al., 2012). Insect UGTs also prefer glucose as their cofactor, whereas UGTs in mammals mainly conjugate glucuronic acid (Bock 2016).

Gunn rats were the first animal model that was developed to study human diseases that are caused by a failure in bilirubin glucuronidation, because these rats have a mutation that
makes them hyperbilirubinemic and jaundiced (Gunn 1944). Even though the Gunn rat is used for developing treatments for UGT1A1 glucuronidation deficiency such as in the Crigler-Najjar syndrome (Tolosa et al., 2015), the Gunn rat mutation affects all members of the UGT1A subfamily. The mutation in these rats leads to a premature stop codon in the fourth exon, which is a common exon in the rat UGT1As, similarly to human UGT1As (Ikushiro 2010).

In addition to rats, the UGT1A subfamily is alternatively spliced in many other species, such as dogs, mice, chimpanzee, rhesus monkeys, baboons, dogs, chickens and zebrafish (Li and Wu 2007, Mackenzie et al., 2005). The splicing is similar, but different species have different order of UGTs, because they have their own pseudogenes. The latter and some variability in amino acid sequences means that the enzymes that have the same name in two species do not necessarily share their substrate specificities (Yang et al., 2017). The human amino acid sequence of the common exons, 2-5, of UGT1A is aligned with the corresponding dog exons in Figure 3. Based on the UGT1As amino acid sequences of human and 16 animal species, the conserved amino acids are marked in the human protein sequence by different colors (Figure 3).

![Amino acid sequence alignments of the four common exons at the C-terminus of human (upper sequence) and dog UGT1As (lower sequence, text in gray color). Differences are marked with red boxes around the amino acids. The conserved amino acids in human and various animal species are shaded on the human sequence in color codes that describe the amino acid characteristics. Green is for amino acids that have a side chain that contains hydrogen, hydroxyl, sulphydryl or amine. Orange is for small and hydrophobic side chain, blue color is for acidic, and gray is for basic amino acids. Compared sequences are from the rat, dog, rabbit, cow, sheep, rhesus monkey, chimpanzee, baboon, pig, mouse, guinea pig, horse, dog, lizard, chicken and zebrafish UGTs. Animal and human sequences are from UGT Nomenclature Committee Website (http://prime.vetmed.wsu.edu/resources/udp-glucuronosyltransferase-homepage, accessed Nov 2018).](image-url)

When preclinical trials are done in animals, the nonrodent species is usually a beagle dog ([https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078933.pdf](https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078933.pdf), site visited on Nov 2018). The complete dog genome (of a female boxer) was published a little over ten years ago (Lindblad-Toh et al., 2005, Kirkness et al., 2003, and few years after that Li and Wu reported the cDNAs of the dog UGT1A
subfamily (Li and Wu 2007). The homology between the dog and human UGT1A protein sequences are presented in a phylogenetic tree in Figure 4.

Only one individual dog UGT from the dog UGT1A family, dog UGT1A6, was cloned and partly characterized earlier (Soars et al., 2003a, Soars et al., 2001b). The activity towards some common substrates was similar to the human UGT1A6 activity (Soars et al., 2001b), whereas the only other cloned dog UGT, UGT2B31, was not an ortholog of any human UGT (Soars et al., 2003a). The dog UGT2B31 is likely the main enzyme in the carnivore UGT2B subfamily (Kondo et al., 2017, Heikkinen et al., 2015).

![Figure 4](image)

**Figure 4**  Phylogenetic tree of human and dog UGT1As. Lower case “d” in front of the UGT name indicates dog UGT and “h” indicates human UGT. In the phylogenetic tree amino acid sequences were first aligned using ClustalW and then further analyzed using Mega version 7 program (Kumar et al., 2016).

The dog is omnivorous and less of an obligate carnivore than the cat. Dogs eat more plants and starch alongside meat, bringing their exposure to dietary xenobiotics a bit closer to human in comparison to cats. However, comparisons of glucuronidation activities between microsomes from dog and human revealed many differences between the two
species (Soars et al., 2001a, Itäaho et al., 2008, Kondo et al., 2017). These differences in glucuronidation across species are not limited to dogs. Despite many species have highly homologous UGT protein sequences and gene arrangements, their glucuronidation rates and substrate specificities differ largely. In a study of regioselectivity in estradiol and epiestradiol glucuronidation, a rat, rabbit, pig, bovine and elk liver microsomal activities were compared with human liver microsomes (Itäaho et al., 2008). The results showed that only human and rat liver UGTs favored the 17-OH position in estradiol glucuronidation, whereas other species glucuronidated mainly at the 3-OH position. Still, there was a difference between the rat and human microsomes, because the human liver glucuronidated epiestradiol at higher rate than estradiol, while in rat it was reversed. Interspecies differences in drug glucuronidation were reported to be even higher in the case of N-glucuronides (see Section 2.2.1 for N-glucuronidation), where the conjugation rates were much higher in humans than in preclinical test animals (Kaivosaari et al., 2011).

2.1.4 Polymorphism and its influences

A polymorphic variant is a variant allele that occurs in the population at a frequency of one percentage or higher in a given population (Brookes 1999). Polymorphisms could affect UGT expression or amino acid sequence, thereby altering glucuronidation activity. These days we can study human frequencies of polymorphism using databases such as the 1000 Genomes Project (Auton et al., 2015), as the progress in whole genome sequencing and bioinformatics is fast. The UGT Allele Nomenclature Committee maintains a dedicated website for human UGT polymorphism (Laval University in Quebec, Canada, https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature/, accessed Nov 2018).

Three different ways were used in this thesis to refer to UGT polymorphism. The first is by the older star system, the second is by the reference single nucleotide polymorphism (SNP) numbers (rs) and the third is by the change in amino acid (non synonymous exonic mutation). In the latter reference method, the two characters indicate the original and altered amino acids, and the number indicates the amino acid’s location within the protein sequence.

Polymorphism in different UGTs is common, but their effects may not result in major drug clearance changes, because other UGTs compensate for the altered UGT activity. However, there are some cases, where the substrate is glucuronidated mainly by one or few enzymes, such as bilirubin by UGT1A1 or zidovudine by UGT2B7. In these cases, if the polymorphism reduces or inhibits enzyme’s activity, then the polymorphism may have a clinical outcome. For example, when unexpected high plasma levels of an investigational drug candidate, RO5263397, were encountered in three African patients in a drug study. Those patients had SNP rs2942857, which affected protein splicing and lowered the correctly spliced UGT2B10 mRNA levels (Fowler et al., 2015). In another case, the UGT2B17 variant had an effect on the pharmacokinetics of the drug candidate MK-7246 in healthy subjects (Wang et al., 2012). For UGT1A1*28 polymorphism, US Food and Drug Administration recommends to genotype the patients that receive irinotecan (SN-38)
therapy. UGT1A1*28 lowers SN-38 glucuronidation rates and is the cause for drug’s toxic effects (Perera, Innocenti and Ratain 2008, Liu et al., 2014). This means that information of patient’s polymorphism could be used when predicting drug dosing for patients (Inoue et al., 2016). For detecting variants, PCR-based methods are often used, such as in the case of genotyping UGT2B7*2 polymorphism (Holthe et al., 2002).

At least 5 kbp upstream from the transcription initiation site and 500 bp downstream of the last exon are included in the UGT polymorphism website (https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature/), which indicates that the gene SNP sites are not limited to the gene transcript. On the contrary, large part of the polymorphic sites are located outside the exons (the protein coding regions), either in the introns or in regions upstream the 5’-end (regulatory region where transcription factors bind) and downstream at the 3’-end (where miRNAs bind). In UGT2B7, the 23 SNPs in the promoter region were reported to be linked to different responses in patients (Hu et al., 2014). The polymorphisms in the promoter region of UGT1A9 and UGT2B7 were reported to lead to variability in exposure to mycophenolic acid, an immunosuppressive drug that is often used in organ transplantations (Fukuda et al., 2012, Guo et al., 2013).

SNPs may occur within the signal peptide of different UGTs. In that case the SNP is in the transcribed messenger RNA (mRNA) but the affected amino acid is subsequently cleaved off the nascent protein during its translocation across the ER membrane. If a signal peptide sequence is altered, then it might fail to interact properly with the ER translocation machinery. This was suggested to occur in the UGT1A1*30 polymorphism that is located in the middle of the signal peptide of UGT1A1 (Seppen et al., 1996). UGT1A1*30 causes hyperbilirubinemia, even if in its less severe form, namely Crigler-Najjar type 2 syndrome. In the case of UGT1A4-P24T, the mutation is near the end of a signal peptide, but it was still suggested to be a functional polymorphic variant (Edavana et al., 2013). Moreover, the effect of UGT1A4-P24T was reported to depend on the substrate. The polymorphic variant had lower trans-androsterone and lamotrigine glucuronidation rates, but its dihydroteosterone and tamoxifen glucuronidation rates were not affected (Zhou, Argikar and Remmel 2011).

The effects of polymorphic variants on activity could be informative for studies of UGT structure and function. UGT1A1-Y486D reduces bilirubin glucuronidation, but affects also the activities of other UGT1A enzymes, because the site of the mutation is in exon 5, one of the common exons of the UGT1A subfamily (Ito et al., 2002, Yamamoto et al., 1998). Consequently, glucuronidation rates of scopoletin, 1-naphthol and serotonin were reported to be reduced with corresponding UGT1A6 (Y485D) mutant (Kurkela et al., 2007). Mutation lowered the UGT1A6 enzyme’s affinity for specific substrate serotonin, but this effect could be reversed by co-expressing the mutant enzyme with other wild-type UGTs, such as UGT1A4 (Kurkela et al., 2007). On the contrary, a polymorphic variant of the nearly inactive UGT1A5 was recently found, UGT1A5*8 (G259R in exon 1), which lead to elevated N- and O-glucuronidation activities (Yang et al., 2018).

Several SNPs were reported to be more common in patients that suffer different types of cancer (Hu et al., 2016). For example, a direct association between the UGT2B15-D85Y polymorphism and prostate cancer was reported (Grant et al., 2013). An intronic SNP in
comprehensive meta-analysis (Figlioli et al., 2016). *UGT1A7* polymorphisms may affect the risk in tobacco-related cancers (Zheng et al., 2001, Wallig 2004) and, furthermore, *UGT1A7*3 was reported to be associated with pancreatic, hepatic and colorectal cancers (Ockenga et al., 2003, Zhang et al., 2017). However, some of the the *UGT1A7*3 cancer association might be due the linkage disequilibrium of *UGT1A7*3 and *UGT1A1* polymorphisms (Tang et al., 2005, Urawa et al., 2006). *UGT1A1* polymorphisms was shown to be associated with a risk for a premenopausal breast cancer in African Americans (Guillemette et al., 2000).

The UGT2B7 is an important UGT in drug metabolism and it has several polymorphic variants that potentially affect drug glucuronidation. For example, among sickle cell disease patients, 35% had a UGT2B7 rs7438135 or rs73823859 variant that might have an influence on opiate response (Joly et al., 2012). The UGT2B7 polymorphism rs7668258 (intrinsic) was reported to effect valproic acid treatment in epilepsy patients (Inoue et al., 2014). This polymorphism is in complete linkage disequilibrium to another common polymorphism *UGT2B7*2a (Saeki et al., 2004, Jin et al., 1993). The *UGT2B7*2a variant has been connected to poor responses in analgesics buprenorphine and morphine (Sastre et al., 2015, Sawyer et al., 2003). Other SNPs that were suggested to alter morphine glucuronidation rates are also in linkage disequilibrium with the *UGT2B7*2 variant (Duguay et al., 2004). It means that the reason for variability in morphine glucuronidation could be caused not by the originally reported polymorphic, but by variant UGT2B7*2. The results have not been consistent, however, because another study did not find a difference in morphine glucuronidation between the variant and the wild-type UGT2B7 (Ross et al., 2005).

2.2. UGTs have overlapping substrate specificity and tissue distribution

2.2.1 Glucuronidation reaction mechanism

In this thesis, the abbreviation UGT stands for a UDP-glucuronosyltransferase enzyme (EC 2.4.1.17), but the same abbreviation is used in literature more broadly for UDP-glycosyltransferase enzymes. The members of this large glycosyltransferases superfamily are grouped based on their nucleotide sequences (Campbell et al., 1997). Glycosyltransferases catalyze glycosidic bond formation in reactions where a saccharide moiety is removed from one molecule (donor) and attached covalently to another molecule (acceptor). The donor molecule is an activated sugar, often referred to as cofactor or co-substrate, whereas the acceptor molecule referred to as a substrate or aglycone. The substrates of the different members of this superfamily could be oligosaccharides, proteins, nucleic acids, lipids and many natural products, which means that the functions and sequences of these enzymes are highly diverse (Coutinho et al., 2003, Campbell et al., 1997, Hu and Walker 2002).

Despite low sequence homology among glycosyltransferases, sequence analyses and solved 3D structures divide these enzymes into two topologies, which means that
glycosyltransferases seem to fold in only two main ways (Lairson et al., 2008). The two basic structures, GT-A and GT-B, emerged from their two archaea ancestors (Coutinho et al., 2003) indicating that there are only few options for how these enzymes can catalyze the formation of glycosidic bonds (Hu and Walker 2002). GT-A enzyme consists of a single domain with two β/α/β domains and eight β-strands around the enzyme’s core. GT-A enzymes require a divalent metal ion for their catalysis. GT-B enzymes have a highly conserved structure of two Rossmann-like β/α/β domains that are less tightly associated. GT-B enzymes are not dependent on divalent metal ions, even if their activity might be increased in the presence of metal ions (Hu and Walker 2002).

The catalytic mechanisms of glycosyltransferases can be divided into inverting and retaining mechanisms. Inverting glycosyltransferases use nucleophilic substitution reaction mechanism, whereas the mechanism of retaining catalysis is not fully understood (Breton et al., 2006). All the UDP-glycosyltransferases belong to the GT-1 family, have a GT-B protein fold and employ an inverting catalytic mechanism (based on the Carbohydrate Active Enzymes database http://www.cazy.org/, accessed on Nov 2018, Lombard et al., 2014). Unfortunately, no 3D structure of any full-size human UGT is yet available, only part of the C-terminal half of UGT2B7 that includes most of the UDPGA binding site has been studied by X-ray crystallography (Miley et al., 2007). Because all the GT-B proteins are predicted to fold in a highly similar way, the 3D structures of other GT-B enzymes could be used for homology modelling (Laakkonen and Finel 2010, Ghentio et al., 2014). For example, the online tool Phyre2 predicts from the amino acid sequence of the human UGT1A1 that the enzyme folds as a glycosyltransferase in GT-B class (Kelley et al., 2015).

Figure 5 The UGT1A1 structure prediction based on homology modelling online tool program Phyre2 (Kelley et al., 2015).
2.2.2 Tissue distribution

The liver is the central organ of drug metabolism, also for glucuronidation. However, some UGTs are expressed in extrahepatic tissues at the barriers against the outside world, mainly in sites where xenobiotics enter or are removed from our body, such as skin, lungs, kidneys, and intestine.

The small intestine is particularly important in protecting us from harmful xenobiotics, while enabling absorption of beneficial nutrients. The protection is physicochemical, including acidic and basic environment, microbes, and last but not least, enzymes and transporters. UGT1A8 was previously thought to be highly expressed in the intestine and UGT1A10 elsewhere along the gastrointestinal tract (Tukey and Strassburg 2000). Other studies, however, reported that UGT1A10 is an intestinal enzyme and UGT1A8 was expressed in the colon (Mojarrabi and Mackenzie 1998). Nonetheless, recent studies reported that there are only four different UGTs expressed in the intestine at significant amounts at the protein level, results that agree with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) measurements of the expression at the mRNA level (Ohno and Nakajin 2009, Court et al., 2012, Fallon et al., 2013a, Sato et al., 2014). These four main UGT enzymes of the small intestine are UGT1A1, UGT1A10, UGT2B7 and UGT2B17 (Sato et al., 2014) (Figure 6).

![Intestine expression](image)

**Figure 6** UGT expression levels in the intestine. Only UGT1A1, UGT1A10, UGT2B7 and UGT2B17 are highly expressed. The figure is combination of LC-MS/MS measurements from two laboratories (Sato et al., 2014, Nakamura et al., 2016). Bars are the mean values of expression and the range of expression values is given in error bars. In case of UGT1A4 and UGT1A10, only one group determined the protein expression levels (no error bars are visible for these enzymes).
The challenge in determining accurate protein level of intestinal UGTs is high due to interindividual variation in their expression, perhaps even higher than among liver UGTs (Strassburg et al., 2000, Harbourt et al., 2012). Another possible reason for the variability, even if technical in nature, is that it is much more difficult to get sufficient good samples of intestinal epithelium from donors for intestinal samples than hepatic samples.

It may be added that not only expression levels in the intestine vary. The same liver sample resulted different results in proteomic quantitations between the two independent laboratories (Achour et al., 2017). In the same way, kidney cells express only three UGTs, UGT1A6, UGT1A9 and UGT2B7, but their relative abundance varied between two LC-MS/MS publications (Sato et al., 2014, Nakamura et al., 2016).

The expression is less variable in liver microsomes where the number of donors is much higher than the number of donors for other tissue microsomes. In addition, there are more published papers about the UGT expression in hepatocytes. The liver is the most important tissue in drug metabolism and more UGTs are expressed in it (Figure 7). UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15 are perhaps the most important hepatic enzymes (Miners, Mackenzie and Knights 2010). The comparison of several studies on proteomic quantitation of hepatic UGT expression mostly agree with this (Margaillan et al., 2015), but also UGT1A3, UGT2B4, UGT2B10 and UGT2B17 are expressed in the liver and have their roles in hepatic glucuronidation (Figure 7 and Section 2.2.3).

![UGTs protein expression in liver](image)

**Figure 7** Liver UGT expression. Bars are the mean expression values of different LC-MS/MS measurements in publications (Fallon et al., 2013b, Harbourt et al., 2012, Sato et al., 2014, Nakamura et al., 2016, Ohtsuki et al., 2012). The range of expression values is given in error bars.
The determination of UGTs expression level in the liver has successfully relied on mRNA measurements by qRT-PCR (Court et al., 2012, Ohno and Nakajin 2009), protein detection with immunohistochemical labels (Bellemare et al., 2011) or enzyme activity measurements in tissue with enzyme specific probes (Fisher et al., 2000b). The last method would be good for actual UGT glucuronidation level determination, but it requires a specific probe for each UGT (See Section 2.2.3 and Table 1). More recent studies are based on mass spectrometry determinations of UGT protein levels in the tissue (Sato et al., 2014). While the mRNA studies could reveal if UGT may be found in that tissue, the other determination methods show whether or not it is actually expressed, because protein expression is regulated at many levels and mRNA transcription does not yet mean that it will be processed to a mature protein. However, even the protein level determination does not reveal the amount of active UGTs in the tissue, since the low or non-active (splice or polymorphic) variants are equally recognised by the method alongside the active enzymes. Whereas the liver expression levels are quite well known (Margaillan et al., 2015), more information is needed about UGTs expression at protein levels in the intestine. It requires a larger number of tissue donors and samples taken from different parts of the intestine.

2.2.3 Substrate specificity

The substrate molecules of most UGTs are relatively small and hydrophobic compounds that have a hydroxyl or amine group(s) onto which the UDP-sugar could be attached. There are also more hydrophilic substrates such as serotonin and nicotine, or larger molecules like bilirubin, that serve as substrates of certain UGTs. So simply based on the molecule’s structure or physicochemical properties one cannot know whether it is a UGT substrate. Beside size and hydrophobicity, the molecule has to fit into the enzyme’s active site. The 19 UGTs have each their own substrate affinity and partly, but not fully, overlapping specificities toward the substrate molecules. This means that a certain compound, such as 4-methylumbelliferone, could be a substrate for several UGTs. One of the enzymes, however, might glucuronidate the compound at a higher velocity or bind it at a higher affinity than the other UGTs (Figure 8).

Nearly all the human UGTs form O-glucuronides. The substrates in O-glucuronidation are compounds that have hydroxyl group as part of their structure, such as phenols, flavonoids or carboxylic acid. Many of these substrates are CYP metabolites or from hydrolytic reactions especially from ester prodrugs. For CYP metabolites, the hydroxyl groups of compounds are introduced or unmasked in a preceding oxidation (phase I) reaction. In addition to O-glucuronidation, there are other types of glucuronidation reactions that attach the sugar group onto nucleophilic group of different nitrogens (N-glucuronidation), or in few rare cases to sulfur or carbon nucleophile (S- and C-glucuronidation).

Glucuronidation reactions may also be considered as detoxifying reaction, since the glucuronides are usually inactive or less biologically active than the parent compounds. However, there are exceptions to this rule, such as morphine-6-glucuronide or acyl glucuronides (Paul et al., 1989, Regan et al., 2010). An acyl glucuronide is formed when a
Figure 8  An illustration of two glucuronidation reactions that are catalyzed by two different human UGTs. The original compound is transformed into a more water-soluble form by the addition of a sugar group. Top left, the compound is zidovudine that is mainly glucuronidated by UGT2B7. Lower left, bilirubin that is an important and specific UGT1A1 substrate. The pink part of the protein denotes the N-terminal domain and blue is for the C-terminal domain. Transparent yellow box visualizes the ER membrane interaction with C-terminal tail and part of the N-terminal domain. Modified from the homology model of UGT1A1 (Laakkonen and Finel 2010).

carboxylic acid is glucuronidated and the acyl glucuronide product can be a reactive electrophile that covalently binds to nucleophilic molecules. Several UGTs form acyl glucuronides from commonly used drugs, such as ibuprofen, indomethacin, salicylic acid, diclofenac and mycophenolic acid (Bernard et al., 2006, Iwamura et al., 2017). Considering the number of drugs that carry a carboxylic acid, there are less reports on clinical impact of acyl glucuronides than could be expected. The reason for this could be related to the generally efficient excretion of glucuronides, including acyl glucuronides, from the cell (Koga et al., 2011). However, several non-steroidal anti-inflammatory drugs (NSAID) were removed from the market due to allergic reaction or liver toxicity and the mechanism in some cases has been attributed to reactive acyl glucuronide formation.

The rest of this Section, 2.2.3, shortly describes the roles of individual UGTs in the glucuronidation of specific drugs or endobiotic molecules. Some of the substrates that are used as probes or that are mainly catalyzed by a specific UGT are listed in Table 1.

The first UGT that was cloned and characterized was the human liver bilirubin UGT, HUG-Br1, an enzyme that is currently known as UGT1A1 (Ritter, Crawford and Owens 1991). It is the only human enzyme that glucuronidates bilirubin at a large scale. Therefore, UGT1A1 polymorphisms can lead to jaundice and familial hyperbilirubinemia diseases, such as Gilbert’s syndrome and Crigler-Najjar syndromes type 1 (rare but often fatal) and type 2 (less severe). UGT1A1 glucuronidates drugs containing carboxylic acids to form acyl glucuronides such as the NSAID ibuprofen and the antibiotic trovafloxacin (Fujiwara et al., 2015, Iwamura et al., 2017). UGT1A1 also N-glucuronidates primary or secondary
amines, for example a carcinogenic compound 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), which is produced in cooked meat (Malfatti and Felton 2001).

UGT1A3 glucuronidates several of the same reactions as UGT1A1, such as estradiol 3-glucuronidation reaction and thyroxine glucuronidation (Itääho et al., 2008, Yoder Graber et al., 2007). There are, however, some substrates that are regiospecifically glucuronidated by UGT1A3 more than other enzymes (See Table 1). UGT1A3 efficiently catalyzes bile acid conjugation on their carboxylic acid groups.

Table 1. Enzyme specific substrate molecules or drugs. Some of the substrates are UGT specific only when they are assayed in certain tissue. For example, estradiol is 3-glucuronidated only by UGT1A1 in the liver, but both UGT1A1 and UGT1A10 glucuronidate 3-position in intestine. If substrate is mentioned twice in the table, the regiospecificity of the two enzymes differ.

<table>
<thead>
<tr>
<th>UGT enzymes</th>
<th>Enzyme specific substrates (UGT probes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>Bilirubin$^1$, estradiol$^2$, ethinylestradiol$^3$, SN-38$^4$, etoposide$^{13}$</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>Desacetylcinobufagin$^9$, zolarsartan$^{14}$, chenodeoxycholic acid$^{17}$, R-lorazepam$^{14}$</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Trifluoperazine$^2$, dexmedetomidine$^7$, desacetylcinobufagin$^9$, lamotrigine$^{10}$, imipramine$^{15}$, midazolam$^{20}$</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>Serotonin$^2$, naphthol$^6$, deferiprone$^{16}$, 4-hydroxyindole$^{18}$</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Propofol$^{2,6}$, entacapone$^{11}$, psoralidin$^{27}$</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>Ethinylestradiol$^5$, SN-38$^4$, dopamine$^{12}$, estrone$^{19}$, 16α-hydroxyestrone$^{19}$</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Zidovudine$^2$, morphine$^6$, epitestosterone$^8$, epirubicin$^{21}$, carbino$^{22}$</td>
</tr>
<tr>
<td>UGT2B10</td>
<td>Levomethadomidine$^7$, nicotine$^{23}$, cotinine$^{25}$</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>S-oxazepam$^2$, androstanediol$^6$</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>Testosterone$^6$, dihydrotestosterone$^{24}$, 17-dihydroxemestane$^{26}$</td>
</tr>
</tbody>
</table>


The two UGTs that favor the N-glucuronidation are UGT1A4 and UGT2B10. When N-glucuronidation of primary, secondary or tertiary amines is expected, UGT1A4 is often the main enzyme catalyzing the glucuronidation reaction (Green and Tephly 1996, Kairosaari et al., 2011, Kamdem et al., 2010, Seo et al., 2010). The significance of UGT2B10 in N-glucuronidation reactions for many tertiary amines was discovered only just over ten years ago, when UGT2B10 was reported to be the most active human UGT to glucuronidate nicotine and cotinine (Kairosaari et al., 2007, Chen et al., 2007). Until then UGT1A4 was the prime candidate to catalyze any N-glucuronidation reaction. However, UGT1A4 has undeniably role in the N-glucuronidation of certain drugs, such as the antiepileptic drug lamotrigine. This enzyme’s ability to catalyze tertiary amines such as lamotrigine and
trifluoperazine was proposed to result from the amino acid sequence difference in UGT1A4 from most UGTs at position 40, where it has a proline instead of catalytic histidine (Kubota et al., 2007). UGT2B10, the other \(N\)-glucuronidating UGT, has a leucine instead of histidine at this position (Kaivosaaari et al., 2007, Patana et al., 2008). UGT1A4 and UGT2B10 differ in \(N\)-glucuronidation of medetomidine, a veterinary analgesic drug. UGT1A4 catalyzes the \(N\)-glucuronidation of dexametomidine, whereas UGT2B10 glucuronidates its enantiomer levomedetomidine (Kaivosaaari et al., 2008). It appears, at least with the medetomidines and some tricyclic antidepressants, that while glucuronidation velocities are higher with UGT1A4, UGT2B10 has much higher affinity for substrates (Zhou et al., 2010).

No good substrates are known for UGT1A5 and UGT2B11 and it is currently unclear if they are fully active UGTs. Both UGTs have been sequenced and shown to be poorly expressed at the mRNA level (Section 2.2.2). The very few reports about UGT2B11 glucuronidation activity suggested that UGT2B11 exhibited glucuronidation activity toward androsterone and metabolites of arachidonic and linoleic acid (Turgeon et al., 2003, Jin et al., 1997). However, when UGT2B11 was tested toward over 100 potential UGT substrates, it did not show any glucuronidation activity (Beaulieu et al., 1998).

UGT1A6 glucuronidates small and planar compounds, such as phenols, coumarins and indoles (Manevski et al., 2010, Hanioka et al., 2001). It is also the major UGT in glucuronidation of salicylic acid and the only UGT that glucuronides neurotransmitter serotonin, even if at low affinity (Krishnaswamy et al., 2003, Agundez et al., 2009). Although most UGTs can glucuronidate 1-naphthol, UGT1A6 catalyzes this reaction at a much higher rate than the other UGTs and, therefore, 1-naphthol is often considered as a non-selective substrate for UGT1A6 in the liver (Miners et al., 2010).

UGTs 1A7, 1A8, 1A9 and 1A10 have broad overlapping substrate specificity and highly homologous DNA and amino acid sequences, but only UGT1A9 is expressed in the liver. UGT1A7 glucuronidates cyclic aromatic compounds and was reported to play a role in the detoxification of tobacco carcinogens (Strassburg et al., 1999). UGT1A8 glucuronidation activity was proposed to be clinically significant in response to the anti-cancer drug raloxifene (Sun et al., 2013). UGT1A9 glucuronidates carboxylic acids and it is the most important hepatic UGT in mycophenolic acid glucuronidation (Bernard and Guillemette 2004). A well-known substrate for UGT1A9 is propofol, an important anesthetic drug (Ebner and Burchell 1993). Even higher specificity substrate for UGT1A9 was entacapone, a drug that increases the bioavailability of levodopa in treatment of Parkinson’s disease (Lautala et al., 2000). In addition, UGT1A9 is the only UGT that \(C\)-glucuronidates phenylbutazone, even if at a low rate (Nishiyama et al., 2006). Dihydroartemisinin, which is used for malaria treatment, is glucuronidated by UGT1A9 and UGT2B7 (Ilett et al., 2002). UGT1A9 also glucuronidates an anticancer agent flavopiridol and NSAID acetaminophen (Ramirez et al., 2002, Court et al., 2001).

UGT1A10 can conjugate heterocyclic amines (Dellinger et al., 2007). It also acyl glucuronidates diclofenac and indomethacin (Zhang et al., 2012b). There has been some controversy in the literature about UGT1A10 activity levels. In mammalian cells, UGT1A10 was reported to be a highly active enzyme, whereas the reports about the activity of the commercial recombinant UGT1A10 were different and only low activity was found (Lv et al., 2015b, Oguri et al., 2004, Basu et al., 2004, Lepine et al., 2004, Dellinger et al., 2007).
2006). Recombinant UGT1A10 that was produced in baculovirus-infected insect cells in our laboratory, however, glucuronidates estrogens with very high rates (Itáaho et al., 2008, Sneitz et al., 2013) and it exhibited the highest, by far, estrone glucuronidation rates (Kallionpää et al., 2015).

All the UGT2A family members glucuronidate bile acids. UGT2A1 has a broad substrate specificity and it glucuronidates its substrates at higher rates when compared to UGT2A2 (Sneitz et al., 2009). UGT2A2 was less active toward several other substrates, but it glucuronidated bile acids at a higher rate than UGT2A1 (Perreault et al., 2013, Sneitz et al., 2009). Both UGT2A1 and UGT2A2 are olfactory enzymes, whereas UGT2A3 is expressed in the liver and intestine. Nevertheless, UGT2A3 was not active toward most tested compounds, only some bile acids including hyodeoxycholic acid were substrates for this enzyme (Court et al., 2008). In studies of the metabolism of tobacco carsinogens, UGT2A1 showed activity against polycyclic aromatic hydrocarbons (Bushey et al., 2011).

With this type of compounds, UGT2A2 was active toward 1-naphthol, 1-hydroxypyrene and hydroxylated benzo(a)pyrene, whereas UGT2A3 was active only in 1-naphthol and 1-hydroxypyrene glucuronidation (Bushey, Dluzen and Lazarus 2013).

The substrate specificity of UGT2B4 overlaps UGT2B7 specificity, but it is not as broad as the latter. Attempts to distinguish the glucuronidation activities of UGT2B4 from UGT2B7, as well as to find a probe substrate for UGT2B4, have not yet succeeded (Barre et al., 2007). In a study of naftopidil, a drug that is used in benign prostatic hyperplasia, UGT2B4 was stereoselective and favored one enantiomer over the other, whereas UGT2B7 glucuronidates both of them equally (Liu et al., 2017).

UGT2B7 catalyzes glucuronidation of a wide range of xenobiotic and endobiotic substances. UGT2B7 is involved in the glucuronidation of endobiotic signaling molecules such as estriol, androsterone, bile acids, and retinoic acid (Gall et al., 1999, Sten et al., 2009). Furthermore, UGT2B7 is one of the most important human UGTs in drug metabolism. It glucuronidates drugs from various classes, such as opioids, NSAIDs, the anti-cancer drug ebirubicin and zidovudine (Innocenti et al., 2001, Coffman et al., 1998, Jin et al., 1993). Also, gemcabene, a novel drug that alters lipid composition, is glucuronidated by UGT2B7 (Bauman et al., 2005). While UGT1A9 is the main enzyme in mycophenolic acid glucuronidation, UGT2B7 catalyzes the formation of an acyl glucuronide. This acyl glucuronide is a minor metabolite of mycophenolic acid and may be responsible for some of the drug’s adverse effects (Picard et al., 2005). UGT2B7 is reported to be the main human UGT that metabolizes morphine-based opioids and the only one that conjugates morphine at the 6-hydroxy position, generating morphine-6-glucuronide, which stimulates the analgesic effect of morphine (Stone et al., 2003). The HIV drug zidovudine is also only glucuronidated by UGT2B7. Zidovudine is often used as a UGT2B7 probe compound, alongside morphine (Court et al., 2003). Carbinol, an antihormonal treatment for breast cancer patients, was reported to be a novel probe for UGT2B7 (Precht et al., 2013). UGT2B7, together with UGT2B4, glucuronidates the anti-diabetic drug evoglipin that is used for the treatment of type II diabetes mellitus (Jeong et al., 2015). Metabolites of a potent sedative benzodiazepine, midazolam, are formed by UGT2B7, UGT2B4 and UGT1A4 (Zhu et al., 2008, Seo et al., 2010). The drug sertraline, that is used to treat
symptoms of mania and depression, is also \textit{N}-glucuronidated by UGT2B7, with smaller contributions by UGT1A3, UGT1A6 and UGT2B4 (Obach, Cox and Tremaine 2005).

The amino acid sequences of UGT2B15 and UGT2B17 are 95\% identical and they have overlapping substrate specificities. Dihydrotestosterone is glucuronidated by UGT2B15 from its 17-hydroxy position, whereas UGT2B17 catalyzes 3-hydroxy glucuronidation of dihydrotestosterone (Gauthier-Landry, Belanger and Barbier 2015). Bisphenol A is an endocrine disruptor, which is still commonly used in the manufacture of plastic products, and it is mostly glucuronidated by UGT2B15 (Hanioka, Naito and Narimatsu 2008a). Testosterone glucuronidation is catalyzed primarily by UGT2B17, and its deletion polymorphism, which is a common polymorphism amongst the Asian population, was reported to reduce testosterone excretion (Jakobsson \textit{et al.}, 2006, Sten \textit{et al.}, 2009). The activity of UGT2B28 toward xenobiotics is limited, but there were reports about very low activity rates in steroidal compounds glucuronidation (Levesque \textit{et al.}, 2001).

\subsection*{2.2.4 UGT specific inhibitors}

Sometimes it is difficult to identify with certainty which enzyme catalyzes a given reaction. For example, the UGT1A3 activity often resembles UGT1A1 and UGT1A4 activity, and UGT1A8 activity resembles UGT1A10 activity in the intestine. Such cases might be solved by using enzyme specific inhibitors, if they are available. Unfortunately, even less enzyme specific inhibitors are known than specific substrates and their use does not always solve the issue. For example, experiments with recombinant UGT1A8 and UGT1A10 showed that both enzymes are inhibited by the same inhibitors, either bakuchiol or emodin (Dong \textit{et al.}, 2014, Watanabe, Nakajima and Yokoi 2002).

An inhibitor is a molecule that binds to the enzyme and inhibits its glucuronidation activity. This can occur when the molecule binds directly to the active site of the enzyme or the UDPGA binding site. Some inhibitor could bind to other parts of the enzyme as well and alter its movement. Binding of an inhibitor might affect the microenvironment at the active site of the enzyme, so that glucuronidation reaction does not take place, or is slowed down considerably. Inhibitor molecules do not need to be UGT substrates, but some substrates could act as inhibitors for other glucuronidation reactions. For example, in cholestasis, the bile acid concentrations rise, which inhibits glucuronidation reactions for other UGT substrates (Fang \textit{et al.}, 2013). The UGT1A1 substrate bilirubin inhibits posaconazole glucuronidation, a reaction that is mainly catalyzed by UGT1A4 (Ghosal \textit{et al.}, 2004). Bisphenol A, which is mostly glucuronidated by UGT2B15 (Hanioka \textit{et al.}, 2008a) inhibits UGT2B family members, as well as UGT1A6 glucuronidation of serotonin (Hanioka \textit{et al.}, 2008b, Jiang \textit{et al.}, 2013). There are many reported UGT inhibitors and more are expected to be identified in the near future, but their specificity is rarely tested in full. In Table 2, common UGTs inhibitors are listed, but only few of them can be considered as specific, since in many cases not all the recombinant UGTs were tested in these studies.

Sometimes the inhibitory effect may be substrate dependent. This can be because there is more than one active site in the enzyme, or due to large differences in the glucuronidation rates and their inhibition degrees. For example, flunitrazepam inhibited catechol estrogen
Table 2. Compounds that were reported as UGT specific inhibitors. Certain inhibitors may be specific when liver microsomes are assayed, but not when extrahepatic enzymes are present. The inhibitory effect may depend on the used substrate and the inhibitor concentration.

<table>
<thead>
<tr>
<th>UGT enzymes</th>
<th>Enzyme specific inhibitors</th>
</tr>
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<tbody>
<tr>
<td>UGT1A1</td>
<td>Nilotinib&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>Imipramine&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Hecogenin&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>Magnolol&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Niflumic acid&lt;sup&gt;2&lt;/sup&gt;, magnolol&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Fluconazole&lt;sup&gt;3&lt;/sup&gt;, imipramine, propofol, androstanediol&lt;sup&gt;6&lt;/sup&gt;, gemfibrozil&lt;sup&gt;7&lt;/sup&gt;, isaloxifolol derivative C26b&lt;sup&gt;9&lt;/sup&gt;, atractyleolide&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT2B10</td>
<td>Trifluoperazine&lt;sup&gt;4&lt;/sup&gt;, imipramine&lt;sup&gt;4&lt;/sup&gt;, amitriptyline&lt;sup&gt;4&lt;/sup&gt;, nicotin&lt;sup&gt;6&lt;/sup&gt;, desloratadine&lt;sup&gt;6&lt;/sup&gt;, amitriptyline&lt;sup&gt;4&lt;/sup&gt;, doxepin&lt;sup&gt;6&lt;/sup&gt;, mianserin&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>Valproic acid&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>


glucuronidation by UGT2B7, UGT1A1 and UGT1A3, but only inhibited UGT1A1 and UGT2B7 when the substrate was buprenorphine (Cheng et al., 1998).

In addition, there are compounds that inhibit a certain family of UGTs instead of a single enzyme. These kinds of inhibitors may be useful when studying potential drug-drug interactions, or in reaction phenotyping when using individual recombinant enzymes. The UGT1A family inhibitors are for example the two sodium-glucose transporter inhibitors canagliflozin and dapagliflozin, that potently inhibited UGT1A enzymes, mostly UGT1A1, UGT1A9 and UGT1A10 (Pattanawongs et al., 2015).

2.2.5 Regulation of UGT expression

UGT expression is modified by polymorphism, copy-number variations, alternate splicing and epigenetics (Guillemette et al., 2010). High degree of epigenetic DNA methylation was reported to be the cause for the lack of UGT1A10 from the liver, whereas hypomethylation allow, perhaps support, its expression in the intestine (Oda et al., 2014). The main molecular mechanisms for expression variation between the tissues are differences in the transcription factors that are expressed in them, such as sp1, hepatocyte nuclear factor 1 alpha (HNF1α) that are highly expressed in the liver, in contrast to the intestinal caudal homeodomain transcription factor, Cdx2 (Gregory et al., 2004). Naturally, transcription factors are active mainly on UGTs that have suitable sequences for them in their promoter region. An additional aspect of transcription factor is nuclear receptors. They could be activated by ligands, such as benzo(a)pyrene that activates the aryl hydrocarbon receptor (AhR), which
regulates the transcription of several UGT1As (Mackenzie, Hu and Gardner-Stephen 2010). Regulation could be tissue specific and either down- or upregulation. For example, estradiol induces UGT2B15 and UGT2B17 expression through estrogen response unit (ERU) in breast cancer cells (Mackenzie et al., 2010). If the transcription factors and nuclear receptors work at the level of mRNA transcription, microRNAs are reported to inhibit UGT mRNA translation process (Dluzen et al., 2016).

UGTs expression levels vary throughout life. The liver glucuronidation activity is low in a fetus, because the blood passes through the umbilical cord and the toxins are metabolized in the mother's liver. Glucuronidation begins in the fetus liver after 20 weeks, but the enzyme activity levels stay low (Ekstrom, Johansson and Rane 2013, Krekels et al., 2012). For example, the fetal liver UGT1A1 activity is less than 1% of the corresponding adult’s enzyme (Kawade and Onishi 1981). Neonatal liver has 10-fold less UGT1A4, UGT1A6, UGT1A9 and UGT2B7 than in adult liver, but the levels increase rapidly in the first few days and weeks after birth. The maturation rates of UGTs vary and their abundances in the liver grow throughout childhood (Bhatt et al., 2018, Krekels et al., 2012). After reaching adulthood, the age does not influence activity levels of the UGTs (Court 2010, Miners and Mackenzie 1991).

Hormonal factors have been proposed to affect the glucuronidation of some drugs and to be UGT specific (Miners and Mackenzie 1991). UGT2B15 and UGT1A6 exhibited slightly higher glucuronidation levels in males but, in general, there was no large differences in liver glucuronidation between males and females (Court 2010).

Some herbs and drugs, cigarette smoking and alcohol were shown to induce UGT expression and increase glucuronidation rates in liver microsomes (Court 2010). The levels of hepatic UGT2B7 was reported to be reduced in cases of diabetes mellitus (Dostalek et al., 2011).

2.3 Enzyme sources and protein expression systems

Studies of human glucuronidation activity in vivo are usually done by measuring glucuronides from urine and feces, and combining the data with information on the patient’s genotype. The in vivo glucuronidation could be predicted by calculations, or in more complicated cases by in silico modelling from in vitro data. The in vitro assays are done either with enzymes mixture from a tissue sample (microsomes) or with individual enzymes which are expressed as recombinant enzymes in heterologous expression systems.

2.3.1 Recombinant UGTs expression systems

UGTs have been expressed in many host cells as recombinant proteins. The UGTs expression have some limitations, however, because their catalytic activities require correct folding and protein structure. For the folding, the post translational modifications (PTMs), such as signal sequence cleavage, protein glycosylation and phosphorylation, should be close as possible to the PTMs in the native cells, for example hepatocytes and enterocytes.
There is a report on expression of a full-length human UGT in the bacterium *Escherichia coli* (*E. coli*) (Ouzzine *et al.*, 1994). In that case it was made by using a signal sequence from bacteria, which was not cleaved correctly, but it was improved following mutagenesis. The advantage of *E. coli* is that proteins can be expressed at a rather large amounts, and this system was suitable for getting enough protein for the crystallization of the UGT2B7 fragment of the C-terminal half that was successfully crystallized and its structure solved by X-ray crystallography (Miley *et al.*, 2007). Hence, it seems that while the *E. coli* expression is preferable for water-soluble protein fragments, beyond UGT1A6 it had no successful continuation with full-length UGTs.

Yeast, a simpler eukaryote, differ from higher eukaryotes in its mannose-rich protein glycosylation, making it less optimal system for the expression of recombinant ER and plasma proteins. In addition, the yeast *Saccharomyces cerevisiae* (baker’s yeast) does not produce UDPGA, raising doubt about its ability to correctly fold a recombinant UGT.

*Spodoptera frugiperda* is a moth, the Fall armyworm, from which several insect cell lines were isolated and developed into a protein expression system, following infection by modified baculovirus. The main advantage of these cells, such as Sf9, Sf21 and High Five, in comparison to mammalian cells, is that they can be cultured successfully in suspension. This allows a rather rapid production of high amounts of recombinant protein, particularly when combined with the strong, late promoter of baculovirus. These cells are able to form N-glycosylated proteins, but they are not able to build complex N-glycans with terminal sialic acids (Shi and Jarvis 2007). Another issue seems to be that insect cells lack as strict or efficient quality control system for removal of incorrectly folded proteins from the ER (correct PTMs) as mammalian cells have. This might allow the production of mutations that would not be expressed in mammalian cells (Polgar *et al.*, 2006). The disadvantage is that it could cause an accumulation of a large proportion of inactive enzyme, if the protein expression machinery is pushed too far with a large amount of virus (Zhang *et al.*, 2012, Oda *et al.*, 2012).

The first human recombinant human UGT was expressed in immortalized monkey kidney COS-7 cells in 1988 (Harding *et al.*, 1988). Another mammalian cell line was V79, which is a Chinese hamster lung cell line and its protein expression rate is higher than in COS-7 cells (Fournel-Gigleux *et al.*, 1991). Currently, the most commonly used mammalian cell type for recombinant UGTs expression is human embryonic kidney 293 cells (HEK293) (Coffman *et al.*, 1997, Radominska-Pandya, Bratton and Little 2005, Kozlovich *et al.*, 2015, Girard-Bock *et al.*, 2016). The PTMs in HEK293 cells are correct, however, mammalian cells cultivation takes relatively long time and it is more expensive than insect cells culturing, largely since the cells are grown only as monolayers. The yield of expressed recombinant enzyme is mostly low, but often the amount is enough for kinetic studies, particularly these days, when more laboratories have access to sensitive and accurate detection systems.

The most often used, commercial recombinant UGTs, are expressed in insect cells. They were, and still are, produced by Gentest™ and sold as Supersomes™. The company that sold them was previously BD Biosciences and now it is Corning® (https://www.corning.com/worldwide/en/products/life-sciences/products/adme-tox-research/recombinant-metabolic-enzymes.html, accessed Nov 2018). There are also some
smaller companies selling UGTs that were produced in other expression systems. MyBiosource company sells the recombinant proteins which are produced in yeast, prokaryotes or cell free expression system. UGT1A1 is produced in yeast with a His-tag or N-terminal half of UGT1A1 with N-terminal His-tag in E. coli. UGT1A4 is from E. coli and it has 6xHis-tag in its N-terminus with or without a small ubiquitin-related modifier (SUMO) fusion for improved protein yield and solubility. Also, UGT2B7 is partially expressed in E. coli with N-terminal His-tag (https://www.mybiosource.com, site visited on Nov 2018).

It may be noted here that the N-terminus of the UGTs is predicted to fold close to the active site, whereas the last section of the C-terminus goes through an ER membrane and should not take part in the glucuronidation reaction. This would make the C-terminus a safer choice to attach a tag for the protein purification and expression level determination.

2.3.2 Microsomal fractions and hepatocytes

Often the first step to assess the amount and kind of the formed glucuronide(s) is to use isolated human liver and intestine microsomes, each of which is a typical mixture of UGTs in a certain tissue. For drug glucuronidation, liver microsomes that contain pooled microsomes from human livers are the most important. However, human (or dog) liver microsomes cannot be used as in vitro prediction models for glucuronidation that takes place in the gut because they lack certain extrahepatic UGTs, namely UGT1A7, UGT1A8 and UGT1A10 in human and UGT1A2, UGT1A9 and UGT1A11 in dog (Heikkinen et al., 2015), if to list only the UGT1A subfamily members. There are commercially available intestinal microsomes, but they are usually smaller pools, from 5-10 donors in contrast to 50 donors that make current commercial human liver microsomes pools, and in addition to this their intestinal UGT content may vary depending on the site along the intestine from which the sample was taken.

Another option to study UGT in the liver is to use cultivated liver tissue, particularly primary hepatocytes. These cells differ from other cells in having a large amount of endoplasmic reticulum membranes. Primary hepatocytes do not proliferate in culture, however. There are hepatocyte cell lines that originated from cancer cells, such as Hep3B, HepG2 or Huh7. HepG2 cell lines are the most commonly cultivated, but often fail to expression UGTs at significant levels (Westerink and Schoonen 2007). A breakthrough came with the development of hepatocytes cell line that will be reproducible and express rather correctly the liver UGTs as well as the other drug metabolizing enzymes, namely development of the HepaRG cell line (Aninat et al., 2006), even if the ratio of different enzymes within them does not as accurately represents the in vivo situation in human liver as do both fresh and cryo-preserved hepatocytes (Darnell et al., 2012). Nevertheless, since HepaRG is a commercial system and not cheap, it is not often used in academic laboratories for routine or early stage metabolic studies.
2.3.3 Oligomerization and protein interactions

Moving a substrate to the UGT active site and the glucuronide out from the ER lumen requires facilitation through the membrane, because even if the UGT substrates are to some extent lipophilic, they do not always diffuse through the membrane efficiently enough, particularly not the cofactor UDPGA. There is an ER transporter that effluxes glucuronides from the ER lumen (where UDPGA is concentrated) out to the cytosol. If enzymes that are in the same detoxification pathway, such as CYP and UGT, are close enough to interact, it might enhance the elimination process. With untargeted proteomics, UGTs have been shown to interact with other detoxification proteins, but also with lipids and carbohydrates (Rouleau et al., 2017). Supporting evidence of enzyme interactions were that UGTs had been shown to be expressed as oligomers (Finel and Kurkela 2008). The conformation and membrane topology of oligomeric UGTs is currently undetermined (Figure 9).

In some publications, a specific glucuronidation activity increases when the UGT is coexpressed with another UGT enzyme. UGT2B7 was reported to be much more active when it is coexpressed with a UGT1A subfamily member (Fujiwara et al., 2010). In another paper, the activity of a mutant UGT1A6 could be stimulated by coexpression with nearly each of the other human UGTs, above all when it was coexpressed with UGT2B7 (Kurkela et al., 2007).

The C-terminal transmembrane helix has been studied with truncated proteins and it was proposed to interact between the dimers (Yuan et al., 2016). Also, codons 71 and 268 in UGT2B7 were suggested to be part of the dimeric interaction (Yuan et al., 2015).

![Figure 9](image)

The active site of the UGT is facing the luminal side of the ER. UGTs are type I membrane proteins and have a single C-terminal transmembrane helix. The oligomers are suggested to interact through the C-terminal part of the protein as well as N-terminal parts, but their detailed oligomeric topology is currently unknown.
Another unanswered question is whether UGTs have several active sites? Sometimes it is observed that they have atypical kinetics, such as biphasic kinetic profile, which could be caused by multiple binding sites, or result from the oligomeric nature of UGTs. Examples of atypical glucuronidation kinetics are biphasic kinetics for morphine-6-glucuronide by UGT2B7 and for 4-phenylphenol by UGT2A1 (Stone et al., 2003, Sneez et al., 2009). In addition, enzyme specific heterotropic activation have been reported previously (Mano et al., 2004).

2.4 Activity determination to study enzyme function

Almost seventy years ago, Dutton and Storey discovered the glucuronidation reaction and that it required some element from boiled liver extracts, which two years later they proposed to be UDP-glucuronic acid (Dutton and Storey 1951, Dutton and Storey 1953). UDPGA is the cofactor that is now added the reaction mixture that includes enzymes to initiate the glucuronidation reactions. In kinetic analyses, the substrate concentration is variable (unless UDPGA kinetics is tested), whereas the amounts of enzyme, incubation time, as well as the other conditions of the reaction are usually constant (Walsky et al., 2012).

2.4.1 Glucuronidation screening using recombinant enzymes

Microsomal glucuronidation data gives the total glucuronidation information from the tested tissue, but does not identify the enzyme(s) that are involved in the reaction, and their relative contribution. For this reason, screening assays are performed with individual recombinant enzymes. This is usually done in the presence of a substrate concentration that is a little below or equal to the $K_m$ value that was obtained with liver (or other tissue) microsomes, and then also ten-fold higher substrate concentration. With active enzymes, the individual kinetic profiles could be determined and then compared to kinetics done with microsomes (see Section 4.3.2) (Picard et al., 2005, Street et al., 2017). In the literature, screening assays using recombinant UGTs are usually done with commercial UGTs, with the low activity UGT1A10 (V), or without enzymes such as UGT2B10 (Nishihara et al., 2013) that only became commercially available at a later stage than most other UGTs. In some cases, the high glucuronidation rates in the tissue microsomes could not be explained by the activity of the screened commercial recombinant enzymes, particularly with extrahepatic UGTs (He et al., 2018). Therefore, just on the basis of glucuronidation rates in the screening assays with recombinant UGTs it is unwise to draw conclusions about the UGTs’ relative contribution. The protein concentration in the commercial enzyme preparations cannot be used for direct comparison of the activity rates between two enzymes in screening assay, where the glucuronidation rates are often given as pmol/mg/min. This is both since the membranes are of different origin and protein composition, and mainly because the commercial samples probably include a mixture of active and inactive UGT, making comparisons based on immunodetection, even when a specific antibodies are available, rather meaningless (Oda et al., 2012). This is also an issue with LC-MS/MS
quantitation of tryptic peptides. Hence, while accurate activity comparison should not be based on activity screening, kinetic profiles and calculations such as relative affinity approach could be done and yield meaningful results (Kishi et al., 2016, Gill et al., 2012, Hong et al., 2017, Zhu et al., 2012b). Another option to ensure that a recombinant UGT activity is similar to the microsomal activity is to inhibit its specific probe activity in microsomes, or ensure that the test activity disappears by using enzyme specific inhibitors (Miners et al., 2010).

2.4.2 Kinetic parameters in the comparison of enzyme activities

In UGT studies, the $K_m$ and $V_{\text{max}}$ values, which are derived from the Michaelis-Menten equation, the simplest kinetic model that can be used to describe enzyme reactions (Cornish-Bowden 2013). The equations and kinetic parameters are further introduced in Section 4.5. Measured data in glucuronidation assays is fit into kinetic models, where the simplest fitting model should be chosen for parameters calculations.

The comparison of these parameters cannot be done straightforwardly. First the kinetic models should be the same if the parameters are compared. The $V_{\text{max}}$ values are directly proportional to the protein concentration, and more specifically proportion to the concentration of the active enzyme, which is not easy to determine. A comparison of $K_m$ values is more sensible, especially when the substrate concentration stays below the $K_m$ values (Eisenthal, Danson and Hough 2007). The $K_m$ values should not differ significantly between the measurements or between different enzyme preparations that measure the same activity under the same conditions. The in vitro intrinsic clearance $CL_{\text{int}} (V_{\text{max}}/K_m)$ is used often in the literature to describe the theoretical clearance of a drug in the assay, or with further calculations, in the tissue (Miners et al., 2010, Knights et al., 2016). The comparison of these values to predict in vivo glucuronidation is fine as long as the amount of active enzyme in the enzyme sources is the same as it is in the tissue of interest.

The terms turnover number ($k_{\text{cat}}$) and catalytic proficiency are other terms that are used when enzyme activities are compared. It would be tempting to compare these two values of different UGTs when the aim of the research is to know which is the most active enzyme in catalyzing a given reaction. Problem is that the value of $k_{\text{cat}}$ is dependent on $V_{\text{max}}$ and hence, the amount of active enzyme. Similarly, the catalytic proficiency constant ($k_{\text{cat}}/K_m$) sometimes gives incorrect conclusions, because two enzymes’ rates ratio might vary at different substrate concentrations. So, if two enzymes have different $K_m$ values, the higher catalytic efficiency enzyme can at certain substrate concentration catalyze a reaction at lower rates than the other enzyme, with lower efficiency value. These specificity determinations should be used as they were originally intended, comparing the kinetics of different substrates for the same enzyme (Cornish-Bowden 2012, Eisenthal et al., 2007).
2.4.3 The challenges in glucuronidation in vitro assays

Understanding glucuronidation at the enzyme level is a crucial part of the drug metabolism that is not yet fully understood, making the study of this topic important.

Many efforts have been made to produce enough soluble, human recombinant UGT for structural studies (Kurkela et al., 2003), but thus far only the structure of part of the C-terminal domain, containing residues 285–451 of UGT2B7, have been solved by X-ray crystallography at the high resolution of 1.8 Å (Miley et al., 2007). The structure of this segment, which contains the UDPGA binding site, showed that in the core there is a single parallel β-sheet of six strands and around them there are seven α-helices (Miley et al., 2007).

In addition to lack of high-resolution structures, there are only few highly specific antibodies, few specific substrates, and only few inhibitors currently known (see Sections 2.2.3 for substrates and 2.2.4 for inhibitors). The absence of such tools makes comparison of enzyme activities between different UGT preparations difficult. Even a buffer change in could influence activity by three-fold, such as in the case of zidovudine (Engtrakul et al., 2005, Soars et al., 2003b, Walesky et al., 2012). Moreover, sometimes unpredicted assay conditions or even assay vials can affect the results, as was the selective inhibition of UGT1A7 by liquid scintillation glass vials (Uchaipichat et al., 2006b). Multiple binding and effector sites also add difficulties in studying these enzymes, because sometimes the effect is visible with certain substrate, but not with another (Uchaipichat et al., 2008). In addition, the results vary since not so many laboratories study UGTs and even fewer produce their own enzymes, so they use the same commercial enzymes that are available. Variability between laboratories can be “normalized” by using the commercial enzymes (not in all cases). Different laboratories favor some buffer over another, the mostly used are phosphate and Tris-buffers. The buffer pH is usually adjusted to 7.4, but sometimes the pH is lowered to prevent metabolite hydrolysis (Zhang et al., 2012b).

A widely known and examined problem of in vitro assays is the so called UGT latency. A recently published paper reviewed three different theories that could explain UGT latency (Liu and Coughtrie 2017). Those theories are compartmentation, conformation change and inhibitors, and all the three theories are related to the location of UGT in ER (Liu and Coughtrie 2017). First, the latency could be caused by the treatment during membrane preparation, where membranes form a barrier that polar compounds cannot pass. UDPGA is a charged molecule that is synthesized outside the ER in the cytosol, so it needs to cross the membrane to access the UGT. The product glucuronides also need some transport in the other direction, to get outside the ER. Whilst the transporter for UDPGA is known, the glucuronide transporter in the ER membrane has not been identified. The second theory about the cause of latency was that the physical properties of released phospholipids from preparation of microsomes change the enzyme conformation or interactions, thus inhibiting their activities. The third theory was that some luminal inhibitors are released during the membrane preparation (Ishii et al., 2012, Liu and Coughtrie 2017).

Despite the different reasons for why there is UGT latency, the first solution to overcome the latency was to make the membrane more permeable. This was done by different mild detergents, such as Brij 58 and CHAPS (Ishii et al., 2012, Lett et al., 1992). Another commonly used method is to add alamethicin into the reaction mixture. Alamethicin is an antibiotic peptide that makes holes in the membrane. The magnitude of
latency removal with alamethicin is more reproducible between different UGTs than it is with detergents (Walsky et al., 2012, Fisher et al., 2000a). The amount of alamethicin that should be added into reaction mixtures to remove UGT latency differ between publications. Initially it was suggested that 50 µg of alamethicin should be added per mg of total protein concentration (Fisher et al., 2000a). A later study stated that instead of total protein amount, one should use the volume of microsomes. Walsky and his colleagues suggested in their report that 10 µg alamethicin per ml of microsomes would be a sufficient amount for glucuronidation studies (Walsky et al., 2012). Another option is to optimize the alamethicin concentration that is needed for the assay by checking that the glucuronides formation is within the linear range. The reason why membrane disruption methods overcome the UGT latency is not clear, but it is more likely that membrane disruption in this way accelerates substrate and UDPGA movements to and from the catalytic site (Liu and Coughtrie 2017).

While UGT latency was present only in microsomal preparations, another type of inhibition was found to reduce the activities of recombinant enzymes, as well as microsomes. The solution to overcome this inhibition in in vitro-assays and thus to improve in vitro - in vivo extrapolation was to add albumin into reaction mixture. This was suggested to remove inhibitory fatty acids that are released during the membrane or microsomes preparations. The amount of added albumin varies somewhat between laboratories. Our group published a paper where 4-MU glucuronidation by UGT1A9 was studied with two concentrations of albumin, namely 1% and 0.1% (Manevski et al., 2011). The 0.1% BSA concentration was a good compromise to have an albumin’s inhibitor removal effect but still to be able to perform the studies with a high fraction of unbound substrate. The albumin binding is not that high (and therefore not a problem) if the substrate is less hydrophobic. For example, zidovudine’s binding to 2% albumin is negligible (5%) and this binding is independent of zidovudine concentration (Uchaipichat et al., 2006b). The 0.1% BSA concentration was used also in the albumin study (I), mainly to prevent situations where there would have been only a very small fraction of unbound substrate, as we studied more lipophilic substrates. In addition that the mechanism how albumin affects is not fully clear, the obtained results varied for some enzymes. For example, UGT1A9 was reported to have an increase in Vmax when substrate was entacapone (Manevski et al., 2011) whereas in other studies using different substrates, there was no effect on the Vmax (Rowland et al., 2008, Shiraga et al., 2012, Manevski et al., 2011, Manevski et al., 2012, Walsky et al., 2012).

There are also some aspects that make the UGT studies easier than other drug metabolism enzymes. For example, product formation can be measured, which is not the case with transporters that transfer the same molecule, such as glucuronide, across the membrane. The physicochemical properties of the reaction product, the glucuronide, differ from the aglycone, meaning that the product could be separated from the parent compound and its formation measured. Glucuronide characterization is often done by LC/MS/MS (Ketola and Hakala 2010). For drugs, it is important that they are somewhat hydrophobic to be absorbed and reach the site of effect, but if they are too hydrophobic, they would accumulate inside the cells. Hence, the solubility of both substrates and glucuronides is usually not a serious problem, and when the substrate is hydrophobic, substrate stocks can be prepared in dimethyl sulfoxide (DMSO) because UGTs tolerate few percent of organic
solvent such as DMSO in their reaction mixture (Zhang et al., 2011, Uchaipichat et al., 2004).
3 Aims of the study

The main objective of this study was to learn how individual UGT enzymes differ in substrate preferences and activity. This was done by studying activity differences between variant and wild type enzyme, by measuring glucuronidation activity of different enzymes and by comparing dog and human UGT1A family members.

The comparison of substrate preferences is a tool to learn about the substrate binding, active site and eventually about the 3D-structure of the protein. In addition, in the case of SNPs or species orthologs, the aligned protein sequences could help to detect candidate amino acid residues that modify the enzyme’s glucuronidation activity.

A challenging SNP-related aim was to solve the mechanism of a functional polymorphic variant UGT1A4-P24T that occurs inside the signal sequence of UGT1A4. If that mutated amino acid is cut off from a mature protein, how could it affect the glucuronidation reaction?

Another aim was to identify which enzymes glucuronidate structurally similar substrates bisphenol A, bisphenol F and bisphenol S.
4 Materials and methods

4.1 Substrates, inhibitors and commercial enzymes

The original articles I-V describe in detail the methods, chemicals and tested substrates, as well as the suppliers of each used materials. The list of studied substrates and inhibitors is also given in Table 3. The structures were drawn using the ChemDraw program (ChemDraw® Professional, version 17, PerkinElmer). The purity of the substrates was equal or higher to 98%.

Table 3. Substrates and inhibitors that were used in the different parts of this thesis. The first column presents compound’s name and inside brackets its abbreviation in the thesis. CAS numbers are given for further identification. The abbreviation Publ. in the column on the right indicates the publication number in this thesis, where the substrate has been used.

<table>
<thead>
<tr>
<th>Compound name (abbreviation)</th>
<th>CAS number</th>
<th>Chemical structure</th>
<th>M, g/mol</th>
<th>Publ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylumbelliferone (4-MU)</td>
<td>90-33-5</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>176.17</td>
<td>I, II, V</td>
</tr>
<tr>
<td>1-Naphthol (1-NP)</td>
<td>90-15-3</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>144.17</td>
<td>I</td>
</tr>
<tr>
<td>Epiestradiol (epi-E2)</td>
<td>57-91-0</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>272.39</td>
<td>I, II</td>
</tr>
<tr>
<td>Estradiol (E2)</td>
<td>50-28-2</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>272.39</td>
<td>II, V</td>
</tr>
<tr>
<td>Ethinylestradiol (EE)</td>
<td></td>
<td><img src="image" alt="Chemical structure" /></td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Bilirubin (BIL)</td>
<td>635-65-4</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>584.67</td>
<td>II</td>
</tr>
<tr>
<td>Diclofenac (DIF)</td>
<td>15307-86-5</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>296.15</td>
<td>II, V</td>
</tr>
<tr>
<td><strong>Indomethacin</strong> (IND)</td>
<td>53-86-1</td>
<td>357.79</td>
<td>II, V</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
<td>--------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td><strong>R-propranolol</strong> (<strong>R-PR</strong>)</td>
<td>13071-11-9**</td>
<td>259.35</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><strong>S-propranolol</strong> (<strong>S-PR</strong>)</td>
<td>4199-10-4**</td>
<td>259.35</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><strong>Bisphenol S (BPS)</strong></td>
<td>80-09-1</td>
<td>250.27</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td><strong>Bisphenol A (BPA)</strong></td>
<td>80-05-7</td>
<td>228.29</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td><strong>Bisphenol F (BPF)</strong></td>
<td>620-92-8</td>
<td>200.24</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td><strong>Trifluoperazine</strong> (<strong>TFP</strong>)</td>
<td>440-17-5**</td>
<td>407.16</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td><strong>Estrone</strong> (E1)</td>
<td>53-16-7</td>
<td>270.37</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td><strong>7-Amino-4-methyl-coumarin (AMC)</strong></td>
<td>26093-31-2</td>
<td>175.19</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td><strong>Atractylenolide I (ALI)</strong>*</td>
<td>73069-13-3</td>
<td>230.31</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td><strong>Entacapone</strong> (ETC)</td>
<td>130929-57-6</td>
<td>305.29</td>
<td>I, II, V</td>
<td></td>
</tr>
<tr>
<td><strong>Dexmedetomidine</strong> (DME)</td>
<td>113775-47-6</td>
<td>200.29</td>
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<tr>
<td><strong>Levomedetomidine</strong> (LME)</td>
<td>119717-21-4</td>
<td>200.29</td>
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<td><strong>Testosterone</strong> (TS)</td>
<td>58-22-0</td>
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</tr>
<tr>
<td>Compound</td>
<td>CAS Number</td>
<td>Molecular Weight</td>
<td>UGT</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Epi-testosterone (epi-TS)</td>
<td>481-30-1</td>
<td></td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>6-Hydroxy-indole (6-HI)</td>
<td>2380-86-1</td>
<td></td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>7-Ethyl-10-hydroxy-camptothecin (SN-38)</td>
<td>86639-52-3</td>
<td></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Nilotinib (NIL)*</td>
<td>641571-10-0</td>
<td></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Bavachinin (BAV)*</td>
<td>19879-30-2</td>
<td></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>N-(3-carboxypropyl)-4-hydroxy-1,8-naphthalimide (NCHN)</td>
<td>NA</td>
<td></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Morphine (MRP)</td>
<td>52-26-6**</td>
<td></td>
<td>V</td>
<td></td>
</tr>
</tbody>
</table>

*used as inhibitor in the study  
**hydrochloride  

Commercial recombinant UGTs (Supersomes™), UGT1A1, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15, were purchased from Corning Life Sciences (Corning, NY) or from the former retailer of these enzymes, BD Biosciences (Franklin Lakes, NJ).

Pooled human liver (Lot 18888) and intestinal microsomes (Lot no. 3189756) and pooled male beagle liver microsomes were bought from BD Biosciences (Franklin Lakes, NJ). The pooled male beagle intestinal microsomes were bought from Xenotech, LLC (Lenexa, KS).

### 4.2 Cloning and expression of recombinant enzymes

Usually, an already processed DNA is required for cloning because it includes only the parts of the gene that are translated. This means that we exclude introns, regulatory parts and
other DNA areas that do not encode any amino acids in the DNA. Usually, a good source for start cloning UGTs is either total RNA, mRNA or cDNA libraries from the tissue of interest for cloning. So, if we use liver cDNA library, we get only the enzymes that are expressed in the liver, but not extrahepatic UGTs.

Because dogs UGT1As share exons 2-5, just as human UGT1As do, it was possible to clone all the dog UGT1A family members from liver cDNA and genomic DNA, without knowing in which tissue they are expressed. The only dog 1A enzyme that was previously expressed, dog 1A6, was cloned from dog liver cDNA, so we knew that we could use liver cDNA to get the common part of genes without introns (Soars et al., 2001b). Without that previous work, we would have started with dog UGT1A1, because it is rather certain that the dog UGT1A1 is expressed in the liver, where it has its role in bilirubin glucuronidation. Following the cloning of full-length dog UGT1A6, only the first exon was needed of any other dog UGT1A to make the construct complete. Further details of the method for dog UGT1As clones are described in Figure 10 and detailed in publication II.

**Figure 10** Liver cDNA was used to get the common part (shared exons) of dog UGT1As without introns. Primers were made to match sequences of published members of dog UGT1A subfamily (Li and Wu 2007) and used to “fish” the first exon for each subfamily member out from genomic DNA. These parts were ligated together to complete the clone construct.

Already before the dog UGTs, the majority of human recombinant UGTs were cloned and expressed in our laboratory. They were expressed with a short C-terminal fusion peptide that ends in a His-tag, using baculovirus infected *Spodoptera frugiperda* (SF9) insect cells (Kurkela et al., 2007, Kurkela et al., 2003, Kuuranne et al., 2003). The amount of active UGT in the cells was optimized for each enzyme by adjusting the amount of the virus used in infection. The UGTs expression level was mostly determined by dot blot, as described below, and their activities were measured with suitable substrates (Zhang et al., 2012). After determining the optimum virus infection, the selected virus concentration was used for larger scale infection. The insect cells were cultured for 48 h post infection, then they were
collected by centrifugation, washed and stored at -20°C. The washed cell pellets were later
thawed and either suspended for use as cell suspensions or subjected for preparation of
enzyme enriched membrane preparations. Typically, the final total protein concentration of
the membrane preparations was about 20 mg/ml.

Immunoblotting was used to determine the level of recombinant protein expression in
membrane preparations or cells (Kurkela et al., 2004, Kurkela et al., 2007). The dot blot
samples were done in triplicates and an average value was used for the normalization of
glucuronidation rates according to expression level differences. In other words, the
glucuronidation activity rates were divided by the expression level of each enzyme, using
the lowest expression level enzyme in the set as 1.0 for such comparisons. The immunoblots
were done using anti-His-tag monoclonal antibody, which allows to compare the expression
of different UGTs to each other. Since commercial enzymes lack a His-tag, they were not
expression normalized in the assays. Because there is no good antibody for all the UGTs,
and because our laboratory has a good specific antibody against histidine tag, addition of
the c-terminal His-tag to recombinant protein is the most efficient way for us to determine
expression levels of all three subfamily proteins, UGT1A and 2A and 2B. In the expression
optimization step, our laboratory tries to minimize the level of inactive UGT (Zhang et al.,
2012), however, the commercial UGTs might contain considerable amounts of inactive
enzyme (Oda et al., 2012). This means that even if there was an accurate way to compare
UGTs expression in different commercial samples, its results might have been misleading
since such immunodetection mostly measure both the active and inactive proteins.

4.3 Glucuronidation assays

Substrate stock solutions were prepared either in methanol or DMSO. When the solvent
was methanol, the substrate dilutions were pipetted into reaction tubes and the methanol
was evaporated either under vacuum or under a nitrogen flow.

To activate microsomes, 50 μg alamethicin per mg of a microsomal protein were added
into the mixtures (Fisher et al., 2000a). Alamethicin was not added into reactions done with
recombinant enzymes, because previous studies in our laboratory showed that the
recombinant proteins produced in insect cell membranes do not require alamethicin for
activation (Kaivosaari et al., 2008, Zhang et al., 2011). The glucuronidation reaction
mixtures consisted of between 0.02 mg/ml and 0.2 mg/ml total protein as enzyme source
(recombinant or microsomes) and 10 mM MgCl₂ in 50 mM phosphate buffer, pH 7.4. In
diclofenac assays, the pH of the buffer was adjusted to 6.0 (Zhang et al., 2012b) and in the
albumin effect studies, 0.1% albumin was added to the mixtures. The mixtures were pre-
warmed for 5 min in a +37°C thermostated heat block before reaction initiation by the
addition of 5mM UDPGA. The samples were protected from light during the
glucuronidation assay incubation, which lasted between 5 and 60 min, at +37°C. The
reactions were terminated either by addition of 100 μL ice-cold methanol, 60 μL ice-cold 4
M perchloric acid and methanol mixture, 10 μL cold 4 M perchloric acid or 100 μL 5%
acetic acid in methanol. The choice of termination method was based on preliminary assays.
Termination with a strong acid in the presence of methanol resulted in an additional peak
in some cases. This peak had an additional mass of 14 Da, which probably resulted from methylolation of the glucuronic acid (Kuuranne et al., 2002). The termination method is further discussed in the supplementary material of publication III. Following termination, the reaction tubes were kept at -20°C for 30 to 60 min and then centrifuged at 16000g for 5 to 10 min. Aliquots of the resulting supernatant were transferred to HPLC or UPLC analysis.

4.3.1 Preliminary assays

The first glucuronidation assays were usually done with a mixture of enzymes, such as liver microsomes. If the most active UGT was already known from the literature, then the preliminary glucuronidation tests and method development were performed with that recombinant UGT.

The main purpose of preliminary assays was to ensure a linear range of product formation with respect to time and protein concentrations. This could have been done neither in two parts, or combining them together in the Selwyn’s test (Selwyn 1965, Cornish-Bowden 2012). The second purpose was to find reaction conditions so that the substrate consumption will be kept below 10%, a prerequisite for correct use of Michaelis-Menten kinetics. The third purpose of preliminary assays was to obtain an estimate of the $K_m$ value for the actual enzyme kinetic assays. The preliminary kinetic assays were done with a broad substrate concentration range, often using a single or duplicate samples per substrate concentration point. In the actual kinetic assays, triplicate samples were used for each concentration point.

Substrate binding to recombinant enzymes or microsomes is often reported to be negligible (Boase and Miners 2002, Manevski et al., 2011). Nevertheless, I kept the enzyme concentrations low to minimize the possible unspecific binding of the drug. Moreover, unspecific substrate binding was still monitored in the assays by calculating the substrate concentrations from substrate peak areas in control samples without UDPGA, and comparison to the peak areas without membranes. Similarly, if cells were used in the assay, the binding of substrate was checked with the highest concentration of cells’s protein.

Five types of controls were used in the assays, the most important and frequent was the minus UDPGA control. Then was a control without substrate but with UDPGA, to see if one of the peaks is a background peak. A substrate concentration control was just substrate in buffer, that would also reveal any of the (small) peaks from possible contaminations in the substrate. A reaction stopping control, where immediately after initiation the reaction was stopped, and finally but importantly, a baculovirus control sample. These baculovirus control cells or membranes were prepared from insect cells that had been infected with baculovirus that does not encode any human or other recombinant UGT. This control was used instead of uninfected cells since they also include proteins that the insect cells express in respond to the baculovirus attack.
4.3.2 Glucuronidation screening and kinetics

The purpose of the screening assays was to determine which enzymes have activity toward the test substrate. Enzyme amounts and incubation times were selected based on preliminary assays or information from the literature. The screening was done in triplicates of each recombinant enzyme in a selected substrate concentration with controls without UDPGA and without a substrate. There were usually two substrate concentrations, one low and one high. The high was about 10 times the estimated $K_m$ from preliminary assays with microsomes or from the literature, and the low was approximately the value of $K_m$. From such two-point screening assays I also got some information about the potential kinetic profile of the active enzymes.

The kinetic assays had a minimum of eight concentration points, of which four concentrations points were below $K_m$ and four were higher than the estimated $K_m$ from preliminary assays. The lowest concentration point was at least below the half of the estimated $K_m$ and the highest concentration was at least five times the $K_m$. The incubation times and protein concentrations were adjusted based on the results obtained from preliminary kinetics.

4.3.3 Drug binding assays

The drug binding to albumin was determined using Rapid Equilibrium Dialysis (RED) devices, as previously described (Manevski et al., 2011). In short, the concentration of the unbound drug (F_u) needs to be determined at a pre-decided concentration range in the study. The drug can bind to albumin in a concentration dependent manner, such as in the case of 4-methylumbelliferone, or concentration independent, such as estradiols (I). The main thing is to incubate the device sufficiently long time, so that in the control sample, which is a sample without albumin, the drug is divided equally between the two compartments of the device, through its membrane. Also, the total amount of drug that is placed into the apparatus needs to be compared to amounts of detected drug concentrations from the samples, to determine if the drug has an unspecific binding to device and its membrane, or not.

The fraction unbound ($F_u$) of a drug is calculated from the formula below, where the concentrations of the drug are analyzed from both sides of the chambers in the RED device.

$$F_u = \frac{[S]_{buffer}}{[S]_{sample}}$$

4.4 Analytical methods

The HPLC was my main analytical tool and methods were constantly optimized for assays employing it (see Table 4 for the list of HPLC methods that were used in the thesis studies). When it was important to determine whether a peak is a glucuronide or not, it was done by
mass spectrometry after initial HPLC analyses, and separation methods were developed to collect glucuronide fractions for such MS analyses. Also, to analyze RED results, a short and fast separation method was developed, because there was no need to separate the glucuronide peak from the parent compound peak. Each different method had its own standard curve and limits of detection and quantification. The eluents were dependent on the purpose of the run, but most commonly I employed 0.1 mM formic acid or phosphate buffer (pH 3) as eluent A, and acetonitrile as eluent B.

Table 4. Analytical conditions in the separation and quantification of glucuronides. Entacapone (in article I) was analyzed using UPLC. Detection wavelengths for a UV absorption or fluorescence excitation and emission are given in the third column. The flow was 1 ml/min and the column temperature +40°C. Injection volumes varied between 5 to 80 μL, depending on sensitivity. The limits of detection and quantification were estimated based on signal-to-noise ratios of 3 and 10, respectively. G is an abbreviation for glucuronide, P is for product, FA is formic acid, ACN is acetonitrile and PB is for phosphate buffer.

<table>
<thead>
<tr>
<th>Analyte (PubL)</th>
<th>Eluents and gradient method</th>
<th>Detection2 nm (LOD, LOQ, nM)</th>
<th>Retention times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-NP (I)</td>
<td>A: 0.1% FA, B: ACN; 0–3 min, 30 → 50% B; 3–4 min, 50% B; 4–4.1 min, 50 → 30% B; 4.1–6 min, 30% B</td>
<td>282/335 (1,3)</td>
<td>G: 2.3</td>
</tr>
<tr>
<td>4-MU (I)</td>
<td>A: 0.1% FA, B: ACN; 0–3 min, 20 → 50% B; 3–3.1 min, 50 → 20% B; 3.1–5 min, 20% B</td>
<td>316/382 (1,3)</td>
<td>G: 3.3</td>
</tr>
<tr>
<td>4-MU (II)</td>
<td>A: 50 mM PB, pH 3.0, B: ACN; 0–3 min, 20 → 50% B; 3–3.1 min, 50 → 20% B; 3.1–5 min, 20% B</td>
<td>316/382 (1,3)</td>
<td>G: 1.7</td>
</tr>
<tr>
<td>4-MU (V)</td>
<td>A: 0.1% FA, B: ACN; 0–1.5 min, 10% B; 1.5–5 min, 10 → 50% B; 5–6 min, 50% B; 6–6.1 min, 50 → 10% B; 6.1–8 min, 10% B</td>
<td>316/382 (4,14)</td>
<td>G: 4.7</td>
</tr>
<tr>
<td>6-HI (I)</td>
<td>A: 0.1% FA, B: ACN; 0–6 min, 10 → 50% B; 6–6.8 min, 50% B; 6.8–6.9 min, 50 → 10% B; 6.9–10 min, 10% B</td>
<td>268 (290,960) 268/350 (1,3)</td>
<td>G: 4.0</td>
</tr>
<tr>
<td>6-HI (I)</td>
<td>A: 0.1% FA, B: ACN; 3 min, 40% B</td>
<td>268 (60,200)</td>
<td>G: 1.7</td>
</tr>
<tr>
<td>AMC (V)</td>
<td>A: 20 mM PB, pH 7.0, B: ACN; 0–1 min, 10% B; 1–3 min, 10 → 50% B; 3.5–5 min, 50% B; 5–5.1 min, 50 → 10% B; 5.1–7 min, 10% B</td>
<td>346 (120,410)</td>
<td>G: 2.5</td>
</tr>
<tr>
<td>BAV (V)</td>
<td>A: 0.1% FA, B: ACN; 0–4 min, 50 → 95% B; 4–5 min, 95% B; 5–5.2 min, 95 → 50% B; 5.2–7 min, 50% B</td>
<td>320 (200,660)</td>
<td>G: 2.9</td>
</tr>
<tr>
<td>BIL (II)</td>
<td>A: 0.1% FA, B: ACN; 0–6 min, 35 → 80% B; 6–6.3 min, 80 → 95% B; 6.3–12.5 min, 95% B; 12.5–12.7 min, 95 → 35% B; 12.7–15 min, 35% B</td>
<td>449</td>
<td>G1: 3.2  G2: 4.8</td>
</tr>
<tr>
<td>BPA (III)</td>
<td>A: 0.1% FA, B: ACN; 0–2 min, 30% B; 2–6 min, 30 → 70% B; 6–8 min, 70% B; 8–8.2 min, 70 → 30% B; 8.2–10 min, 30% B</td>
<td>276</td>
<td>G: 4.2  P: 6.5</td>
</tr>
<tr>
<td>BPF (III)</td>
<td>A: 0.1% FA, B: ACN; 0–2 min, 15% B; 2–6 min, 15 → 60% B; 6–8 min, 60% B; 8–8.2 min, 60 → 15% B; 8.2–10 min, 15% B</td>
<td>279</td>
<td>G: 5.6  P: 6.9</td>
</tr>
<tr>
<td>BPS (III)</td>
<td>A: 0.1% FA, B: ACN; 0–2 min, 10% B; 2–6 min, 10 → 55% B; 6–8 min, 55% B; 8–8.2 min, 55 → 10% B; 8.2–10 min, 10% B</td>
<td>260</td>
<td>G: 6.3  P: 7.2</td>
</tr>
<tr>
<td>DME (IV)</td>
<td>A: 50 mM PB, pH 3.0, B: ACN; 0–7 min, 10 → 45% B; 7–7.1 min, 45 → 10% B; 7.1–10 min, 10% B</td>
<td>215 (210,710)</td>
<td>G: 4.8  P: 6.3</td>
</tr>
<tr>
<td>DME (II)</td>
<td>A: 50 mM PB, pH 3.0, B: ACN; 0–7 min, 10 → 45% B; 7–7.1 min, 45 → 10% B; 7.1–10 min, 10% B</td>
<td>215</td>
<td>G1: 4.8  G2: 5.0</td>
</tr>
<tr>
<td>DIF (II)</td>
<td>A: 50 mM PB, pH 3.0, B: ACN; 0–4 min, 35 → 50% B; 4–9 min, 50% B; 9–9.1 min, 50 → 35% B; 9.1–11 min, 35% B</td>
<td>276</td>
<td>G: 3.8</td>
</tr>
<tr>
<td>DIF (V)</td>
<td>A: 20 mM PB, pH 3.0, B: ACN; 0–4 min, 35 → 50% B; 4–9 min, 50% B; 9–9.1 min, 50 → 35% B; 9.1–11 min, 35% B</td>
<td>276 (40,130)</td>
<td>G: 3.9  P: 8.6</td>
</tr>
<tr>
<td>ETC (I)</td>
<td>A: 50 mM PB, pH 3.0, B: ACN; 0–3 min, 20 → 30% B; 3–3.2 min, 30 → 80% B; 3.2–4 min, 80% B; 4–4.1 min, 80 → 20% B; 4.1–6 min, 10% B</td>
<td>309 (52,172)</td>
<td>G: 2.2</td>
</tr>
<tr>
<td></td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–1 min, 15 → 45% B; 1–3 min, 45 → 50% B; 3–4.5 min, 50% B; 4.5–4.6 min, 50 → 15% B; 4.6–6 min, 15% B</td>
<td>309 (52,172)</td>
<td>G: 2.2</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>Epi-E2 (I)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–2 min, 25% B; 2–5 min, 25 → 50% B; 5–9 min, 50% B; 9–9.1 min, 50 → 25% B; 9.1–10 min, 25% B</td>
<td>230 (78,283)</td>
<td>3-G: 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230/308 (17,68)</td>
<td>17-G: 5.7</td>
</tr>
<tr>
<td>Epi-E2 (II)</td>
<td>A: 20% ACN; B: ACN; 0–2 min, 25% B; 0–2.5 min, 55% B</td>
<td>268/206 (5.16)</td>
<td>G: 1.8</td>
</tr>
<tr>
<td>Epi-E2 (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–2 min, 20 → 40% B; 2–5 min, 40 → 50% B; 5–8 min, 50% B; 8–8.1 min, 50 → 20% B; 8.1–11 min, 20% B</td>
<td>281 (273,326)</td>
<td>3-G: 3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281/306</td>
<td>17-G: 3.8</td>
</tr>
<tr>
<td>Epit-TS (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–2 min, 32% B; 2–5 min, 32 → 50% B; 5–9 min, 50% B; 9–9.1 min, 50 → 25% B; 9.1–10 min, 25% B</td>
<td>225 (66,220)</td>
<td>G: 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>225/312</td>
<td>17-G: 5.3</td>
</tr>
<tr>
<td>E2 (I)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–2.5 min, 60% B</td>
<td>216/313</td>
<td>G: 2.1</td>
</tr>
<tr>
<td>E2 (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–2 min, 20 → 40% B; 2–5 min, 40 → 50% B; 5–8 min, 50% B; 8–8.1 min, 50 → 20% B; 8.1–11 min, 20% B</td>
<td>281/306</td>
<td>G: 2.8</td>
</tr>
<tr>
<td>E2 (V)</td>
<td>A: 20 mM PB, pH 3.0; B: ACN; 0–2 min, 20 → 40% B; 2–5 min, 40 → 50% B; 5–8 min, 50% B; 8–8.1 min, 50 → 20% B; 8.1–11 min, 20% B</td>
<td>281/306 (20,75)</td>
<td>G: 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281/306</td>
<td>P: 8.6</td>
</tr>
<tr>
<td>E1 (V)</td>
<td>A: 20 mM PB, pH 3.0; B: ACN; 0–5 min, 22 → 42% B; 5–5.5 min, 42 → 50% B; 5.5–9 min, 50% B; 9–9.1 min, 50 → 22% B; 9.1–11 min, 22% B</td>
<td>282 (230,780)</td>
<td>G: 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P: 8.8</td>
</tr>
<tr>
<td>EED (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–6 min, 25 → 50% B; 6–9 min, 50% B; 9–9.1 min, 50 → 25% B; 9.1–11 min, 25% B</td>
<td>281/306</td>
<td>G: 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281/306</td>
<td>17-G: 4.0</td>
</tr>
<tr>
<td>IND (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–4 min, 40 → 50% B; 4–9 min, 50% B; 9–9.1 min, 50 → 40% B; 9.1–11 min, 40% B</td>
<td>319</td>
<td>G: 3.3</td>
</tr>
<tr>
<td>LME (II)</td>
<td>A: 50 mM PB, pH 3.0; B: ACN; 0–7 min, 10 → 45% B; 7–7.1 min, 45 → 10% B; 7.1–10 min, 10% B</td>
<td>215 (G1: 4.7</td>
<td>G2: 5.0</td>
</tr>
<tr>
<td>MRP (V)</td>
<td>A: 20 mM PB, pH 2.4 B; MeOH ; 0–2 min, 3% B; 3–5 min, 3 → 10% B; 5–6 min, 10% B; 6–7 min, 10 → 20% B; 7–7.1 min, 20 → 3% B; 7.1–9 min 3% B</td>
<td>235 (100,300)</td>
<td>G: 4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P: 6.0</td>
</tr>
<tr>
<td>NCHN (V)</td>
<td>A: 0.1% FA; B: ACN; 0–4 min, 15 → 50% B; 4–6 min, 50% B; 6–6.2 min, 50 → 15% B; 6.2–8 min, 15% B</td>
<td>362/450</td>
<td>G: 3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2/0.7</td>
<td>P: 4.8</td>
</tr>
<tr>
<td>R-PR (II)</td>
<td>A: 0.1% FA; B: ACN; 0–1.5 min, 23.5% B; 1.5–4 min, 23.5 → 28% B; 4–5.5 min, 28% B; 5.5–5.65 min, 28 → 50% B; 5.65–7 min, 50% B; 7–7.15 min, 50 → 23.5% B; 7.15–10 min, 23.5% B</td>
<td>227 (227/344)</td>
<td>G: 3.5</td>
</tr>
<tr>
<td>S-PR (II)</td>
<td>A: 0.1% FA; B: ACN; 0–1.5 min, 23.5% B; 1.5–4 min, 23.5 → 28% B; 4–5.5 min, 28% B; 5.5–5.65 min, 28 → 50% B; 5.65–7 min, 50% B; 7–7.15 min, 50 → 23.5% B; 7.15–10 min, 23.5% B</td>
<td>227 (227/344)</td>
<td>G: 4.0</td>
</tr>
<tr>
<td>SN-38 (V)</td>
<td>A: 0.1% FA; B: ACN; 0–4 min, 10 → 48% B; 4–5.3 min, 48% B; 5.3–5.4 min, 48 → 10% B; 5.4–7 min, 10% B</td>
<td>381 (20,80)</td>
<td>G: 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P: 5.1</td>
</tr>
<tr>
<td>TS (II)</td>
<td>A: 50 mM PB, B: ACN; 0–1.5 min, 27% B; 1.5–5 min, 27 → 50% B; 5–8 min, 50% B; 8–8.1 min, 50 → 27% B; 8.1–10 min, 27% B</td>
<td>244</td>
<td>G: 4.8</td>
</tr>
<tr>
<td>TFP (IV)</td>
<td>A: 0.1% FA; B: ACN; 0–1 min, 35% B; 1–4 min, 35 → 80% B; 4–5 min, 80% B; 5–5.1 min, 80 → 35% B; 5.1–7 min, 35% B</td>
<td>259 (10,40)</td>
<td>G: 3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P: 4.2</td>
</tr>
</tbody>
</table>

1LOD, limit of detection; LOQ, limit of quantification; values were calculated assuming a maximal injection volume.

2Two values are for fluorescence detection, excitation and emission. Only one value represents the UV or the visible light absorbance maximum.

The standard curves were prepared based on the glucuronide’s fluorescence or UV absorbance, or UV absorbance of the parent compound (see Table 4 and detection wavelengths). The use of parent compound’s UV absorbance gives a good glucuronide concentration estimation (Court 2005). However, this is not the case with fluorescence, where glucuronide and its parent compound may have very different fluorescent properties.
The standard curves were prepared based on the glucuronide’s fluorescence or UV absorbance, or UV absorbance of the parent compound (see Table 4 and detection wavelengths). The use of parent compound’s UV absorbance gives a good glucuronide concentration estimation (Court 2005). However, this is not the case with fluorescence, where glucuronide and its parent compound may have very different fluorescent properties. For this reason, using fluorescence required the use of a glucuronide standard, either purchased or biosynthesized. If glucuronide standard or enough material for their biosynthesis was not available, two alternative routes could be used. The first was to use radiolabeled UDPGA, and the second was to use the correlation of UV and fluorescence detection (FLD). The idea in the latter method is that the first standard curve is done using the parent compound and UV detection. Next step is to analyze the glucuronide peak resulting from an injection series of different volumes of reaction sample, which is recorded by both UV and fluorescence detectors. The glucuronide concentration can be calculated using the standard curve (of parent compound), and from FLD we get the fluorescence intensity for the corresponding concentration. This sort of standard curve leads to a glucuronide concentration estimate, because it relies on the assumption that a parent and its glucuronide have similar UV absorbance maxima. Standard curve preparations were often repeated when a new substrate stock was prepared and normally forced through the zero (Dolan 2009).

The HPLC equipment was an Agilent 1100 in most assays (Agilent Technologies, Palo Alto, CA). It was equipped with both UV and fluorescence detectors. The column used in most assays was a C18-column from Agilent (Poroshell 120 EC-C18, 4.6x100mm, 2.7μm; Agilent Technologies, Palo Alto, CA).

### 4.5 Data analysis

Data were analyzed using the GraphPad Prism program, version 5.04 (GraphPad Software, San Diego CA) and then fitted to the best nonlinear regression model, which was chosen by a visual inspection of the Eadie-Hofstee plot, calculated r² value and Akaike’s criteria. Data were fitted to the following kinetic models:

**Michaelis-Mentel model:**

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

**Substrate inhibition:**

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S](1 + \frac{[S]}{K_{st}})} \]

**Sigmoidal model (Hill equation):**

\[ v = \frac{V_{\text{max}} [S]^h}{S_{50}^h + [S]^h} \]
In these models, $v$ is the initial velocity, $[S]$ is the substrate concentration, $V_{\text{max}}$ is the limiting velocity, $S_{50}$ is a substrate concentration at half of the $V_{\text{max}}$, which is similar to $K_m$ in the Michaelis-Menten model. $K_i$ is a substrate inhibition constant, $h$ is the Hill coefficient and $CL_{\text{int}}$ in this formula is a ratio of $V_{\text{max}}^2/K_m^2$. For clearance calculations two formulas were used, depending on whether the kinetics obeyed Michaelis-Menten kinetics or if they followed sigmoidal kinetics. For the Michaelis-Menten kinetics, it was:

$$CL_{\text{int}} = \frac{V_{\text{max}}}{K_m}$$

And for sigmoidal kinetics:

$$CL_{\text{max}} = \frac{V_{\text{max}} (h - 1)}{S_{50} h (h - 1)}$$

Enzyme kinetic constants are given with standard errors from three parallel samples (standard deviations) and 95% confidence intervals. Extra sum-of-squares (F-test in GraphPad Prism) gave the P values for significance of difference between two kinetic constants. In the albumin study (I) the difference in kinetic parameters due to the addition of BSA was calculated as $X = \frac{B}{A} * 100$, where the $B$ and $A$ represent kinetic parameters with and without BSA, respectively. The error calculations for $X$ were therefore done by the general equation for the propagation of independent and random errors.

$$dX = X \sqrt{\left(\frac{dA}{A}\right)^2 + \left(\frac{dB}{B}\right)^2}$$

Where $X$, $A$ and $B$ are the kinetic constant averages and $dX$, $dA$ and $dB$ are their errors (Taylor 1997).

For $CL_{\text{max}}$ error calculations, the general formula for the propagation of uncertainty in a function of several variables was used, in which all the errors are independent and random.

$$\delta x = \sqrt{\left(\frac{\partial x}{\partial a} \delta a\right)^2 + \left(\frac{\partial x}{\partial b} \delta b\right)^2 + \left(\frac{\partial x}{\partial c} \delta c\right)^2}$$

The $a$ is for $V_{\text{max}}$, $b$ is $S_{50}$, $c$ is $h$ and $\delta a$, $\delta b$ and $\delta c$ are corresponding errors (Taylor 1997).
5 Results

The Results section presents the findings from thesis articles (I-V) together with some data that have not yet been presented elsewhere.

5.1 Activity differences between polymorphic variants of UGT1A4 and different preparations of UGT1A10 (IV and V)

This section focuses on the potential causes for activity differences between the same polymorphic variant from different preparations, and of very small differences in the preparation of a UGT that ended up in large activity differences. When expressed in our insect cells, with a short C-terminal fusion peptide, the UGT1A4-P24T variant and UGT1A4 exhibited large activity differences (IV) and our UGT1A10 was highly active in comparison to the commercial UGT1A10 (V).

5.1.1 The two extra amino acids in the beginning of mature protein decrease $V_{\text{max}}$ values (IV)

The single amino acid change in UGT1A4-P24T occurs close to the signal sequence cleavage site, so this mutation could affect the this cleavage. To test this idea, an online prediction tool SignalP (http://www.cbs.dtu.dk/services/SignalP/, accessed Nov 2018, Petersen et al., 2011) was used to predict the cleavage site of the variant and wild-type UGT1A4. The program predicted that the mutation will result in two cleavage sites, one at the wild-type site, between amino acids S28 and G29, and another two residues upstream of it, between amino acids A26 and E27 (Figure 11A). To study if this prediction is correct, the mutation was inserted to our cloned His-tagged UGT1A4, the mutant was expressed in S9 cells and its activity was tested in parallel with the wild-type UGT1A4. In addition, the mutant and wild-type UGT1A4 were purified by affinity chromatography, taking advantage of their C-terminal His-tag. The purified proteins were sent for N-terminal sequencing, to test if the cleavage occurred as the program predicted. As the N-terminal protein sequencing results show, there were two parallel sequences in the UGT1A4-P24T. One was similar to wild type enzyme, whereas the other was in full agreement with the prediction, starting two amino acids upstream the wild-type cleavage site (Figure 12).

The SignalP prediction and N-terminal sequencing analysis revealed that the mutation in UGT1A4-P24T altered more than one amino acid in the protein sequence. The mutation also lengthened the produced protein by two amino acids, but probably only in about half of the proteins. Because the N-terminus is close to proposed catalytic histidine, or in case of UGT1A4 catalytic proline (Kerdpin et al., 2009), these two extra amino acids may have an effect on enzyme activity.

As shown in Figure 11B, we tried to develop some UGT1A4 mutants that would express only the longer version of UGT1A4-P24T. At least two mutants were found, that according
to the SignalP program prediction, would have a single cleavage site, namely UGT1A4-ATIP and UGT1A4-QPdel (Figure 11B). The UGT1A4-ATIP had amino acids 19-21 (SVQ) altered into ATI and QPdel had amino acids 23 and 24 (QP) deleted. These two mutants were prepared by site-directed mutagenesis within UGT1A4, in the same manner as UGT1A4-P24T, but failed to be expressed in our expression system.

**Figure 11** Signal sequence prediction results by the online program SignalP 4.1 for (A) UGT1A4 and its polymorphic variant UGT1A4-P24T. With wild-type UGT1A4 (left panel), a signal peptide is predicted to be cut off clearly and at a single position, G30. The prediction for the variant UGT1A4-P24T (right panel) suggests two cleavage sites with almost equal probability, at E28 and G30. The height of the lines is just below the cut-off value, but this might be a result of the complexity of the site. (B) Two attempts to create UGT1A4 mutants that will have a single cleavage site, two amino acids upstream the beginning of N-terminus in wild-type UGT1A4. These mutants, 1A4-ATIP and 1A4-Qpdel were not expressed properly.

Our His-tagged wild-type and UGT1A4-P24T variant enzymes were purified at small scale, in the presence of SDS, for protein sequencing. Figure 12 shows the purified protein fractions after Western blotting (stained membrane) that were subjected to N-terminus sequencing at the Proteomics Unit (Proteomics Unit, Biotechnology Institute, University of
Helsinki, http://www.biocenter.helsinki.fi/bi/protein/pro.html, accessed Nov 2018). The analysis yielded two parallel signals from UGT1A4-P24T of similar intensity, suggesting equal amounts of two N-termini. From UGT1A4, on the other hand, only one clear sequence was obtained that fully matched the predicted signal sequence cleavage site. The UGT1A4 results also helped to interpret the overlapping and rather weak signals from the mutant. Thus, in UGT1A4-P24T, one sequence is cleaved as wild-type, while the other is cleaved two residues upstream (Figure 12, right side), as predicted by the program for this variant (Figure 11A).

![Image](attachment:image.png)

**Figure 12** The purified UGT1A4 (1A4-XHC) and its polymorphic variant UGT1A4-P24T (1A4-P24T), with their N-terminal analysis results. The results for UGT1A4-P24T revealed two overlapping sequences (lower right panel).

To analyze the effect of mutation on glucuronidation activity, the activity of UGT1A4-P24T was examined, in parallel with wild-type, toward two UGT1A4 substrates, dexametomidine (DME) and trifluoperazine (TFP). Even if the variant had much lower (expression normalized) activity level, it was active enough for kinetic analyses. The results revealed that the main difference was in the $V_{\text{max}}$ values (Table 5), while the other kinetic parameters, including kinetic model and $K_m$ values were nearly the same.

| Table 5. Kinetic parameters of UGT1A4 and its polymorphic variant UGT1A4-P24T. MM is for Michaelis-Menten kinetics and HE is for Hill equation. |
|---|---|---|---|
| | **DME** | | **TFP** |
| | **1A4** | **P24T** | **1A4** | **P24T** |
| **$V_{\text{max}} \pm S.E.$** | $312.0 \pm 7.8$ | $34.1 \pm 1.0$ | $366.8 \pm 11.3$ | $29.1 \pm 1.4$ |
| **$K_m \pm S.E.$** | $387.7 \pm 17.4$ | $371.0 \pm 20.8$ | $52.0 \pm 4.5$ | $42.7 \pm 6.4$ |
| **Model, $r^2$, $h \pm S.E.$** | HE, 0.99; $h = 1.8 \pm 0.1$ | HE, 0.98; $h = 2.1 \pm 0.2$ | MM, 0.99 | MM, 0.95 |
5.1.2 The UGT1A10 expressed in our laboratory is highly active in comparison with the commercial enzyme

Previous reports about UGT1A10 glucuronidation activity varied a lot, from high to low. The variation seemed to depend, at least in part, on whether the enzyme was expressed in mammalian or insect cells. In our system, we express UGT1A10 with a C-terminal His-tag in Sf9 cells and obtain a highly active enzyme, with suitable substrates. For example, in a study on estradiols glucuronidation, the UGT1A10 that we prepare exhibited much higher rates of estradiol-3-glucuronide formation than any other human UGT (Itäaho et al., 2008). At first, this result was confusing, because it differed from a previous study that did a similar assay, but employing the commercial UGT1A10 (Lepine et al., 2004). After several years and accumulation of more examples for problems in the activity of the commercial UGT1A10, we undertook a more systematic study. In this study, I tested the glucuronidation activity of our UGT1A10 and its commercial counterpart, to determine which of them better represents the intestinal UGT1A10 (V). In Table 6, the activity of commercial UGT1A10 is shown alongside the His-tagged UGT1A10 that we express, both preparations are from baculovirus-infected insect cells. It is clear from the results (not-expression normalized) that the activity of the commercial UGT1A10 is very low, at least ten-fold lower than our UGT1A10. For comparison, screening of AMC glucuronidation by UGT1A9 yielded 186.1 ± 8.8 pmol/min/mg and 103.1 ± 3.9 pmol/min/mg for the commercial UGT1A9 and our UGT1A9, respectively (Figure 6 in Study V). This UGT1A9 result is a normal situation, as our enzymes usually have similar range of activity or lower than the commercial enzymes. The reason for the latter is that the UGTs produced in our laboratory are not optimized for maximal activity, but for good activity and low level of inactive UGT, meaning good normalized activity.

Table 6. The activities of two different recombinant UGT1A10s, both were expressed in baculovirus-infected insect cells. The measured velocities, in nmol/min/mg of total protein concentration, are not corrected for expression level.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (µM)</th>
<th>1A10 Activity (nmol/min/mg)</th>
<th>Commercial 1A10 Activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>100</td>
<td>0.14</td>
<td>N.D.</td>
</tr>
<tr>
<td>SN-38</td>
<td>5</td>
<td>0.049</td>
<td>0.005</td>
</tr>
<tr>
<td>4-MU</td>
<td>50</td>
<td>9.1</td>
<td>0.02</td>
</tr>
<tr>
<td>AMC</td>
<td>200</td>
<td>2.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>NCHN</td>
<td>350</td>
<td>5.2</td>
<td>0.02</td>
</tr>
<tr>
<td>DIF</td>
<td>100</td>
<td>2.6</td>
<td>(0.05)</td>
</tr>
<tr>
<td>E2</td>
<td>50</td>
<td>16</td>
<td>0.1</td>
</tr>
<tr>
<td>E1</td>
<td>20</td>
<td>16</td>
<td>N.D.</td>
</tr>
<tr>
<td>BAV</td>
<td>10</td>
<td>2.5</td>
<td>N.D.</td>
</tr>
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5.1.3 Overexpression of poor activity enzymes in insect cells do not correlate with activity rates

When expressed in our insect cell system, the UGT1A4-P24T mutation decreased glucuronidation velocities toward the two studied drugs. The difference in kinetic parameters was limited to a large decrease in $V_{\text{max}}$, approximately ten times, but the $K_m$ value remained unchanged (Study IV, Table 5 above). This result could have been explained by expression level differences. To test this, I needed to determine the expression levels of the recombinant UGT1A4-P24T preparations and the results are shown in Figure 13A. The situation for UGTA10 was somewhat different since this time I could not rely on His-tag antibodies. Fortunately, a monoclonal antibody for the members of UGT1A subfamily is now available. This antibody enabled me to analyze the expression levels of our enzyme and commercial UGT1A10 in a Western blot (Figure 13B). When the amount of a loaded sample is considered, it is clear from the figure that UGT1A4-P24T had more enzyme expressed per total protein than a wild-type UGT1A4. In the case of UGT1A10, the commercial sample had much more enzyme expressed per total protein than in our membrane sample (Figure 13).

![Western blot analysis of (A) wild-type UGT1A4 and the mutant UGT1A4-P24T. Note the difference in loading amounts between the two samples. (B) UGT1A10s, commercial versus our His-tagged UGT1A10. In this case 10 µg total protein of each preparation was loaded.](image)

Western blots of UGT1A10 showed slightly different migration of the commercial enzyme. Theoretically the size of our His-tagged enzyme could have been slightly larger in a gel. The observed difference in migration, however, could also result from the much larger amount of protein in the commercial UGT1A10 lane. The expression levels of UGT1A4 and UGT1A4P24T were calculated more accurately from the dot blot (see methods 4.2 for further details). The expression of the UGT1A4-P24T was 5.1 times higher than the wild-type enzyme.
5.2 Comparison of glucuronidation velocities of individual enzymes

The first step in identifying which enzyme catalyzes conjugation reaction of a new test compound is to do a glucuronidation screening assay. In these assays, the activity toward the studied compound is screened with recombinant enzymes and these activities are then compared to tissue microsomes.

The same was done in the bisphenols glucuronidation study. Bisphenols are toxins from the plastic industry that were known to be largely secreted as conjugates, mainly glucuronides, but little was known about the UGTs that catalyze their glucuronidation. In another study, UGT1A10 activity was determined by screening its activity together with other UGTs. In the albumin study, it was compared how different UGTs were affected by the presence of BSA and found that this is a more complicated phenomenon than previously assumed.

5.2.1 Two highly similar enzymes glucuronidate different bisphenols (III)

The 19 human UGTs were screened for the glucuronidation of three bisphenols and the results are shown in Figure 14. It may be added that UGT2B15 was a commercial enzyme, while the rest were His-tagged enzymes from our laboratory. UGT2B10 and UGT2B11 were screened as cell homogenates, other were enriched membrane preparations. Bisphenols A, bisphenol S and bisphenol F were screened at 20 and 200 μM concentrations, and results from the latter screen are presented in Figure 14.

UGT1A9 was the most active enzyme in bisphenol S glucuronidation, followed by UGT2A1. In addition, UGT1A10, UGT1A8 and UGT2B7 exhibited some activity, whereas UGT2A2 and UGT1A1 activities were detectable, but very low. For bisphenol F, UGT1A10 and UGT2A1 were the most active enzymes, while UGT1A9 did not conjugate this bisphenol.

The screening results showed that while BPF is expected to be readily glucuronidated in the intestine, there is much lower BPS glucuronidation activity in intestinal UGTs. Based on this prediction, BPS and BPF glucuronidation were examined in both liver and intestinal microsomes, and the results are shown in Figure 15.

The active enzymes were further subjected to kinetic analyses and the kinetic parameters are presented in Table 7. The apparent affinity, as suggested by the $K_m$ values, of UGT1A10 and UGT2A1 for BPF were high up to 100 μM. Whereas the apparent affinity of both UGT1A9 and UGT2A1 for bisphenol S was not that high. However, they followed Michaelis-Menten kinetics, whereas UGT1A10 with bisphenol F exhibited substrate inhibition kinetics.
Figure 14  Screening of bisphenol A, bisphenol S and bisphenol F glucuronidation activities by the recombinant human UGTs. UGT2B15 was commercial enzyme (c2B15). The substrate concentrations were 200μM in each case.

Figure 15  The activities of UGT1A9 and UGT1A10 toward the 20μM and 200 μM bisphenols S and F. The liver and intestinal microsomes results are shown in the same scale. The lighter color is for lower concentration and the darker color bar is for higher concentration of the substrate.
Table 7. The parameters for the glucuronidation kinetics of bisphenol S and bisphenol F by UGT1A9, 1A10 and 2A1. Velocities are given as nmol/min/mg per normalized protein concentration and Km and Ki are μM. MM is for Michaelis-Menten kinetics, SI is for substrate inhibition.

<table>
<thead>
<tr>
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<th>BPS</th>
<th>BPF</th>
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<tr>
<td></td>
<td>1A9</td>
<td>2A1</td>
</tr>
<tr>
<td>Vmax ± S.E.</td>
<td>0.54 ± 0.01</td>
<td>1.46 ± 0.05</td>
</tr>
<tr>
<td>Km ± S.E.</td>
<td>163 ± 11</td>
<td>799 ± 51</td>
</tr>
<tr>
<td>Model, r², Kᵢ</td>
<td>MM, 0.99</td>
<td>MM, 0.99</td>
</tr>
<tr>
<td>± S.E.</td>
<td></td>
<td></td>
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</table>

It may be added that BPS and BPF are two new bisphenols that were developed to replace the previously used bisphenol A (BPA). Not much attention was paid to BPA since its glucuronidation, mainly by hepatic UGTs, was previously studied (Hanioka et al., 2008a). Nevertheless, since some extrahepatic UGTs, such as UGT1A10 were not tested, we briefly also tested BPA glucuronidation. The results showed that BPA could be glucuronidated by UGT1A10, but not at very high rates. In addition, results supported the previously published results about UGT2B15 being the main enzyme in BPA glucuronidation, but also added new information about UGT2A1 being active in BPA glucuronidation (Figure 14). As conclusion, all the three bisphenols had their favorite enzyme, but UGT2A1, an extrahepatic enzyme that is mainly found in the airways, could catalyze the glucuronidation of all three. By looking at the structures of the three bisphenols, it is difficult to rationalize the results when it comes to which enzyme glucuronidates which bisphenol, and at what relative rates.

5.2.2 UGT1A10 is an active intestinal enzyme (V)

As I screened the N-glucuronidation of AMC, UGT1A10 had a high activity, which explains that intestine microsomes glucuronidate the compound at a relatively high rate (Figure 6 of Study V). The interesting point in this case is that UGT1A10 also catalyzes an N-glucuronidation reaction, a reaction type that is rather rare for UGT1A10. Moreover, with this coumarin, UGTA10 was by far the most active enzyme in catalyzing this N-glucuronidation reaction amongst the human UGTs.

In a recent study from our laboratory, estrone was found to be a good UGT1A10 substrate and suggested to be used as a useful probe substrate for this enzyme (Kallionpää et al., 2015). In the UGT1A10 study (V) I returned to estrone, partly because in a previously published study (Lepine et al., 2004), almost no estrone glucuronidation activity was found for UGT1A10, but high activity was found with the commercial UGT1A8. Our UGT1A10 glucuronidated this estrogen with high rates, but not our UGT1A8 or the commercial UGT1A8 (Kallionpää et al., 2015) (V). Since qRT-PCR and proteomic studies suggested
that the expression of UGT1A10 is much higher than UGT1A8 in small intestine (Court et al., 2012, Ohno and Nakajin 2009, Sato et al., 2014), I tested estrone glucuronidation kinetics with our UGT1A10, the commercial UGT1A10 (marked as c1A10), as well as human liver and intestine microsomes (Figure 16). The results clearly show that the estrone glucuronidation activity, at least under the assay conditions, was below detection limit with the commercial UGT1A10, as well as in HLM. On the other hand, there was a good activity in HIM and in our recombinant UGT1A10.

![Graph showing glucuronidation activity comparison]

**Figure 16** Estrone glucuronidation activity by human liver and intestine microsomes, the commercial recombinant UGT1A10 (c1A10) and our recombinant UGT1A10. The substrate concentration was 20μM.

Further examination of the similarity between our UGT1A10 and the active UGT in human intestine microsomes was done by kinetic analyses (Figure 17). As can be seen from Figure 16, there is a difference about 6-fold in the $V_{\text{max}}$ value since that particular batch of UGT1A10 was highly active, but the difference in $K_m$ values is much smaller and they are clearly in the same range, even if not identical (Figure 17).

In the UGT1A10 publication (V) I was able to show that in intestinal microsomes, besides UGT2B7, there is at least one more enzyme that catalyzes morphine-3-glucuronidation, and that this enzyme is not sensitive to the inhibitor ALI. In the liver, however, morphine-3-glucuronidation is catalyzed by a single enzyme that is sensitive to this inhibitor. Similarly, estradiol-3-glucuronide formation by UGT1A1 is inhibited by nilotinib in the liver, but not in the intestine, where another enzyme catalyzed it at even higher rates. In a similar manner, glucuronidation activity of N-(3-carboxypropyl)-4-hydroxy-1,8-naphthalimide (NCHN), a fluorescence probe for hepatic UGT1A1 (Lv et al., 2015a) was inhibited by nilotinib in the liver and not inhibited in the intestine (Figure 18).
Figure 17  Estrone glucuronidation kinetics by intestinal microsomes (top left panel) and our recombinant UGT1A10 (top right panel). Eadie-Hofstee plots and kinetic parameters are presented below the kinetic curves. The goodness of fit ($r^2$) is 0.92 for HIM and 0.94 for UGT1A10 when the results are fitted into the Michaelis-Menten equation.

Figure 18  Inhibition studies with either 50 µM estradiol glucuronidation (at the 3-position) by 5 µM nilotinib, 350 µM NCHN glucuronidation inhibition by 1 µM and 10 µM nilotinib, and 100 µM morphine glucuronidation (at the 3-OH) using 50 µM atractylenolide I (ALI) as the inhibitor. In the upper part of the figure, commercial UGT1A1 and UGT2B7, and our recombinant UGT1A10 are presented as controls for the effects of the inhibitors on individual UGTs. In the lower part of the figure the effects of the same substrates and inhibitors combination in microsomal tissues are shown.
5.2.3 BSA addition affects UGTs differently and the effect is also dependent on the used substrate (I)

The albumin study (I) examined the BSA effects on the glucuronidation activity of individual enzymes. In addition to that study, two other kinetic studies on the BSA effect on kinetics were done and not yet published, DME glucuronidation by UGT1A4 and levomedetomidine (LME) glucuronidation by UGT2B10.

In general, the results suggest that the albumin effect is more dependent on the enzyme than on the substrate. The exception for this was UGT1A8, in which $K_m$ differed depending on the substrate. I did not detect unspecific binding to the membranes of UGT preparations.

For two unpublished kinetic studies, the medetomidines’ fraction unbound measurements were performed as previously described in the BSA study (I). The fraction unbound were not dependent on the substrate concentration and they were 0.85 for DME and 0.83 for LME.

Originally, based on the first results with UGT2B7, the albumin effect was reported to be strictly a $K_m$ decrease (Rowland et al., 2007). Our albumin paper (I) showed that while both UGT2B7 and UGT2B17 glucuronidated estradiol and 4-methylumbelliferone, they reacted differently to BSA addition. The effect is much larger in UGT2B7 than it is for UGT2B17 (I).

5.3 The presence of albumin increased $V_{\text{max}}$ more clearly in the members of subfamily UGT1A (I)

The strongest increase of $V_{\text{max}}$ was observed in the members of UGT1A subfamily (Study I and Figure 19). To further complete the albumin story, at least from the point of view of testing all the active UGTs, the activity of UGT1A4 and UGT2B10 were tested with and without BSA (unpublished results, Figure 20 and Table 8). These results (using only one substrate) suggest that UGT1A4 is not one of the UGT1As that are strongly affected by BSA addition in either $V_{\text{max}}$ or $K_m$, whereas UGT2B10 were in line with other UGT2B family members (Figures 19 and 20, Table 8).

Table 8. Kinetic parameters of UGT1A4 (dexametomidine) and UGT2B10 (levomedetomidine) glucuronidation with and without 0.1% BSA.

<table>
<thead>
<tr>
<th></th>
<th>UGT1A4 (DME)</th>
<th>UGT2B10 (LME)</th>
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<tbody>
<tr>
<td>$V_{\text{max}} \pm \text{S.E.}$</td>
<td>0.31 ± 0.01</td>
<td>0.0095 ± 0.0004</td>
</tr>
<tr>
<td>$K_m \pm \text{S.E.}$</td>
<td>388 ± 17</td>
<td>269 ± 19</td>
</tr>
<tr>
<td>Model, $r^2$, $h$ or $K_i \pm \text{S.E.}$</td>
<td>HE, 0.99; $h = 1.8 \pm 0.1$</td>
<td>SI, 0.99; $K_i = 74.8 \pm 10.0$</td>
</tr>
</tbody>
</table>

65
Figure 19  A visual summary of the albumin effects on the $V_{\text{max}}$ of 12 human UGTs, including UGTs that were not studied previously in this context. The BSA effect is presented in the figure as the change in $V_{\text{max}}$ values percentages upon BSA addition, the value 100 in X-axis corresponds the $V_{\text{max}}$ value without albumin addition. Empty space between bars in the figure is for enzyme not tested.

Figure 20  The DME glucuronidation kinetics of UGT1A4 with and without 0.1% BSA (top left panel) and the LME kinetics of UGT2B10 with and without 0.1% BSA (top right panel). The respective Eadie-Hofstee plots are presented below. The kinetics of DME glucuronidation by UGT1A4 was fitted to the Hill equation, whereas LME glucuronidation by UGT2B10 was fitted to substrate inhibition kinetics. The derived kinetic constants are presented in Table 8.
5.4 Dog and human UGT1As (II)

5.4.1 The dog and human UGT1A sequences are highly homologous

Dogs share four out of the five exons that encode a UGT1A, exons 2-5, similarly to human UGT1As, and only the first exon differs among members of this subfamily (see materials and methods 4.2 and Figure 1).

The deduced amino acid sequences were used for making a phylogenetic tree model, because if nucleotide sequences are compared, perhaps somewhat larger differences are observed between different enzymes due silent mutations. In addition, it is the amino acid sequence differences that actually influence the enzyme’s activity. The closest sequences in the phylogenetic tree are perhaps the human UGT1A7-1A10, and because even they differ in their substrate specificities, it gives a reason to assume that there are activity differences between dog and human enzymes. Despite this, the dog and human UGT1A6s have been reported to resemble each other in activity (Soars et al., 2001b) and since dogs serve as one of the preclinical animals, I wanted to test how similar are the substrate preferences of other UGTs. We selected the UGT1A subfamily since their cloning is simpler and therefore a better place to start. The UGT1As could be divided into four groups (Figures 21 and 4), the UGT1A1 group, the UGT1A2-1A5 group, the UGT1A6 group, and the UGT1A7-1A11 group.

Figure 21 Differences and similarities in amino acid sequences between dog and human UGT1As. The analysis was conducted using the Poisson correction model and analyzed with Mega version 7 (Kumar et al., 2016). The intensity of the green color represents the similarities between two sequences, from light green (less similar) to darker green (high similarity). The protein sequences are from the UGT Nomenclature Committee Website (http://prime.vetmed.wsu.edu/resources/udp-glucuronosyltransferase-homepage, accessed Nov 2018).
5.4.2 Substrate specificity of the recombinant dog and human UGT1As

The recombinant dog and human UGT1As were tested for their activity toward 14 different substrates. The purpose was to determine, qualitatively which enzymes take part in a compound’s glucuronidation, but without going to the actual kinetic analyses. Therefore, the results will not provide kinetic model parameters and clearance. An enzyme’s contribution to activity cannot be determined from this single point analysis, primarily since we knew very little about the expression levels and tissues distribution of these UGTs. On the other hand, the results will reveal if the activity pattern is similar, and how well it correlates with sequence homology.

In Figure 22A, the UGT1A1 glucuronidation activities were quite similar between the species. The dog UGT1A1 glucuronidated R-propranolol that was not a substrate for human UGT1A1, but it did at very low rates. Also, diclofenac was glucuronidated more by the dog UGT1A1. The human UGT1A1 glucuronidated entacapone, ethinylestradiol and bilirubin at higher rates.

**Figure 22** Activity screening results with dog (upper panel) and human (lower panel) UGT1A1s (A) and UGT1A6s (B). Tested substrate concentrations were 50μM for E2, epi-E2, EE, TS, epi-TS and BIL, 100μM for DF and IND, 200μM for 4-MU and ETC, 500μM for LME, R-PR and S-PR, and 1000μM for DME.
The activities in the UGT1A6s group are shown in Figure 22B and the activity pattern was similar between two enzymes, of tested substrates both glucuronidated only 4-MU at high rates. The difference was that entacapone was not glucuronidated by dog UGT1A6. In humans this compound was glucuronidated at low rates.

For the group UGT1A2-5, the results in Figure 23 show that the substrate specificity of the dog UGTs 1A2 and 1A3 is highly similar. The dog UGT1A4 also appeared quite similar to dog UGT1A2 and dog UGT1A3, but it exhibited a bit lower glucuronidation rates and it glucuronidated bilirubin at (very) small amounts. In addition, it glucuronidated indomethacin more than dog UGT1A2 and dog UGT1A3. The human UGT1A3 differed in dexametomidine glucuronidation. It did not glucuronidate R-propranolol as the dog UGT1A3 did. The human UGT1A4 was highly different from the corresponding dog UGT, much more than UGT1A3. The human UGT1A4 did not glucuronidate estradiol, epiestradiol or ethinylestradiol at their 3-positions, nor 4-MU or bilirubin. It did glucuronidate estradiols at the 17-position and a small amount of testosterone. Dex- and

![Graphs showing activity screening results for dog UGT1A2, UGT1A3, UGT1A4, and human UGT1A3, UGT1A4, and UGT1A5.](image)

**Figure 23** Activity screening results with the dog UGT1A2, UGT1A3 and UGT1A4, and human UGT1A3, UGT1A4 and UGT1A5. The tested substrate concentrations were 50μM for E2, epi-E2, EE, TS, epi-TS and BIL, 100μM for DF and IND, 200μM for 4-MU and ETC, 500μM for LME, R-PR and S-PR, and 1000μM for DME. The two glucuronides for E2, epi-E2, DME and LME are marked in separate columns. For estradiols, the first column represents the 3-glucuronide and the second column 17-glucuronide.
levomedetomidine were as well glucuronidated by the human UGT1A4, but not by the canine’s enzyme, a result that was somewhat surprising since such activity was observed in dog liver microsomes (Kaiosaari et al., 2008). The human UGT1A5 was only able to glucuronidate a small amount of entacapone, and the values were so low, that it might be a background peak. A similarly unactive enzyme than human UGT1A5 was not found among the dog UGT1As.

The results for the last group of enzymes is shown in figure 24. All the five dog enzymes glucuronidated estradiols at position 3, the dog UGT1A8 also attached glucuronic acid into position 17 of estradiol and epiestradiol at very low rates. The dog UGT1A7, UGT1A8 and UGT1A10 glucuronidated less entacapone and for epiestradiol 3-glucuronide UGT1A7 and UGT1A9 catalyzed the reaction at higher rates. Dog UGT1A9 did not glucuronidate 4-MU almost at all. UGT1A11 did glucuronidate some bilirubin, indomethacin, diclofenac, R-propranolol and S-propranolol. Dog UGT1A7 glucuronidated indomethacin, diclofenac and S-propranolol. The human UGT1A7 catalyzed glucuronidation of entacapone, small amounts of estradiols 3-glucuronides, indomethacin, diclofenac and S-propranolol. The activity pattern of human UGT1A8 was perhaps the most similar to the activities of the corresponding dog enzymes, but all the measured rates were very low. The human UGT1A9 had a huge peak for entacapone. When this is scaled down, the glucuronidation of R- and S-propranolols, indomethacin and diclofenac are visible. UGT1A9 formed a little bit ethinylestradiol-3-glucuronide and even less epiestradiol-3-glucuronide. It did not catalyze estradiol-3-glucuronidation. The human UGT1A10 conjugated estradiol-3-glucuronide and 4-MU at very high rates. When these were scaled down, epiestradiol and ethinylestradiol glucuronidation at the 3-OH, entacapone, indomethacin, and diclofenac glucuronidation at rather high rate is visible. The human UGT1A10 also glucuronidated estradiol at the 17 position, as well as both propranolols. The largest differences were the huge glucuronide peaks with human UGT1A9 of entacapone and with human UGT1A10 of estradiol at the 3-OH. Only dog enzyme within this group catalyzing R-propranolol glucuronidation at considerable rate, was UGT1A7. In addition, dog UGT1A7 formed 3-glucuronides with all three estrogens, which was the main difference when compared to human UGT1A7 or UGT1A8. Perhaps the substrate specificities of dog UGT1A11 and UGT1A9 were the closest to the human UGT1A10.

**Figure 24** The glucuronidation activity toward the tested substrates by the dog UGT1A7-UGT1A11 and human UGT1A7-UGT1A10 (on the next page). The tested substrate concentrations were 50μM for E₂, epi-E₂, EE, TS, epi-TS and BIL, 100μM for DF and IND, 200μM for 4-MU and ETC, 500μM for LME, R-PR and S-PR, and 1000μM for DME. The two glucuronides for E₂, epi-E₂, DME and LME are marked in separate columns. For estradiols, the first column represents the 3-glucuronide and the second column 17-glucuronide.
5.4.3 Similarities and differences in enzyme functions

The dog paper studied individual enzymes of UGT1A subfamily of dog and human and tried to find corresponding enzyme orthologs (II). The enzyme’s location in a phylogenetic tree and their amino acid sequence alignment corresponded reasonably well with substrate specificities, but there were some surprises in this respect.

One of them was the lack of $N$-glucuronidation activity among the dog UGT1As. Another interesting finding was estrogens diglucuronides formation by the dog UGT1A8 and UGT1A10, even if at low rates (II).

The most similar enzymes between dog and human, from activity and substrate specificity points of view, were UGT1A1 and UGT1A6 (Figure 22). This result for UGT1A6 is consistent with a previously published study on the dog UGT1A6 (Soars et al., 2001b).

Small amounts of estradiol-17-glucuronide could be observed with dog UGT1A2 and UGT1A3, highlighting their similarity to human UGT1A3 and UGT1A4 in this respect. However, they did not glucuronidate $N$-glucuronidation substrates, such as dex- and levomedetomidine. The reason might be that only the human UGT1A4, but none of the dog UGT1As, has a proline replacing the catalytic histidine at position 39 (Kerdpin et al., 2009). Nevertheless, this could not be a full answer, since some human UGTs that carry histidine at this position, like UGT1A9, UGT1A10 (V) and UGT2B7 also catalyze $N$-glucuronidation reactions, even if only with few suitable substrates.

Entacapone is glucuronidated at very high rates by the human UGT1A9, and at lower rates by UGT1A1, UGT1A7, UGT1A10 and UGT1A8. In the dogs, UGT1A11 mainly glucuronidated entacapone, even if not at as high rates as the human UGT1A9 (Figure 24). The human UGT1A9 and dog UGT1A11 differ in the glucuronidation of $S$-propranolol. In dogs, UGT1A7 catalyzes $R$-propranolol glucuronidation similarly to the human UGT1A10 but differs from the latter in having only a rather low rate of estradiol glucuronidation at the 3-position (Figure 24). Hence, it could be concluded that the large majority of UGT1A enzymes exhibit both similarities and differences in activities with enzymes in the same group (Figure 21).

This leads to unclear expectations in activity differences between human and dog microsomes. They contain mixtures of different UGTs, the composition of which is still unknown, and they contain UGTs of subfamily UGT2 that I did not get a change to study. This means that the activities of individual dog UGT1As may clarify some of the tissue expression of these dog enzymes, but we could not explain all the microsomes activity results.

5.4.4 Glucuronidation rates in liver and intestinal microsomes differ between dogs and humans

Results on glucuronidation rate differences of the tested substrates between human and dog microsomes are shown in Figure 25 (note the scale difference between the two panels), and they are in line with previous results from liver microsomes (Soars et al., 2001a). Particularly clear differences between the species in my study were the much higher
glucuronidation rate of entacapone in HLM than in DLM, but higher testosterone glucuronidation rate in DLM than in HLM. Interestingly, the opposite for both compounds was found in HIM and DIM, namely higher entacapone glucuronidation in the dog, but higher testosterone in human, alongside almost similar rates of estradiol-3-glucuronidation in both species (Figure 25, II).

There were also clear differences in estrogens glucuronidation, the largest of which is the much higher rate of estradiol-17-glucuronide formation in DLM than in HLM, and of both estradiol-3-glucuronide and estradiol-17-glucuronide formation in DIM than in HIM (Figure 25). On the other hand, testosterone, and to some degree also epitestosterone, both of which are glucuronidated at the 17-OH, were glucuronidated at higher rate in HIM than in DIM. The results also revealed that bilirubin was glucuronidated in HIM, but (almost) not in DIM. The latter results from bilirubin, testosterone and epitestosterone glucuronidation in HIM are all in agreement with the reported expression of UGT1A1, UGT2B7 and UGT2B17, in addition to UGT1A10, in the human small intestine at considerable amounts also at the protein level (Sato et al., 2014).

For the dog, the results suggest that UGT1A11 is expressed in the intestine, based on the entacapone glucuronidation activity. They also support the expected activity of estradiol glucuronidation at the 17-OH, and indicate that it is catalyzed by one or more of the UGT2B enzymes. The results on formation of epiestradiol-3-glucuronide in DIM (Figure 25), in combination with Figure 24, support the expression of UGT1A11 in the intestine.

It may be rather speculative to extend such guessing too far, but the above analysis indicates that even without knowing all the details about the dog UGT2Bs and the tissue expression specificity of the dog UGT1As, one could learn quite much about them from knowing the activities of individual dog UGT1As and the activity results of the microsomes. The main question that was the reason to undertake this study, whether or not the beagle dog is a suitable preclinical animal, from drug metabolism point of view, could not be replied with a simple yes.
Figure 25  Glucuronidation rates comparison between dog and human liver (A) and intestine (B) microsomes. The dog glucuronidation rates are indicated by blue columns, while the corresponding human rates are marked by gray columns.
6 Discussion

The glucuronidation activity and substrate preference of individual UGTs have been further analyzed in this part of the thesis. Accordingly, the potential UGT subfamily difference in BSA effect was assessed against values from the literature and the role of UGT1A10 in the intestine was re-evaluated. Our laboratory has been producing recombinant UGTs in baculovirus-infected S9 cells, and different mutants of these UGTs, since 2001. This knowledge and facilities enabled the cloning and expression of a polymorphic variant of UGT1A4 (UGT1A4-P24T) and all the dog UGT1A subfamily members, none of which is commercially available. His-tag at the C-terminus, enabled determination and comparison of the expression levels of different UGTs, including the many UGTs against which there are no good specific antibodies, and use these relative expression level values to normalize the measured glucuronidation activities. Commercial recombinant enzymes, as well as commercial pooled microsomes of different tissues and species, enabled comparison of our data to results from other laboratories. The analyses of activity differences in this thesis begin from polymorphic variants and end in species differences.

6.1 Activity differences between UGT1A4-P24T and UGT1A4, and between UGT1A10 preparations

6.1.1 Mutations in the UGT signal sequences

It was previously reported that the polymorphic mutation UGT1A4-P24T lowers glucuronidation activity in comparison with the wild-type UGT1A4 (Ehmer et al., 2004), even if the effect was not always found (Edavana et al., 2013). This amino acid change is located still inside the signal sequence, even if close to the beginning of the N-terminus of the mature protein. The signal sequence is cleaved off right after translation and transport across the ER membrane. Hence, it is not present in the mature protein. This raises the question, how the mutation could affect activity if it is not present in the active enzyme? One option is that mutation could affect the cleavage site so that the cleavage does not occur correctly. This hypothesis was examined, and the results showed that a cleavage of the variant is indeed different, even if only for about half of the translated protein (Study IV, Figure 11). To my knowledge, the influence of UGT1A4-P24T, to generate a double cleavage site, is unique or at least not studied much in other UGTs (Table 9). It would be easy to understand if there would not be a cleavage at all and the protein just would have been longer, probably causing an effect on the enzyme folding or activity. Not much is known about the folding process of UGTs and would it be disturbed by an extra protein segment. On the other hand, it is known that the catalytic site is situated close to the N-terminus of the mature protein, so a modified N-terminus could have an even large effect on activity (Laakkonen and Finel 2010).
The UGT variants that were found in the literature and have mutations within the signal sequence, exhibit variable effects (Table 9). An interesting polymorphism inside the signal sequence is UGT1A1-L15R (UGT1A1*30), a variant that significantly lowers the enzyme’s activity, thereby leading to (mild) hyperbilirubinemia and Crigler-Najjar type 2 disease (CN2) (Seppen et al., 1996). The SignalP prediction program (Petersen et al., 2011) failed to recognize a signal sequence in that mutation (Figure 26), which means that the mutation abolished recognition of the cleavage site, at least according to this prediction method. This mutation also reduced the activity of UGT1A1 in vitro, when expressed as recombinant protein in COS cells, the expression was observed to be only 0.5% of the wild-type enzyme (Seppen et al., 1996). It would have been interesting to express this UGT1A1 variant in insect cells and see how it will be expressed in this system and how its activity will be, keeping in mind that high expression level in combination with low activity was one of the signs of poorly folded protein, or combination of correctly and wrongly folded proteins. Another group made UGT1A3 polymorphic mutants inside signal sequence and expressed them in COS cells, but in these experiments the mutants were expressed more or less at the same level as a wild-type enzyme (Iwai et al., 2004).

Table 9. A list of mutations within the signal sequence of different human UGTs.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino acid change</th>
<th>Enzyme activity</th>
<th>References</th>
<th>Effect on signal peptide prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1*30</td>
<td>L15R</td>
<td>Reduced in vivo and in vitro*</td>
<td>(Seppen et al., 1996)</td>
<td>Not recognized</td>
</tr>
<tr>
<td>1A3*2b</td>
<td>W11R and V47A</td>
<td>Increased in vitro</td>
<td>(Iwai et al., 2004)</td>
<td>Same as WT</td>
</tr>
<tr>
<td>1A3*5a</td>
<td>Q6R and W11R</td>
<td>Slightly decreased activity in vitro</td>
<td>(Iwai et al., 2004)</td>
<td>Same as WT</td>
</tr>
<tr>
<td>1A4*2</td>
<td>P24T</td>
<td>Lower in vitro, substrate dependent</td>
<td>(Ehmer et al., 2004, Wiener et al., 2004)</td>
<td>Additional cleavage site</td>
</tr>
<tr>
<td>1A4*4</td>
<td>R11W</td>
<td>Unknown</td>
<td>(Saeki et al., 2005)</td>
<td>Same as WT</td>
</tr>
<tr>
<td>1A6*3a</td>
<td>S7A</td>
<td>Increased in vitro activity</td>
<td>(Nagar et al., 2004)</td>
<td>Same as WT</td>
</tr>
<tr>
<td>1A9*2</td>
<td>C3Y</td>
<td>No significant effect in vitro, located in ER</td>
<td>(Villeneuve et al., 2003)</td>
<td>Same as WT</td>
</tr>
</tbody>
</table>

*CN2

Not all these variants in Table 9 were studied at the enzyme activity level. In Figure 26, I present for the first time, using the same SignalP program that was used for UGT1A4-P24T, the analysis of the CN2 causing variant UGT1A1-L15R (Figure 26). The result suggest that this mutation would prevent, or lower dramatically, signal sequence cleavage,
a suggestion that in this case is a retrospective addition to what was already found experimentally, namely normal RNA transcription, but very poor production of an active UGT1A1, when studied in COS cells (Seppen et al., 1996).

Figure 26  SignalP prediction for the UGT1A1*30 variant that carry a mutation within the signal sequence. Homozygous carriers of this variant suffer from hyperbilirubinemia that could be reduced by treatment with phenobarbitals (Seppen et al., 1996).

6.1.2 One enzyme preparation is active while another preparation of the same enzyme has low activity

The commercial UGT1A10 exhibits much lower activity than our UGT1A10, even though both were expressed in baculovirus-infected insect cells systems. This was demonstrated previously, first indirectly (Itäaho et al., 2008, Lepine et al., 2004) and later more directly (Zhu et al., 2012b). However, since the latter study was not done in a systematic way and focused on magnolol, which is a specific but not common substrate, it was barely cited.

Researches, including drug companies, have used the commercial UGT1A10 before and after my work, and many continue to this day since it is (still) approved and accepted when the metabolism of a new drug candidate is presented to the authorizing organizations, the FDA and EMA. Since our group was aware of the poor activity of the other commercial UGT1A10, something that could not be said on the activity of the other commercial UGTs, it was important to finally perform a systematic comparison between the two preparations and, above all, to test which of them provides a better model system for the native human UGT1A10 in intestinal microsomes.

Our laboratory has previously reported that dopamine is a low affinity but high specificity substrate for UGT1A10 (Itäaho et al., 2009). More recently, in a study on the glucuronidation of estrone and 16α-hydroxyestrone that included recombinant UGTs, HLM and HIM, it was found in our laboratory that estrone is an almost specific substrate for UGT1A10 (Kallionpää et al., 2015). Based on this, we have suggested to use estrone as a
more suitable than dopamine as a specific substrate for UGT1A10 due to its relatively high affinity, suitability for common reverse phase chromatography methods and rather easy detection. Having a good specific substrate, it was easy to go on and ask, as I did in this thesis work (V) which of the two recombinant UGT1A10s that are made in insect cells, the broadly used commercial or ours, better represents the native enzyme in the human intestine. The results were quite clear, the activity of the commercial UGT1A10 does not represent the native enzyme in the intestine, whereas our does, even if several preparations had up to six-fold higher activity, per mg protein, than in HIM (Figure 7A of Study V, and Figure 16). That excess activity was not present in each preparation batch (see, for example (Kallionpää et al., 2015) and when it was present, as in the UGT1A10 batch that I used for Study V, it could be removed by dilution with control membranes. I did not do such a dilution, however, because the original batch was useful for glucuronides biosynthesis (outside the scope of this thesis). In addition to glucuronidation rates, also the enzyme kinetics in estrone glucuronidation of our UGT1A10 and HIM were tested, and found to be similar, with the exception of $V_{\text{max}}$ that, in this particular batch, was nearly six-fold higher in our UGT1A10 (Figure 17).

It is interesting to ask why there is such a clear and large activity difference between ours and the commercial UGT1A10? Finding the correct answer(s) could be valuable both for understanding recombinant UGTs expression and, particularly in this specific case, to revealing how the high activity of UGT1A10 in the intestine, an activity that is surely important in detoxification and first pass metabolism of many phenols and flavonoids containing compounds, have been overlooked for so long.

There is a small difference in the insect cell lines that we use in our UGT preparation and the one used by the commercial producer. Our insect cells are Sf9, a line that originates from the moth Spodoptera frugiperda pupal ovarian tissue and could be cultivated in suspension without serum addition. The cells used by the commercial producer of “Supersomes” are a bit newer type of insect cells, called High five cells, or BTI-TN-5B1-4, from the ovarian cells of the cabbage looper, Trichoplusia ni. Both of these insect cell lines are similarly infected by baculovirus and are able to form human-like complex N-linked glycans that are often required for correct protein folding. Unlike Sf9, however, High five cells may add a non-human and immunogenic N-linked α-1,3-fucose into recombinant protein (Rendic, Wilson and Paschinger 2008, Hancock et al., 2008). Nevertheless, High five cells yield higher expression levels and usually commercial enzymes are expressed to a higher level and often higher activities than we get with our system for many enzymes.

In the case of hepatic UGTs, for most of them there is a specific substrate and the commercial UGTs were developed to reach similar activity per total mg protein, whereas our laboratory is trying to reach a good activity while minimizing the fraction of inactive UGT. This often leads to differences in $V_{\text{max}}$ between the commercial and our UGTs. Nevertheless, the substrate specificity of the UGTs from both preparations, the kinetic models with each of the tested substrates, as well as the ratios in $V_{\text{max}}$ between different substrates for the same UGT, have all been very similar (for example Figure 6 in Study V).

It might be noted, however, that at some stage, before this thesis study was started, the comparisons with commercial UGTs made our laboratory switch from our UGT2B15 to commercial UGT2B15, since in this particular case the rate differences between the

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preparations were much larger than among other UGTs. Commercial UGT2B15 is used for example in Study III.

While the commercial producer could use specific substrates for developing the preparation of hepatic UGTs, they lacked a way to prepare certain extrahepatic enzymes, such as UGT1A7, UGT1A8 and UGT1A10 that resemble each other. The problem from the research field point of view is that many laboratories, including those in drug companies, were not aware that the activity of the commercial extrahepatic UGTs, unlike hepatic UGTs, does not resemble the activity in the native tissue, in this case the small intestine. For our way of preparing a batch of recombinant UGTs, on the other hand, this does not cause problems, as long as we have a substrate to measure its glucuronidation activity to optimize activity in our method that aims at lowering the level of inactive UGTs (Zhu et al., 2012b).

Another difference, which is perhaps the main one, between our recombinant UGT1A10 and the commercial one, is the C-terminal fusion peptide that end with a histidine tag in our UGT1A10. While the reason for its effect is not clear and our laboratory has previously demonstrated that such a fusion peptide do not affect the activity and kinetics of UGTs 1A1, 1A9 and 2B7 significantly (Zhu et al., 2012b), it seems to have an effect on UGT1A10 expression in our system. We have tried several times in our laboratory to produce an active UGT1A10 without a His-tag but failed to get an expressed protein.

There is one similarity between the poorly active preparations of UGT1A4-P24T and commercial UGT1A10, each in comparison to the high activity counterpart. It is the high concentration, per total protein, of the less active UGT in them (Figure 13). This phenomenon may be because of, at least in part, lower capacity of the quality control system in the ER of insect cells, a system that is probably less efficient than in mammalian cells (Polgar et al., 2006), particularly when it has to face high expression level “stress” that the strong baculovirus promoter initiates.

6.1.3 Differences in the effect of UGT1A4-P24T, in vitro versus in vivo

If half of the UGT1A4-P24T signal sequence cleavage was the same as wild-type UGT1A4, and if the two residues longer version of the enzyme is inactive, then the activity should have been half of the wild-type activity. According to my results, the activity was highly affected by the mutation, lowering the $V_{\text{max}}$ values by ten-fold (Table 5). This suggests that the inactive enzyme somehow inhibited the active enzyme. Interestingly, the UGT1A4-P24T mutation effect was much higher in my study (IV) than in a previous work, in which it was expressed in the mammalian HEK293 cells system (Zhou et al., 2011). Moreover, also in human carriers of the polymorphic variant, its effect was minor, much less than in my study (Reimers et al., 2016, Wiener et al., 2004).

The main explanation that I can currently give for these differences is the low capacity of the quality control system in the insect cells, particularly in combination with the strong promoter of baculovirus. Therefore, the findings of this study highlight a limitation in the insect cells expression system, particularly when trying to express proteins that could have difficulties in correct folding process.
6.2. Comparison of glucuronidation activities of individual UGTs

6.2.1 Determining the enzyme that is responsible of glucuronide formation

One of the main challenges in glucuronidation studies is the lack of sufficient specific substrates and inhibitors for individual UGTs, that will assist in determining which enzyme glucuronidates the drug in question. If we would have specific substrates, relative affinity factor (RAF) measurements could be done at higher reliability, as was suggested previously (Zhu et al., 2012b). Activity could be tested by a combination of microsomes and recombinant enzymes, if we would have enzyme specific inhibitors, as it was done in the UGT1A10 study (V).

When there is a conflict between reports, one needs to evaluate which of them is more reliable. Literature results does do not always mean much, if they were all obtained using the same enzyme source in their assays, for example the commercial low activity UGT1A10. Much more trustworthy are reports from in vitro assays with commercial enzymes that match results obtained using human microsomes, particularly when considering the expression levels differences of the different enzymes between the liver and small intestine (Sato et al., 2014). It should also be added that recombinant UGTs that were expressed in mammalian cells, such as different COS and HEK293 cell lines often provide more accurate model for the native human enzymes, but since the main source of commercial UGTs is baculovirus-infected insect cells, and the majority of the literature results are from assays using this type of recombinant UGTs, our laboratory has decided long time ago to focus on producing this type of recombinant UGTs.

When two UGTs have high amino acid sequence similarity, that is, they are highly homologous to each other, they may be expected to have similar substrate specificity. In the case of UGT1A9 and UGT1A10 this is rarely the case, even if these proteins are 93% identical in their protein sequence (Itäaho et al., 2010). There is a rather long list of substrates that in which they differ, particularly different estrogens, or catalyze the other diastereomer, as in propranolol (Sneitz et al., 2013, Sten et al., 2006, Itäaho et al., 2008, Itäaho et al., 2010). The study on bisphenols glucuronidation (III), highly similar compounds, added two more compounds to this list. It was somewhat surprising since the structure of the bisphenols are very similar, and largely different from estrogens. Therefore, the findings of this study are interesting since they further enlarge the understanding how different the substrate specificity, as well as kinetic parameters, between two UGTs, the hepatic UGT1A9 and the extrahepatic UGT1A10 in this case, could be despite the very high sequence similarity between them.

6.2.2 When glucuronidation results from individual UGTs do not match microsomes results

A good example for how UGT activity screening can identify a wrong UGT as a main enzyme in a reaction was the bavachinin glucuronidation in the intestine. In the study of
bavachinin, the authors failed to find the UGT1A10 activity because they used the commercial recombinant enzymes in their glucuronidation screening, leading to the conclusion that bavachinin is a specific substrate for UGT1A8 (Lv et al., 2015b). It would have been good to have a substrate distinguishing between UGT1A8 and UGT1A10 activities. However, when I tested bavachinin glucuronidation by our UGT1A10, the results were different and our high activity (the super active in this case) UGT1A10 catalyzed bavachinin glucuronidation at nearly three-fold higher rates than the commercial UGT1A8, strongly suggesting that it is UGT1A10, much more than UGT1A8, that is responsible for the intestinal glucuronidation activity (Lv et al., 2015b). Since it is now known that UGT1A8 is not that highly expressed in the intestine, based on both qRT-PCR and proteomic studies (Ohno and Nakajin 2009, Court et al., 2012, Fallon et al., 2013a, Sato et al., 2014), the bavachinin results raise an important question: how many other UGT1A10 substrates were wrongly attributed to UGT1A8, or at least the sometime high contribution of UGT1A10 to their glucuronidation in the intestine was left unrecognized? This, however, does not include the very few laboratories that have expressed UGT1A10 in mammalian cell lines.

Unfortunately, not enough groups include intestine microsomes in their screening assays. When this is done, along with recombinant UGTs, there are often interesting finding with respect to UGT1A10. Perhaps picroside II glucuronidation could have been a case test in future experiments, since even with the low activity commercial UGT1A10, it exhibited the highest glucuronidation rates, even if not that much higher than UGT1A9 (Li et al., 2011). Could this be a good probe substrate for UGT1A10 in the intestine?

### 6.2.3 Logic in expression and why UGTs catalyze the same reactions

The human UGTs have developed to protect us from different toxins and waste products, but do we need all the different UGTs? Because they have broad enzyme activities, could we survive, for example, without UGT1A7? The answer is probably yes for some UGTs, but no for another. The answer might have been given already, at least in part, by the very low expression levels of some UGTs.

One enzyme could compensate for another, if it does not work properly, but not always. An analogy for this might be the road, if one lane is jammed, a driver may move to the next line, or choose another route. However, sometimes in the rush hour, all the lanes in the same direction are blocked, and there is not always an alternative route, or the decision to take it should have been made at an earlier stage. The “rush hour” situation may resemble a compound for which there is only one metabolic pathway, for example glucuronidation, and it is catalyzed by a single enzyme, or few enzymes. An accident happening in the middle of the road during the rush hour could then symbolize a mutation in the responsible enzyme. And when the enzyme is essential for a particular reaction, a failure to remove the substrate (open road quickly) means accumulation of a toxic compound and possible damages of different kind. This does not take place in UGTs very often, but it does in quite many cases with drugs that are not well adjusted to the patient, such as SN-38, and in extreme cases, when UGT1A1 is fully malfunctional and unconjugated bilirubin is accumulated at early
childhood, causing severe neurological outcomes, a condition called CN1 that is often fatal. CN1 is a rare disease that is now also treated by liver transplantation, if possible.

6.3. BSA addition to improve in vitro - in vivo correlation

6.3.1 Improved assay conditions improve in vitro-in vivo correlation

The reasons why in vitro glucuronidation assays tend to underestimate in vivo glucuronidation are not yet fully understood. It was suggested that fatty acids are released from disrupted cells, during microsomes preparation, and inhibit the UGTs, an inhibition that could be relieved by the addition of BSA (Rowland et al., 2007). Nonetheless, no difference in the effect of BSA addition was observed between microsomes and recombinant enzymes (Manevski et al., 2011), even if the membranes from insect cells preparation contain different amounts and composition of fatty acids than mammalian cells (Marheineke et al., 1998).

When BSA is added to the UGT assay, it improves the in vitro – in vivo -correlation, but it also introduces another, new, variable in the experiment. If BSA is used, the drug binding to it needs to be determined since it could cause large errors in the calculations of kinetic parameters. A comparison between microsomes and recombinant enzymes does not necessarily need BSA addition, it depends on the study purpose and the enzyme activity. On the one hand it is good to keep the test conditions as simple as possible, because every additional factor in the assay adds a source of error. When BSA is added, one should measure and calculate the fraction unbound of the substrate (See material and methods and Study I). On the other hand, BSA addition brings the assay closer to in vivo activity, which is often the ultimate goal in the glucuronidation studies.

6.3.2 Finding the optimal BSA concentration for the assays

In many cases, 2% BSA could be added into reaction mixture, but if the fraction unbound gets very low, the error rate is likely to increase significantly. The concentration of 0.1% BSA was sufficient to get the same effect (Manevski et al., 2011, Shiraga et al., 2012), so this concentration was used in our BSA studies.

Nonspecific BSA binding was visible with UGT2A1 in the albumin study, when the total protein concentration in the assay was above 0.5 mg/ml (I). This is a good example that BSA effect can also depend on the used enzyme amount. For this reason, BSA binding to the membrane, in addition to the substrate binding to BSA, should be tested before the actual glucuronidation measurements.

The drug binding to 0.1% BSA was negligible in the case of 6-hydroxyestradiol (below 10%), but with estradiol it was rather high, almost 40%. Because the estradiol binding to BSA is concentration-independent, this does not have an effect on the results. However, the
actual drug concentration should be calculated based on the free drug concentration (Section 4.3.3 and Study I).

### 6.3.3 UGT subfamilies and the BSA effect

Following the finding that not all UGTs respond to BSA addition similarly (Manevski et al., 2011), the BSA effect on most human UGTs, each glucuronidating several different substrates was tested (Study I). The results are presented in Figure 19 and Figure 27 below, and they may largely allow grouping the UGTs into the known subfamilies (also) based on the effect of BSA (Figures 20 and 27, Study I).

![Graph showing the BSA effect on various UGT subfamilies](image)

**Figure 27** The BSA effect on the $V_{\text{max}}$ values of the human UGTs. Figure 19 is here updated with literature values, where the color of the bar indicates which BSA concentration was used in the study. The red color is for 2% BSA, blue is for 1% BSA and lilac is for 0.1% BSA. The substrates are 4-MU for UGT1A1 and UGT1A6; 4-MU, ETC (highest $V_{\text{max}}$ increase) and darexaban for UGT1A9; codeine for UGT2B4; codeine, AZT, and 4-MU for UGT2B7. The BSA effect is presented in the figure as the $V_{\text{max}}$ values, in percentages units, upon BSA addition. Hence, the value 100 in X-axis corresponds to the $V_{\text{max}}$ value without albumin addition. The literature values are from: (Rowland et al., 2008, Rowland et al., 2007, Raungrut et al., 2010, Shiraga et al., 2012, Uchaipichat et al., 2006b, Manevski et al., 2011). Empty space between bars in the figure is for enzyme that were not tested or there is no available data on them.
The BSA effect was earlier studied in UGT1A1, UGT1A6, UGT1A9, UGT2B4 and UGT2B7 (Uchaipichat et al., 2006b, Rowland et al., 2007, Rowland et al., 2008, Raungrut et al., 2010, Walsky et al., 2012, Shiraga et al., 2012). Figure 27 includes these literature results, as well as those presented in Figure 19 (I). In Figure 27, I used a color code to indicate the BSA concentration that was used in the original study. The highest $V_{\text{max}}$ increase was observed with UGT1A7, followed by UGT1A10 and UGT1A1. $V_{\text{max}}$ increase was more substrate dependent than the $K_m$ differences were in the BSA study (I), a result that contradicted the general trend (best described in Rowland et al., 2007). Moreover, a correlation in the BSA effects between the $K_m$ and $V_{\text{max}}$ values difference was not found (I).

At the individual enzyme level, perhaps the most interesting observation was the decrease in $K_m$ of UGT1A8. The $K_m$ decrease was obvious when the tested substrate was large, such as entacapone and estradiol, whereas with smaller substrates such as 4-MU and 1-NP, the albumin effect on $K_m$ was smaller (I).

Finding the reasons why BSA affects different UGT subfamilies dissimilarly could lead us researchers to potential inhibitor sites. Could the possible explanation for the inhibition removal being similar among UGT1A subfamily is that they share exons 2-5? Perhaps their structure is more similar in the place where the inhibitor attaches or is placed? And could pronounced $V_{\text{max}}$ increase in some UGTs be related to certain amino acids? Moreover, could this information be used to study the site in the UGT where the inhibitor binds? I have some preliminary results that one amino acid difference between variant enzymes has an impact on the albumin effect, even though the original activity of the enzymes is similar. This effect seems to be substrate dependent as well (unpublished, results not shown).

6.4. Species differences in glucuronidation

6.4.1 Some species have higher glucuronidation capacity than others

There are several so-called preclinical animals that are used in different steps of testing new drug candidates. When the toxicity of a drug is tested, particularly liver toxicity, it is important to consider both reactive metabolites formation, as well as their neutralization and excretion. UGTs mostly contribute to the neutralization, even if in some cases they may generate active metabolites. The beagle dog is one of the second-round preclinical animals, after rodents, but its glucuronidation capacity and individual UGTs have only been poorly studied before our dog UGT1As article (II).

The glucuronidation rates in dog and human liver and intestinal microsomes were examined and compared in study II, using a set of different substrates. There are clear differences, such as the much higher entacapone glucuronidation rate in human liver microsomes than in the dog counterparts (II). There are also similarities, at least in general, between the two species, like significantly higher glucuronidation rates in liver microsomes than in intestinal microsomes for most substrates. Exceptions to the latter were the higher glucuronidation of entacapone in the dog intestine and of estradiol-3-glucuronidation in the
human intestine than in the liver of the corresponding species (Figure 17 of article II). The relatively higher entacapone glucuronidation rate in the dog intestinal than liver microsome could indicate that the dog UGT1A11 is expressed in the intestine, a suggestion that got later some support from the work of another laboratory (Heikkinen et al., 2015).

There was a study of dog intestinal microsomes, which reported higher clearance in the dog intestinal glucuronidation compared to humans (Furukawa et al., 2014). The clearance was studied in the presence of albumin, and it was observed that BSA addition affects the microsomes of these two species in different ways (Furukawa et al., 2014). Their results of entacapone and indomethacin glucuronidation were similar to our observations (II). However, this sort of comparison of microsomal glucuronidation rates might differ, besides the diverse expression levels and UGT polymorphisms in donors, also because the quality differences in microsomal preparation procedures. However, when buying the microsomes from a commercial source, as was done in my studies with microsomes, the activity of each preparation is checked and reported.

Results from dog UGTs expression studies suggest that dogs glucuronidation activity differ significantly from human in tissue expression pattern. At the mRNA level, the dog UGT1A6 was expressed all the way in the intestine and its abundance was two or three times as high as the expression of CYPs (Haller et al., 2012). The proteomics study in the dog liver and intestine showed, however, that dog UGT1A6 was not found in the small intestine, but in colon, and that dog UGT1A9 and dog UGT1A11 are intestinal enzymes that are absent from the liver (Heikkinen et al., 2015). In conclusion, there are three enzymes that are present in the dog intestine but not in the liver, namely UGT1A2, UGT1A9 and UGT1A11 (Heikkinen et al., 2015), whereas only extrahepatic UGT1A10 was observed at protein level in the human intestine (Sato et al., 2014).

6.4.2 Dogs and humans have only few UGTs that share substrate specificities

The activities of the ten dog UGT1As were tested and compared to the nine human UGT1As (II). Results revealed some similarities and many differences. These results could help to understand and interpret the published reports on the interspecies differences in glucuronidation between dog and human. Because human and dog UGT1A amino acid sequences resemble each other, it is interesting to compare the substrate specificities of the two species. The conserved C-terminal sequence is presented in Figure 28, and a substrate specificity scheme of all the dog and human UGT1As toward the tested substrates (II) is presented in Figure 29.

All the N-terminal sequences of the first (variable) exons of the dog (Li and Wu 2007) and human UGT1As are aligned in Figure 30, together with their relative activity rates toward the tested substrates (II). Humans and dogs have similarly functioning UGT1A1, but only partly (Figure 30A). Also, the previously reported dog UGT1A6 seemed to glucuronidates similar substrates as the human UGT1A6 (Figure 30B) (Soars et al., 2001b). The glucuronidation activities of the dog UGT1A11 resembles the human UGT1A10 (II, Figure 30D), also by its expression site, which is in the intestine (Heikkinen et al., 2015). It may be pointed out here that the name of specific dog UGT1As do not necessarily match
their human counterparts, due to the difference in number and location of pseudogenes (see Figure 1 of Study II).

Figure 28  Amino acid sequence alignment between the dog and human C-terminal halves of their UGT1As, each includes four shared exons. The aligned sequences consists of amino acids 289 to 533 of the human UGT1A1. The dog sequence is the upper one and differences in amino acid sequence are highlighted in yellow color.

Figure 29  Substrate specificity scheme of the dog and human UGT1As. Dark blue color represents the most active enzyme for each substrate, and lighter the blue gets, less active the enzyme is in glucuronidating the indicated test compound. Both human and dog UGT1As glucuronidation activities are compared together.
Figure 30  Alignments of the first exons of dog and human UGT1A. The dog sequences are above the human sequences. The substrate specificity of an enzyme is indicated by the color code (see Figure 29). In addition, the lowest number indicates the substrate that has the highest glucuronidation rate (pmol/min/mg) of the enzyme. Dog and human UGT1A1s (A), dog and human UGT1A6s (B), dog UGT1A2-UGT1A4 and human UGT1A3-UGT1A5 (C) and dog UGT1A7-UGT1A11 and human UGT1A7-UGT1A10 (D, on next page). The small d before enzyme’s name indicates the dog enzyme.
One difference between human and dog UGT1As was in the formation of different types of diglucuronides. Another one, perhaps more significant, was the lack of \(N\)-glucuronidation activity in the dog UGT1A family members, at least using the current test substrates.

The difference in estrogens diglucuronide formation between dog and human has been reported previously (Murai, Iwabuchi and Ikeda 2005). The results in Study V supported the linked diglucuronide formation with 3- and 17-estradiol. The two structures for formed diglucuronides, bisglucuronides or linked diglucuronides, are presented in Figure 31 (Argikar 2012).

![Figure 31](https://via.placeholder.com/150)

Figure 31  The second sugar is added to dog estradiol glucuronide as a linked glucuronide (right). (Murai et al., 2005).
6.4.3 Studying glucuronidation in animals is important, even if they differ from human glucuronidation

Dogs, as well as cats, require their own medications and one reason for this is differences in drug metabolism between them and humans. For development of veterinary drugs, it is important to study dog glucuronidation, thereby to learn more about dog’s drug metabolism.

Despite the species differences in drug glucuronidation, it is still needed to use test animals in drug development because we cannot proceed to clinical tests in humans straight from in vitro and in silico assays. When a drug candidate is tested in a whole animal instead of microsomes and other cell fractions, there could be some unexpected and unpredictable events. But because of the differences, if dog microsomes are assumed to be a reliable model system for human drug glucuronidation, the kinetic studies and clearance rate determinations are likely to be wrong. Dogs may be used for these experiments only if we want to develop a drug for these animals, or to study if the glucuronide itself is toxic.

6.5 Future goals and challenges in in vitro glucuronidation assays

6.5.1 Finding the selective substrates and inhibitors for each UGTs

If an enzyme specific substrate is available, we could aim at expressing levels of recombinant enzymes that match the liver or intestine activity. Sometimes it is not easy to predict from the compound’s structure if it is a substrate or an inhibitor for a certain UGT, and substrates can also act as inhibitors. In this situation, no or little product is formed, but the substrate occupies the active site of the enzyme, inhibiting enzyme’s activity toward other substrates. It was reported that bisphenol A inhibits the UGT2B family members (Jiang et al., 2013), but it is also glucuronidated by several UGTs (III). Also, BSA can act as an inhibitor but also as a competitive substrate (I) (Rowland et al., 2007, Tsoutsikos et al., 2004, Turgeon et al., 2003). Especially in the case of UGT1A8, the enzyme was differently affected depending on the substrate size, which either means that there are different binding modes (I), or then it could be speculated that the albumin removal of an inhibitor allows the enzyme to move more freely and enhances the binding of a substrate. The $V_{\text{max}}$ increase could be explained also by removing of mixed-type inhibitors (Manevski et al., 2012).

6.5.2 Solving the UGT structure would benefit glucuronidation studies

The current knowledge of UGTs has a large gap in having details on how they are embedded in the membrane, or what sort of interactions a UGT has with its environment. My experience taught me that they are extremely difficult to purify from this environment as monodispersed, active enzymes, even if this has been partly succeeded in one case (Kurkela
et al., 2003). If we could more easily extract the protein from its membrane environment, we would have a better chance to purify it and perhaps to solve its structure by X-ray chromatography, or even at somewhat lower resolution by cryo-EM. Presently, however, many missing pieces in UGT study are waiting for a real progress due to this lack of high-resolution structure. The *E. coli* expression system that was used to produce the segment of the C-terminal domain (Miley et al., 2007) was mainly good for water-soluble fragment of the protein and solving the structure of the UDPGA binding site. Ultimately, the goal should be to have much more detailed information about the enzyme active site and its entire structure, since parts away from the active site could also affect its activity. The current expression systems of recombinant proteins do not produce enough high quality protein for crystallization studies at the moment (see Section 2.3.1), but perhaps in the future there will be no need for such amount of protein, or other recombinant protein expression systems will contribute to this and allow intact UGT purification. One candidate may be LEXSY (Jena Bioscience, https://www.jenabioscience.com/lexsy-expression, accessed Nov 2018), which is a system based on *Leishmania tarentolae*, a parasite of lizard that is harmless to humans and it could be utilized for protein production.

Once the structure would be solved, it would stimulate inhibitor and substrate studies *in silico*. Drug design *in silico* could be improved and potential drug-drug interactions could be detected earlier than from the *in silico* data. This data could also help to find good enzyme specific inhibitors and substrates.

Overall, it comes back to the main question about which UGT plays the major part in the glucuronidation of a new test drug, and which enzymes are inhibited by the drug and food additives. The study with bisphenols further highlighted our inability to predict the substrate specificity of individual UGTs. Highly similar compounds are glucuronidated by different enzymes (III). At the moment, we are mostly unable to predict which UGT will be responsible for the glucuronidation of a new drug candidate just by looking the drug structure.

### 6.5.3 The future of UGT assays and drug safety studies

If there are many UGTs that glucuronidate a drug, then probably there will not be a problem, but if only one does, SNPs or expression differences between individuals might be dangerous. Several studies tried to search whether there are any inhibitors that comes from food and affect UGTs’ activity, such as grapefruit for CYP3A4.

Inhibiting enzyme’s activity would mean that it takes longer time for a drug, or its phase I metabolite, to circulate and influence the human body, what could sometimes be a positive thing if the drug is otherwise easily excreted. However, this is potentially dangerous, for example if the inhibited enzyme has other important substrates and the concentration of their unconjugated form should be kept low, such as bilirubin. Instead of modifying the enzyme activity, it would be safer to alter the structures of the drug candidate in a way that it would not be so easily glucuronidated, if this is possible. In a toxic situation, induction is an option, as done with phenobarbital for CN2 patients. In such a case, the drug should be changed or at least its doses should be lowered to avoid repeated toxicity.
The issue of UGTs regulation is interesting and a less understood field, particularly the possible role of short, incomplete variant transcripts. In addition, there are the variants with wrong exon that contains premature stop codons, variants that lead, if translated, to inactive proteins. Could such inactive proteins “shut down” the activity of active UGTs by forming dimers with them, as suggested (Girard et al., 2007)? Several recent studies reported the involvement of micro-RNAs in UGTs expression regulation, for example, miR-216b-5p that regulates UGT2B7 and UGT2B4 expression (Dluzen et al., 2016) and miR-141-3p that downregulates UGT1As protein expression (Tatumi et al., 2018). So perhaps in the near future we could affect missing enzyme activity by expression regulation, causing inhibition or induction of UGT activity in this way.

Once again, it is important to remember that in most cases these enzymes do not act alone in drugs metabolism and excretion, they work in homeostasis with other drug metabolism enzymes. There are also other important parts of the big picture that were not discussed in this thesis, namely the CYPs that often “prepare” the substrates for the UGTs, and the efflux transporters that excrete them from the cell. All these, together with the UGTs and other conjugating enzymes, such as sulphotransferases, participate in what started as protecting us from environmental toxins and developed or extended to include drug metabolism.
7 Conclusions

This thesis brings new information about glucuronidation activity in screening \textit{in vitro} assays to answer the questions: which enzyme glucuronidates a compound and at what rate.

The role of UGT1A10 has often been underestimated, while it is an important UGT in intestinal glucuronidation reactions (V).

The mutation in the signal peptide in polymorphic variant UGT1A4-P24T leads to an additional signal sequence cleavage site. The variant enzyme had lower glucuronidation rates when tested with two UGT1A4 substrates, dexmedetomidine and trifluoperazine.

Regardless the similarities among the three studied bisphenols, bisphenols S, F and A, bisphenol S is glucuronidated at the highest rate by UGT1A9, bisphenol F by UGT1A10, and bisphenol A is a UGT2B15 substrate.

The BSA effect is dependent on the used substrate, not just on the enzyme. The BSA addition increased $V_{max}$ values more profoundly in the members of UGT1A subfamily.

The ten dog subfamily UGT1As enzymes were cloned and expressed as recombinant proteins in insect cells. The glucuronidation activities of the dog UGT1A members were compared with corresponding human enzymes. The results revealed some similarities at the level of individual enzymes activities, but also many differences were found, both in the enzyme activity rates and the site of the glucuronidation.
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