Functional Expression of Uridine Diphospho Glucuronosyltransferases in Fission Yeast

Kumulative Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

der Universität des Saarlandes

von

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Saarbrücken

September 2011

Tag des Kolloquiums:	05. Dezember 2011	
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"*Siehst du, Momo*", sagte er dann zum Beispiel, "*es ist so:*
Manchmal hat man eine sehr lange Straße vor sich. Man denkt, die ist so schrecklich lang;
das kann man niemals schaffen, denkt man."

Er blickte eine Weile schweigend vor sich hin, dann fuhr er fort:
"*Und dann fängt man an sich zu eilen. Und man eilt sich immer mehr. Jedes Mal, wenn man aufblickt, sieht man, dass es gar nicht weniger wird, was noch vor einem liegt.*
Und man strengt sich noch mehr an, man kriegt es mit der Angst, und zum Schluss ist man ganz außer Puste und kann nicht mehr. Und die Straße liegt immer noch vor einem.
So darf man es nicht machen."

Er dachte einige Zeit nach. Dann sprach er weiter:
"*Man darf nie an die ganze Straße auf einmal denken, verstehst du? Man muss nur an den nächsten Schritt denken, an den nächsten Atemzug, an den nächsten Besenstrich. Und immer wieder nur an den nächsten.*"

Wieder hielt er inne und überlegte, ehe er hinzufügte:
"*Dann macht es Freude; das ist wichtig, dann macht man die Sache gut. Und so soll es sein.*"

Und abermals nach einer langen Pause fuhr er fort:
"*Auf einmal merkt man, dass man Schritt für Schritt die ganze Straße gemacht hat. Man hat gar nicht gemerkt wie, und man ist nicht außer Puste.*"
Er nickte vor sich hin und sagte anschließend:
"*Das ist wichtig.*"

Beppo Straßenkehrer in „Momo“ von Michael Ende

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Abbreviations

API	active pharmaceutical ingredient
CYP	cytochrome P450
DME	drug metabolizing enzyme
ER	endoplasmic reticulum
FDA	Food and Drug Administration
GRAS	generally regarded as safe
GT	glycosyl transferase
NAD^+	nicotinamide adenine dinucleotide
UDP-GA	uridine diphospho-α-D-glucuronic acid
UDP-Gal	uridine diphospho-α-D-galactose
UDP-Glc	uridine diphospho-α-D-glucose
UTP	uridine triphosphate
UDP	uridine diphosphate
UGDH	uridine diphospho glucose-6-dehydrogenase
UGT	uridine diphospho glucuronosyltransferase

Abstract

Uridine diphospho (UDP) glucuronosyltransferases (UGTs) catalyze the transfer of a sugar moiety from UDP-sugar to endogenous or exogenous compounds. The water solubility of these substances is increased thereby and their excretion from the human body is facilitated. The resulting glycosides, mostly glucuronides, can be pharmacologically relevant and are therefore needed as purified metabolites as reference standards or for toxicity studies. In this work, a whole-cell biotransformation system using recombinant *Schizosaccharomyces pombe* for the production of glycosides is described. It is based on the coexpression of a single UGT isoform and human UDP glucose-6-dehydrogenase, which delivers UDP-glucuronic acid. The system existed for UGT1A9 and has been extended in this work to be established now for each of the 19 human (and one further polymorphic variant of UGT2B7) and one rat isoform. The system was applied for different purposes. In one study, the usage of alternative UDP-sugar cofactors was investigated and the glucosidation of ibuprofen was shown. Furthermore, enhanced glucoside production rates could be obtained by overexpression of the fission yeast gene *fyu1*, which was discovered to act as UDP glucose pyrophosphorylase. Investigations of glucuronidation properties of single isoforms and those of different functional groups were studied as well. So, the glucuronidation of two substrates, which just differed in their functional group, being a hydroxyl group or a thiol, was compared.

Zusammenfassung

Uridin-diphospho (UDP) Glukuronosyltransferasen (UGTs) katalysieren den Transfer eines Zuckers von UDP-Zucker auf endogene oder exogene Stoffe. Dies erhöht deren Wasserlöslichkeit und erleichtert somit ihre Ausscheidung aus dem Körper. Die resultierenden Glykoside, meist Glukuronide, können pharmakologisch relevant sein und werden als Referenzstandards oder in Toxizitätsstudien benötigt. In dieser Arbeit wird ein Ganzzell-Biotransformationssystem mit rekombinanten *S. pombe*-Zellen für die Produktion von Glykosiden beschrieben. Es basiert auf der Koexpression einer einzelnen UGT-Isoform mit humaner UDP-Glukose-6-Dehydrogenase, die UDP-Glukuronsäure liefert. Das System war bereits im Vorfeld für UGT1A9 etabliert worden und wurde in dieser Arbeit erweitert auf alle 19 humane UGT-Isoformen (und eine weitere Variante von UGT2B7) sowie eine Ratten-UGT. Es wurde eingesetzt, um den Gebrauch alternativer UDP-Zucker Kofaktoren zu untersuchen. So konnte die Glukosidierung von Ibuprofen gezeigt werden. Weiter konnte die Glukosid Produktion durch die Überexpression des Spalthefe-Gens *fyu1* erhöht werden. Dieses fungierte dabei als UDP-Glukose Pyrophosphorylase. Untersuchungen von Glukuronidierungs-Eigenschaften von einzelnen Isoformen und von verschiedenen funktionellen Gruppen wurden ebenfalls durchgeführt. So wurde die Glukuronidierung eines Substrates mit einer durch ein Thiol ersetzten Hydroxyl-Gruppe durch verschiedene UGT Isoformen verglichen.

1 Introduction

1.1 Drug metabolism and the role of glucuronides

Metabolism describes all biochemical procedures, proceeding in the plant, animal or human organism or in parts of these, and conducing to the assembly, conversion and maintenance of the body substance as well as the maintenance of the body functioning (der Brockhaus, 2003). A very important part is the metabolism of xenobiotics including drugs. Many of those are lipophilic compounds which accumulate in the human body if they are not converted to more water soluble metabolites. If those compounds additionally have toxic properties, an accumulation in the human body would be fatal. Hence, detoxification mechanisms have developed increasing the water soluble properties of foreign compounds which facilitates their elimination mainly via bile or urine from the human body. This detoxification process occurs mainly in the liver and is schematically classified in two phases. In principle, the molecule is functionalized in phase I by the introduction of a functional group, which in turn is conjugated with a further molecule moiety in phase II. But this classification nowadays is obsolete to some extent, since many molecules are metabolized by conjugation without undergoing a phase I process, or in turn, many molecules are already excreted after a phase I reaction. Enzymes participating in this detoxification mechanism are shown in Figure 1.

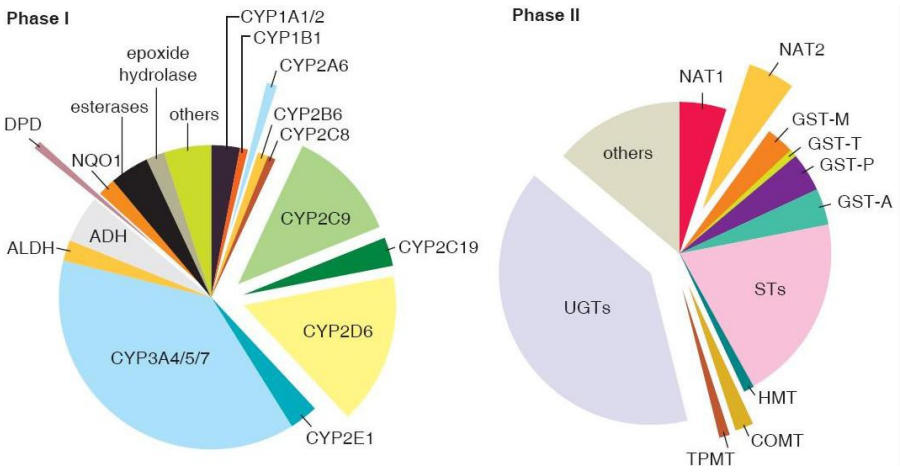

Figure 1: Overview of enzymes participating in phase I and phase II of human drug metabolism. The percentage of phase I and phase II metabolism of drugs that each enzyme contributes to, is estimated by the relative size of each section of the corresponding chart. Those enzyme polymorphisms that have already been associated with changes in drug effects are separated from the corresponding pie charts. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH:quinone oxidoreductase or DT diaphorase; COMT, catechol *O*-methyltransferase; GST, glutathione *S*-transferase; HMT, histamine methyltransferase; NAT, *N*-acetyltransferase; STs, sulfotransferases; TPMT, thiopurine methyltransferase; UGTs, uridine diphospho glucuronosyltransferases; taken from (Evans and Relling, 1999) with modifications.

Three quarters of the 200 most prescribed drugs in the USA in 2002 are getting metabolized before they leave the human body. Three quarters of those again are substrates of cytochrome P450 (CYP) enzymes and one eighth are substrates of uridine diphospho (UDP) glucuronosyltransferases (UGTs), the major enzymes of metabolism phase II (Williams et al., 2004). The huge majority of phase I enzymes are cytochrome P450s (CYPs) (Evans and Relling, 1999), external monooxygenases that catalyze the introduction of a single atom of molecular oxygen to a substrate while the other oxygen atom is reduced to water (Bernhardt, 2006). UGTs catalyze the transfer of a glucuronic acid moiety from UDP-α-D-glucuronic acid (UDP-GA) to their substrates (Mackenzie et al., 2005). The fact that one in ten prescribed drugs is converted by an UGT enzyme before it leaves the human body (Williams et al., 2004) illustrates the enormous relevance of this class of enzymes for drug metabolism.

In contrast, glucuronidation does not always lead to detoxification (Ritter, 2000). Pharmacologically active metabolites are described as well, as for example the 6-glucuronide of morphine (van Dorp et al., 2008) or retinoyl β-glucuronide (Formelli et al., 1996); hence, the usage of a glucuronide as active pharmaceutical ingredient (API) is cogitable. Further approaches study the applicability of glucuronides as prodrugs in cancer therapy, since a higher activity of β-glucuronidase (the UGT antagonist hydrolyzing the glycosidic bond of glucuronides) in cancer cells could release the cytotoxic drug in the tumor (Chen et al., 2003). In addition to those metabolites with direct pharmacological activity, the formation of chemically reactive glucuronides is also known (Ritter, 2000). Glucuronides that result from the addition of the glucuronic acid group to a carboxylic acid group of the substrate are referred to acyl glucuronides. They are of great pharmacological interest because of their special properties and risks. Besides their chemical instability caused by hydrolysis and acyl migration, acyl glucuronide metabolites are reactive electrophiles, which are discussed to have toxic properties due to their ability to bind covalently to proteins, which may lead to immunogenic products (Bailey and Dickinson, 2003; Regan et al., 2010) The subject of toxicity of acyl glucuronides is discussed controversial, with some scientists suggesting that the approval of new drugs that are known to be metabolized to acyl glucuronides should be called into question generally. On the other hand, knowledge about general acyl glucuronide toxicity is not sufficient enough to draw such rigorous conclusions; not least since there are many drugs

forming acyl glucuronides, as e.g. ibuprofen, which emerged to be safe (Adams, 1992; Boelsterli, 2011). Another mechanism of UGT caused toxicity brings toxic substances in form of glucuronides to their site of action, like *N*-hydroxy arylamines, released from their glucuronides by the acidic pH value in the bladder, are thought to be involved in the initiation of bladder cancer (Babu et al., 1995). There is a huge demand on the part of the pharmaceutical industry on purified glucuronide metabolites, since the occurrence of pharmacologically or chemically reactive glucuronides implicates their requirement for toxicity studies. A further market for purified glucuronides is founded by a recently published guidance for industry concerning the safety testing of drug metabolites from the Food and Drug Administration (FDA). It recommends that "metabolites identified only in human plasma or metabolites present at disproportionately higher levels in humans than in any of the animal test species should be considered for safety assessment. Human metabolites that can raise a safety concern are those formed at greater than 10 percent of parent drug systemic exposure at steady state" (US Department of Health and Human Services, 2008). In this document, a disproportionate drug metabolite is defined as "a metabolite present only in humans or present at higher plasma concentrations in humans than in the animals used in nonclinical studies." The glucuronidation of amine groups leads to *N*-glucuronides. Their production is preferred by humans and higher primates, whereas this phenomenon is mainly restricted to the glucuronidation of tertiary amines (Chiu and Huskey, 1998). A hypothesis for an explanation of this occurrence concerns the UGT isoform which is mainly responsible for *N*-glucuronidation of tertiary amines; in humans this seems to be UGT1A4. Since the gene cluster for the *UGT1* family is highly conserved, a mutation in the first exon of *UGT1A4* may be responsible for the deficiency of some organisms to form charged quarternary N^+-glucuronides (Chiu and Huskey, 1998; Green and Tephly, 1998). Since there are many APIs nowadays on the market with a tertiary amine which is metabolized to a quarternary N^+-glucuronide (Green and Tephly, 1998), the issue of disproportionate UGT metabolites could be of severe importance for the pharmaceutical industry due to the required toxicity studies of the respective metabolites. As for active metabolites, purified glucuronides are needed. A further market for the production of pure glucuronides as fine chemicals is justified in the requirement as reference standards, e.g. in doping control (Saudan et al., 2006).

1.2 UGTs

UTGs (EC 2.4.1.17, see Table 1) increase the hydrophilicity of their substrates by conjugating them with a glucuronic acid moiety deriving from UDP-GA and thereby facilitating their renal or biliary excretion (Mackenzie et al., 2005). Substrates can be different kinds of endogenous or exogenous compounds like bilirubin, steroids, bile acids or hormones and drugs, environmental toxicants or carcinogens (Tukey et al., 2000). They are expressed in the liver, gastrointestinal tract, esophagus, brain, kidney, olfactory epithelium, pancreas, mammary gland, prostate, lung and skin (Tukey et al., 2000). UGTs belong to the family of glycosyltransferases (GTs, EC 2.4, see Table 1), which catalyze the generation of a glycosidic bond using an activated sugar donor with a phosphate leaving group. Nucleotide sugar dependent GTs can be classified in two groups, GT-A or GT-B, according to their structural fold (Lairson et al., 2008). Further categorization can be done into an inverting or retaining reaction mechanism concerning the configuration of the bondage at the anomeric carbon of the sugar. Those result in four clans: GT-A and GT-B inverting enzymes compose clan I and clan II and GT-A and GT-B inverting enzymes are clan III and clan IV. Human UGTs belong to the GT1 family; they are inverting enzymes that use UDP coupled sugars and show a GT-B structural fold which makes them members of Clan II (Lairson et al., 2008; Radominska-Pandya et al., 2010). Within the GT1 family, UGTs belong to the UDP glycosyltransferase superfamily (which is also abbreviated by UGT, EC 2.4.1, see Table 1), which is based on a characteristic signature sequence and consists of 4 families with totally 22 members in humans (Mackenzie et al., 1997; Mackenzie et al., 2005). An overview of the EC number definitions is given in Table 1.

Table 1: EC number derivation of UGTs.[1]

EC number	Definition
2	Transferase
2.4	Glycosyltransferase
2.4.1	Hexosyltransferase
2.4.1.17	Glucuronosyltransferase

[1] http://www.chem.qmul.ac.uk/iubmb/enzyme/

The 19 human members from UGT families 1 and 2 (subfamily A and B) prefer UDP-GA as sugar cofactor. Therefore they are named glucuronosyltransferases. The UGT3 family consists of two isoenzymes. While UGT3A1 catalyzes *N*-acetylglucosaminidations of endogenous and exogenous substrates (Mackenzie et al., 2008), UGT3A2 uses UDP-glucose and UDP-xylose for the conjugation of endogenous and exogenous substrates. However, due to the low expression level in the main organs of human metabolism, liver and gastrointestinal tract, UGT3A2 is not supposed to play an important role for drug metabolism (Mackenzie et al., 2011). The UGT8 family just contains one human isoenzyme, which uses UDP-galactose to catalyze the synthesis of galactosylceramide (Bosio et al., 1996). The transfer of a glycoside moiety seems to be an evolutionary conserved reaction, since UDP glycosyltransferase sequences can be found in mammals, fish, worm, virus, yeast, plants, bacteria, and insects (Mackenzie et al., 1997; Bock, 2003).

1.2.1 UGT isoforms and gene arrangement

There are two UGT families mainly acting as glucuronosyltransferases, UGT1 and UGT2, with UGT2 beeing splitted into two subfamilies UGT2A and UGT2B. The human *UGT1* family contains 13 genes with nine functional isoforms (*UGT1A1*, *UGT1A3*, *UGT1A4*, *UGT1A5*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *UGT1A9* and *UGT1A10*) and four pseudogenes (*UGT1A2P*, *UGT1A11P*, *UGT1A12P* and *UGT1A13P*). The genes for all isoforms are arranged in one gene on chromosome 2q37 and mRNAs are produced by alternative splicing. For each isoform, there is an individual promoter and first exon, which is spliced to common four further exons. So, all the 13 transcripts possess an individual N-terminal part and an identical C-terminal part. With respect to sequence homologies of the polypeptides of the first exons, the genes could be clustered into four groups. UGT1A1 and UGT1A6 comprise an own group, respectively; their first exon polypeptides just have a sequence similarity of 50 % to each other and also to the other groups. UGT1A2P – UGT1A5 and UGT1A7 – UGT1A13P comprise groups number three and four, respectively. The polypeptides of their first exons exhibit sequence homologies of 75 – 92 % within each group (see Figure 2, (Mackenzie et al., 2005)).

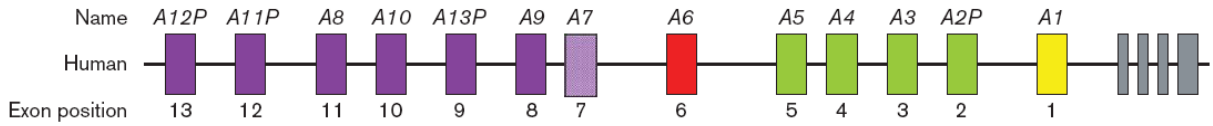

Figure 2: The human *UGT1* family. Each exon 1 is represented by a coloured rectangle and its position relative to exons 2-5 is indicated. Exons 2-5, which are joined to each first exon in the mature transcript, are shown in grey. Pseudogene names end in the label *P*. The exons are not drawn to scale. The human *UGT1* locus extends over approximately 200 kb. Exon clusters are indicated by different colours. The figure is derived from Build 35.1 of the human genome; taken from (Mackenzie et al., 2005) with modifications.

The UGT2 family consists of subfamilies UGT2A and UGT2B. The *UGT2A* gene family comprises three functional isoforms (*UGT2A1*, *UGT2A2* and *UGT2A3*). *UGT2B* is composed of 12 isoforms with seven functional isoforms (*UGT2B4*, *UGT2B7*, *UGT2B10*, *UGT2B11*, *UGT2B15*, *UGT2B17*, *UGT2B28*) and five pseudogenes (*UGT2B24P*, *UGTB25P*, *UGT2B26P*, *UGT2B27P*, *UGT2B29P*). The genes for all *UGT2* isoforms are located on chromosome 4q13. In contrast to the *UGT1* family, there is no usage of common exons in family *UGT2*, with exeption of *UGT2A1* and *UGT2A2*, which also just have an own first exon, respectively, and share exon 2 – 6. Additionally, all genes of family *UGT2* possess 6 exons, respectively (see Figure 3, (Mackenzie et al., 2005)).

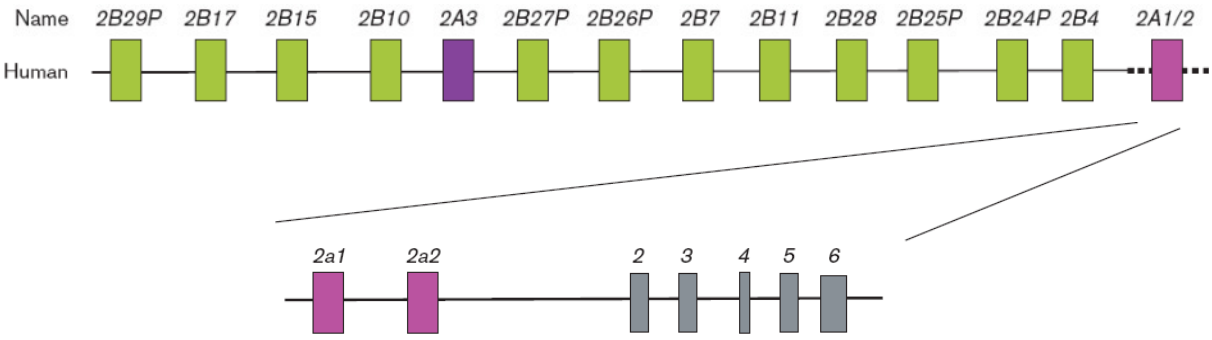

Figure 3: The human *UGT2* family. Each gene, consisting of six exons, is represented by a coloured rectangle, except for that which is labeled "2A1/2", which represents seven exons. The human *UGT2A1* and *UGT2A2* genes contain unique first exons (*2A1* and *2A2*) and a shared set of five downstream exons (exons 2-6 in grey). Pseudogene names end in the label *P*. The genes are not drawn to scale. The human *UGT2* genes extend over approximately 1.45 Mb. The figure is derived from Build 35.1 of the human genome; taken from (Mackenzie et al., 2005) with modifications.

1.2.2 Structure and intracellular organization of UGTs

UGT proteins encompass two domains of approximately the same size; the more conserved C-terminal domain carries the UDP cofactor binding site and is - at least within the UGT1 family and for UGT2A1 and UGT2A2 - identical due to exon sharing. The individual N-terminal domain is responsible for substrate binding and, thus, for substrate specificity (Meech and Mackenzie, 1997; Bock and Kohle, 2009). UGTs consist of approximately 530 amino acids; approximately half of the protein is derived from exon 1 and constitutes the N-terminal domain (Tukey and Strassburg, 2001). There is a characteristic signature sequence (Prosite accession number PS00375) in the C-terminal half, which can be found in all UDP glycosyltransferases and which is thought to be involved in the UDP sugar cofactor binding (Mackenzie et al., 1997). UGT enzymes are located in the endoplasmic reticulum (ER) membrane and the nuclear envelope. They contain a hydrophobic domain near the C-terminus, which anchors the protein in the membrane (Radominska-Pandya et al., 2005). The basic C-terminal part of the enzyme is located in the cytoplasm, the N-terminal part in the ER lumen. Most UGTs possess an N-terminal signal peptide, which directs the protein to the ER during translation and is then cleaved off (Tukey and Strassburg, 2001; Radominska-Pandya et al., 2005). The UGT protein structure is schematically shown in Figure 4.

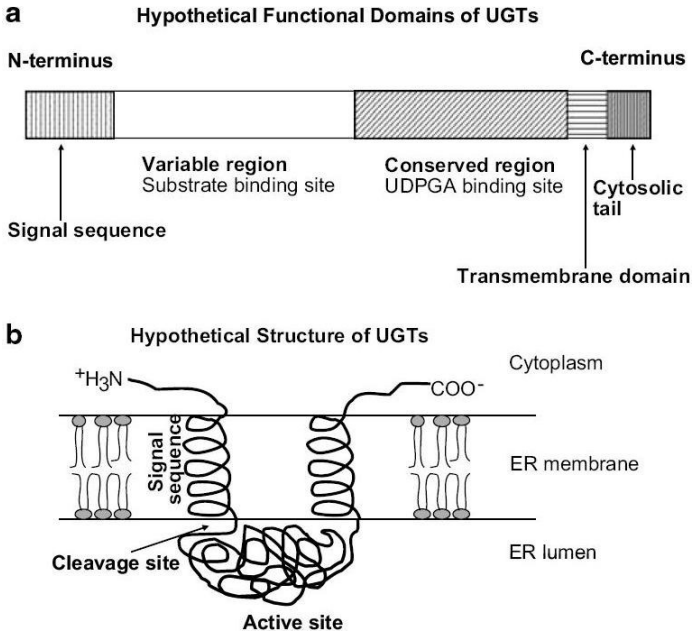

Figure 4: Putative protein structure of UGT proteins. (a) Functional domains are depicted in a linearized protein. (b) The UGT protein as it is presumed to be located within the endoplasmic membrane is depicted. ER: endoplasmic reticulum; taken from (Nagar and Remmel, 2006).

The intraluminal localization of the active site of UGT enzymes requires transporters for the transfer of nucleotide sugars, which are needed as UGT cofactors, through the ER membrane. This is actualized by an antiporter that uses UDP-*N*-acetylglucosamine as counterpart (Bock and Kohle, 2009). Likewise, glucuronide transporters are required for the transport of UGT metabolites out of the ER (possibly organic anion transporters located in the ER membrane) and at the same time into the ER for hydrolysis by ER localized β-glucuronidase (Bock and Kohle, 2009). Multidrug resistance-associated proteins and organic anion transporters are involved in the transfer of glucuronides out of the cell (Ishii et al., 2010a). Several studies indicate that UGTs may act as oligomers; dimers are believed to be responsible for monoglucuronide formation, whereas tetramers are assumed to support diglucuronide formation, e.g. in the case of bilirubin (Bock and Kohle, 2009). The functions of UGTs may be further affected by phosphorylation (Basu et al., 2008), glycosylation (Barbier et al., 2000) and protein-protein association with CYPs (Ishii et al., 2010b).

1.2.3 Requirements for glucuronidation and UGT reaction mechanism

For a glucuronidation reaction UGTs require a substrate with a functional group suitable for glucuronidation as well as the cofactor UDP-GA (or other nucleotide sugars). This cofactor is delivered by UDP glucose-6-dehydrogenase (UGDH, EC 1.1.1.22) in humans (Egger et al., 2010). This soluble enzyme catalyzes the dehydrogenation of UDP-α-D-glucose (UDP-Glc) to UDP-GA, thereby protonating two nicotinamide adenine dinucleotide (NAD^+) molecules (Sommer et al., 2004). UDP-Glc in turn is synthesized from the glycolysis intermediate glucose-1-phosphate and uridine triphosphate (UTP) under cleavage of pyrophosphate by UDP glucose pyrophosphorylase (UGP, EC 2.7.7.9) (Peng and Chang, 1993). UDP-GA has further important functions in the human body, since it is a precursor for the synthesis of extracellular matrix glycosaminoglycans (heparan sulfate, hyaluronan, and chondroitin sulfate) and UDP-xylose, the initial sugar in glycosaminoglycan synthesis on proteoglycans (Moriarity et al., 2002; Viola et al., 2008). UDP-Glc is a key intermediate in the carbohydrate metabolism and has versatile applications, e.g. for glycogen biosynthesis and protein and lipid glycosylation (directly and over a further conversion to UDP-galactose) (Lecca and Ceruti, 2008). The metabolic

pathway from glucose-1-phosphate to a glucuronide, with 4-methylumbelliferone as exemplary substrate, is given in Figure 5.

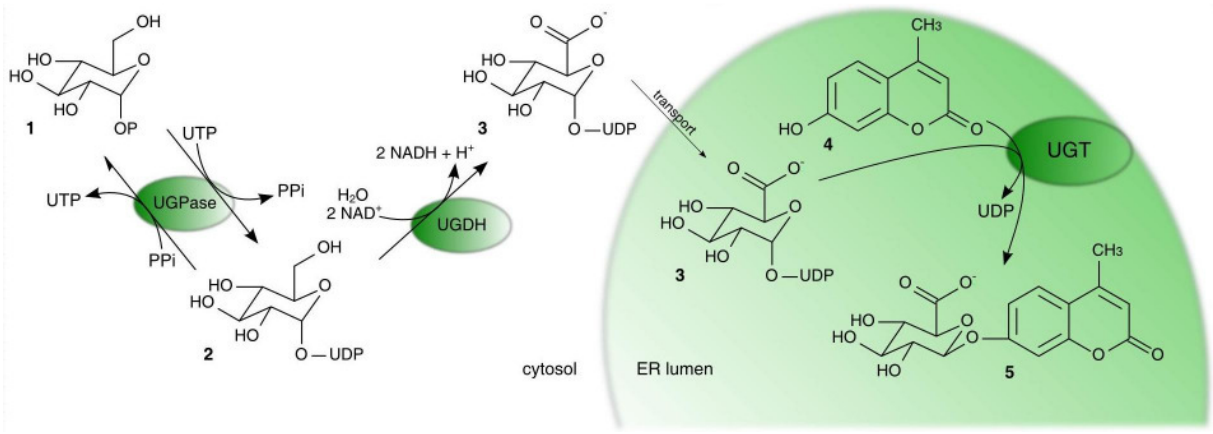

Figure 5: Metabolic pathway from glucose-1-phosphate to a glucuronide in humans. The glycolysis product glucose-1-phosphate (1) is coupled to UTP to build UDP-Glc (2) with a simultaneous splitting off of pyrophosphate (PPi) by UGPase. (2) is then oxidized by UGDH to UDP-GA (3). After the transport of UDP-GA through the ER membrane, it is used by UGT as cofactor for glucuronidation, which releases UDP. Exemplarily, the glucuronidation of 4-methylumbelliferone (4) as substrate to 4-methylumbelliferyl-glucuronide (5) is shown.

The UGT reaction mechanism is supposed to proceed according to a S_N2 reaction mechanism, since an inversion of the configuration at the anomeric carbon of the glucuronic acid moiety takes place. That means UDP-α-D-glucuronic acid is used for the production of an aglycon-β-D-glucuronide (Ouzzine et al., 2003). It is assumed that a nucleophilic group is activated at the substrate by deprotonation by a base. This nucleophile in turn may attack the anomeric carbon of the glucuronide moiety of UDP-GA, which would lead to the formation of the aglycon-β-D-glucuronide and the splitting-off of UDP, which is enabled by an interaction of Mg^{2+} with the pyrophosphate (Ouzzine et al., 2003) (see Figure 6).

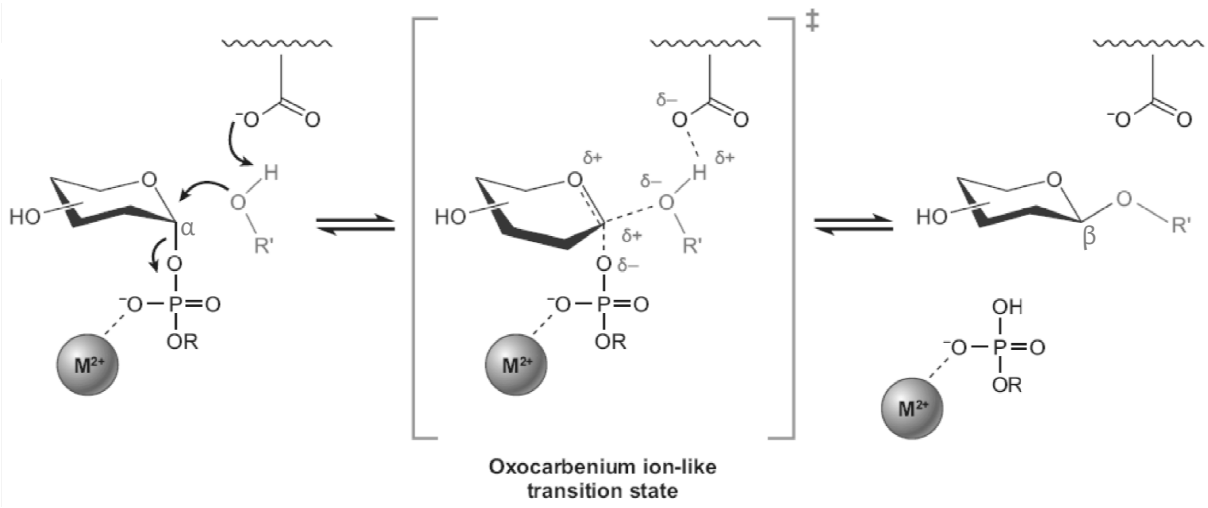

Figure 6: Proposed UGT reaction mechanism. Inverting glycosyltransferases utilize a direct-displacement S_N2-like reaction that results in an inverted anomeric configuration via a single oxocarbenium ion-like transition state. Taken from (Lairson et al., 2008) with modifications.

1.2.4 Substrates and cofactors of UGTs

The transfer of glucuronic acid from UDP-GA results in a glycosidic bond and can take place at different kinds of functional groups, like hydroxyl, carboxyl, sulfuryl, carbonyl or amino functions (Tukey et al., 2000); even *C*-glucuronides already have been reported (Richter et al., 1975). Those glucuronide types can have different properties. *N*-glucuronides can be built from amides or primary, secondary or tertiary amines, with the resultant glucuronide of the tertiary amine, the quarternary ammonium-linked glucuronide, or N^+-glucuronide, carrying a fixed positive charge (Hawes, 1998). Although different UGT isoforms play a role in *N*-glucuronidation, in humans UGT1A4 is considered to be a dominant isoform responsible for their formation (Green and Tephly, 1998). Primary and secondary amines can react spontaneously with CO_2 to yield carbamic acids. Glucuronides linked via this acid group are a special type of glucuronides, *N*-carbamoyl-glucuronides (Schaefer, 2006). *O*-glucuronides can be subdivided in aryl, alkyl, and acyl glucuronides, respectively, (Stachulski and Jenkins, 1998) and can be built from different types of substances like phenols, flavones, opioids, steroids, coumarins, alcohols or carboxylic acids (Tukey et al., 2000), whereupon special attention is paid to acyl glucuronides, which set themselves apart from other *O*-glucuronides due to their special properties. In contrast to *O*-glucuronides, the number of known *S*-glucuronides is rare. *S*-linked glucuronides have been reported from thiols as for example from 4-

nitrothiophenol produced by guinea pig liver microsomes (Smith et al., 1992) and from malotilate, found in rat bile (Nakaoka et al., 1989), or from thiones like the *S*-glucuronides from HMR1098 found in rat, dog and human bile (Ethell et al., 2003) or from AR-C133611XX, built by dog hepatocytes (Martin et al., 2003).

Besides the use of UDP-GA as sugar cofactor for the conjugation of different substrates, UGTs have also been shown to be able to use other UDP-sugar cofactors like UDP-Glc, UDP-α-D-galactose (UDP-Gal) or UDP-α-D-xylose (Senafi et al., 1994; Mackenzie et al., 2003; Toide et al., 2004; Tang and Ma, 2005). This may also be pharmacologically relevant since glucoside metabolites have been reported to occur in humans (Tang, 1990; Paibir and Soine, 1997; Nakazawa et al., 2006). Tang and Ma showed that only one in two substrates is glucosidated or galactosidated in the presence of UDP-Glc or UDP-Gal, respectively, by human liver microsomes, even though both substrates are glucuronidated in the presence of UDP-GA (Tang and Ma, 2005). In a further study, eight human UGT isoforms (five from UGT1 family and three from UGT2B family) were investigated for their ability to glucosidate AS-3201. It was shown that three members from UGT1 and all from UGT2B family were able to glucosidate the substrate, whereas 4 members from solely UGT1 family were able to glucuronidate it (Toide et al., 2004).

1.3 Production of glucuronides

Purified glucuronides are required in milligram amounts as analytical standards and in gram amounts for toxicity studies. Therefore, efficient chemical or biological glucuronide production systems are required. Those have been reviewed by the author of this work in a recent publication (Zöllner et al., 2010). A chemical method, on which many glycoside synthesis procedures including aryl and alkyl *O*-glucuronidations are based on, is the Koenigs-Knorr reaction (Stachulski and Jenkins, 1998; Wimmer et al., 2004). Using this reaction, many *O*-glucuronides could be produced successfully so far, e.g. those of phenols (Goenechea et al., 2001) or steroids (Thevis et al., 2001). Also, the production of acyl glucuronides like those of ibuprofen (Johnson et al., 2007) or diclofenac (Kenny et al., 2004) or of *N*-glucuronides like the secondary *N*-glucuronide of 4-aminobiphenyl (Al-Zoughool and Talaska, 2005), the tertiary *N*-glucuronides of norgallopamil and norverapamil (Mutlib and Nelson, 1990) or the charged quarternary N^+-glucuronide of 4-(2-methyl-1H-imidazol-1-yl)-2,2-diphenylbutanamide (Araya et al., 2007) could be achieved

by chemical synthesis. *S*-glucuronides are not impossible to synthesize chemically as well, as shown by the production of HMR1098-*S*-glucuronide methyl ester (Ethell et al., 2003). However, not all glucuronides can be produced by chemical methods; this is especially true for monoglucuronides of molecules with several functional groups that are potential sites of glucuronidation (Zöllner et al., 2010). To facilitate the production of these metabolites, many biological approaches have been developed. Since the activity of UGT enzymes is highly dependent on their membrane embedment, the purification and use of purified enzymes is difficult (Radominska-Pandya et al., 2005); nonetheless, reports on purifications of human UGTs can be found (Seppen et al., 1995; Kurkela et al., 2004). Widely used enzyme-dependent catalysis of glucuronidation is based on cell lysates or microsomes, which are available from cells natively containing UGTs, like liver cells, or from cells recombinantly expressing UGT enzymes (Zöllner et al., 2010). Interestingly, the UGT reaction side product UDP has been shown to inhibit the UGT reaction (Fujiwara et al., 2008). It has been speculated that the activity of purified enzymes is further decreased by this inhibitory effect of UDP, which is depleted in intact cells by luminal nucleoside diphosphatases. Since the activity of this enzyme in turn is calcium dependent and microsome incubation conditions in general do not contain calcium, even for microsomal incubations this might be relevant (Fujiwara et al., 2008). Microsomes from baculovirus infected insect cells, which overexpress UGT isoforms, are popular. They have excellent prerequisites for metabolite productions, since they lack endogenous UGT and CYP activities and subsequently, the risk of the production of undesired UGT or CYP metabolites due to background activities is low (Brandon et al., 2003). Those methods are capable of small scale metabolite production or metabolism studies in general, but for the production of larger amounts of glucuronide they are not practicable since they all require the addition of the very expensive cofactor UDP-GA. Background activities by other drug metabolizing enzymes (DMEs) or the lack of tissue specific UGT isoforms (UGT1A7, UGT1A8, UGT1A10, UGT2A1, and UGT2A2 are not expressed in human liver (Fisher et al., 2001; Sneitz et al., 2009)) can cause further complications when using microsomes or homogenates from a defined tissue or organism. The preparation of glucuronides from living animals, body fluids or plants also has been tried, but again, the up-scaling is very demanding (Zöllner et al., 2010). The problems of the expensive cofactor UDP-GA and up-scaling can be circumvented by whole-cell biotransformations.

This method uses complete cells expressing the biocatalyst and in case of UGTs, they may produce endogenously UDP-GA. It has been applied in different cells, like mammalian cell lines, but there are also the drawbacks of endogenous background DME activities and tissue dependent UGT isoforms (Zöllner et al., 2010). First results of a promising new technology, showing the suitability of recombinant fission yeast for the production of glucuronides using at least human UGT1A9, already existed at the beginning of this work.

1.4 Fission yeast *Schizosaccharomyces pombe* as a biotechnological tool

The biotechnological production of different kinds of products requires adequate host organisms. The fission yeast *Schizosaccharomyces pombe* was isolated from an East African millet beer in 1893 and was firstly described by Paul Lindner (Lindner, 1893). "Pombe" is the Swahili word for beer. *S. pombe* is a unicellular, eukaryotic, rod-shaped organism which belongs, as the baker's yeast *Saccharomyces cerevisiae*, to the kingdom of fungi and the phylum of ascomycota. Fission yeast and baker's yeast are the two most commonly used yeasts in molecular biology. In contrast to baker's yeast, which reproduces by budding, fission yeast grows at the cell tips and divides by medial cleavage. Concerning the evolution, both yeasts are differing from each other as each of them from man; many proteins of fission yeast even seem to be more similar to higher eukaryotes than their homologues in baker's yeast (Sipiczki, 2000). This is reflected in a number of cellular processes in *S. pombe*, which are more similar to those in higher eukaryotes than to *S. cerevisiae*, e.g. the signalosome, mRNA splicing, RNA interference, chromosome associated proteins or chromosome structure (Forsburg, 2005). In contrast, *S. cerevisiae* has a higher similarity to higher eukaryotes in other aspects, e.g. in the peroxisome (Forsburg, 2005). *S. pombe* is a suitable model organism for the study of molecular and cell biology processes of higher eukaryotes. It gained high profile since Paul Nurse (together with Lee Hartwell and Tim Hunt) received the Nobel Prize for Physiology or Medicine in 2001 for his studies on cell cycle regulation. In addition to its value for basic research, *S. pombe* possesses several properties that qualify it as an excellent tool for biotechnological purposes (Takegawa et al., 2009). Due to decades of basic research, many molecular biology tools and techniques have been developed for fission yeast (Siam et al., 2004; Forsburg and Rhind, 2006). The genome of *S. pombe* is completely sequenced (Wood et al., 2002) and it possesses the "generally regarded as safe" (GRAS) status

allowing a utilization in security level 1 laboratories and the comparatively easy production of substances which are intended to be applied on humans. Furthermore, DNA microarray, proteome and transcriptome data are available (Takegawa et al., 2009; Worner et al., 2009). For the expression of human mitochondrial or ER membrane proteins, *S. pombe* displays advantages to bacteria or baker's yeast. It possesses eukaryotic subcellular structures in contrast to bacteria, and shows more human-like posttranslational modification patterns like protein folding or glycosylation (Chappell and Warren, 1989; Parodi, 1999; Takegawa et al., 2009). This in turn, together with correct protein folding, is difficult or impossible in prokaryotes. However, even better preconditions are given by use of cells from insects or mammalian cell lines, but long generation times and demanding cultivation conditions require alternative tools (Takegawa et al., 2009). In the past, *S. pombe* has been shown to be an excellent host for the functional expression of membrane bound DMEs like microsomal or mitochondrial CYPs (Bureik et al., 2002; Dragan et al., 2005; Peters et al., 2007). Using recombinant fission yeast cells, the production of the respective metabolites was successfully established using the technique of whole-cell biotransformation (Peters et al., 2007; Peters et al., 2009). Moreover, prior to this work, Călin-Aurel Drăgan already showed the functional expression of UGT1A9 in fission yeast. The lack of the essential UGT cofactor UDP-GA in fission yeast cells was compensated by the coexpression of human UGDH, which could be shown to efficiently deliver UDP-GA endogenously, and allowed a self sufficient glucuronide production in whole-cell biotransformations.

2 Aims and scopes

The significant demand for purified glucuronides is illustrated by the above mentioned existence of biologically or chemically active metabolites, the recent FDA guideline on metabolite testing as well as the demand of reference standards including isotope-labeled glucuronides. Toxicity studies require substance amounts of up to hundreds of grams of the purified metabolite. Chemical synthesis of glucuronides is not always possible, and the existing biological approaches suffer from three main disadvantages: (I) the dependency of UGT activity on phospholipids constrains the use of purified enzymes, (II) *in vitro* approaches always depend on the external addition of the expensive cofactor UDP-GA and (III) *in vivo* approaches suffer from difficult up scaling of the production in higher organisms and from restricted UGT expression or background activities in cell cultures. Fission yeast, being an eukaryotic organism, has a high potential to bypass the disadvantages of other biological systems. The functional expression of human UGT1A9 and the glucuronide production in whole-cell biotransformations could successfully be accomplished prior to the beginning of this work. UDP-GA was provided intracellularly by the coexpression of human UGDH. Background activities by other UGTs were not expected since no UGT orthologous open reading frame could be found in fission yeast. The issue of up scaling should be easy to handle with fission yeast, since cultivation is far not as complex, expensive and risky in terms of contaminations as that of cell cultures. A first aim of this study was the application of the remaining 18 human UGT isoforms (and additionally the polymorphic variant UGT2B7*2) and rat UGT1A7 in this self-sufficient glucuronide production system. One recombinant fission yeast strain for each UGT should be established and tested for its ability to produce glucuronides in whole-cell biotransformations. The use of the cells in whole-cell biotransformations was especially important, since the "in-cell" production of UDP-GA and the possibility of easy up-scaling were to be confirmed. Besides the biotechnological utilization for the production of glucuronides, this system also appeared to be suitable to study molecular properties of single UGT isoforms like substrate or cofactor preferences. Since the usage of alternative sugar cofactors is known and fission yeast endogenously contains UDP-Glc and UDP-Gal, one interesting point was the question whether the fission yeast system is suited for the production of glycosides. If so, it could be used to investigate the ability of single UGT isoforms to catalyze those reactions. For this purpose, the coexpression of UGDH had to

be critically considered, since the endogenous UDP-Glc level may be decreased by the conversion to UDP-GA. Moreover, different types of glucuronides (with the glucuronide conjugated to different functional groups) display different properties in terms of charge or stability. A main difference between glucuronides and other glycosides (like glucosides or galactosides) is the charged acidic moiety occurring in glucuronides. Therefore, a focus was set on the applicability of this system on the production of the diverse kinds of glucuronide- and glycoside-metabolites.

3 Publications

The results of this work are described in the following publications.

3.1 Drăgan et al. 2010

Drăgan C-A, **Buchheit D**, Bischoff D, Ebner T and Bureik M: Glucuronide production by whole-cell biotransformation using genetically engineered fission yeast Schizosaccharomyces pombe. *Drug Metab Dispos.* 2010 Mar; 38(3): 509-15. Epub 2009 Dec 11.

0090-9556/10/3803-509–515$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 38, No. 3
30965/3567570
DMD 38:509–515, 2010
Printed in U.S.A.

Glucuronide Production by Whole-Cell Biotransformation Using Genetically Engineered Fission Yeast *Schizosaccharomyces pombe*

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Received October 29, 2009; accepted December 11, 2009

ABSTRACT:

Drug metabolites generated by UDP glycosyltransferases (UGTs) are needed for drug development and toxicity studies, especially in the context of safety testing of metabolites during drug development. Because chemical metabolite synthesis can be arduous, various biological approaches have been developed; however, no whole-cell biotransformation with recombinant microbes that express human UGTs was yet achieved. In this study we expressed human UDP glucose-6-dehydrogenase together with several human or rat UGT isoforms in the fission yeast *Schizosaccharomyces pombe* and generated strains that catalyze the whole-cell glucuronidation of standard substrates. Moreover, we established two methods to obtain stable isotope-labeled glucuronide metabolites: the first uses a labeled aglycon, whereas the second uses $^{13}C_6$-glucose as a metabolic precursor of isotope-labeled UDP-glucuronic acid and yields a 6-fold labeled glucuronide. The system described here should lead to a significant facilitation in the production of both labeled and unlabeled drug glucuronides for industry and academia.

The metabolic steps that lead to drug clearance in the human body are divided into two distinct parts, which encompass chemical modifications of the parent compound (phase I) and conjugations of parent or phase I metabolites with endogenous molecules (phase II). As a huge majority of the 200 most prescribed drugs in the United States are metabolized in the human body (Williams et al., 2004), the synthetic or biosynthetic accessibility of drug metabolites is a prerequisite for drug development and toxicity studies. According to current knowledge, cytochrome P450 (P450) systems are most important for phase I (Bernhardt, 2005; Ingelman-Sundberg et al., 2007) and UDP glycosyltransferases (UGTs) for phase II reactions (Mackenzie et al., 2005), respectively. The human UGT superfamily consists of four families with 22 isoenzymes: the 19 members of the UGT1 and UGT2 families (the latter encompassing subfamilies 2A and 2B) are primarily involved in xenobiotic metabolism and efficiently use UDP glucuronic acid (UDP-GA) for the conjugation of drugs to glucuronic acid (Mackenzie et al., 2005). UGT3A1 was recently shown to be a UDP-*N*-acetylglucosaminyltransferase that also appears to have a function in drug metabolism (Mackenzie et al., 2008); the catalytic activity of UGT3A2 is not yet known; and UGT8A1 catalyzes the transfer of galactose from UDP galactose to ceramide (Bosio et al., 1996). Similar to many P450s, some drug-metabolizing UGT isoforms (e.g., UGT1A1, UGT1A6, UGT1A9, and UGT2B15) display polymorphisms with a demonstrated association between genotype and clinical pharmacokinetics (Katz et al., 2008). Many UGTs are expressed in the liver, but other drug entry points such as epithelial surfaces of the nasal mucosa, gut, skin, brain, prostate, uterus, breast, placenta, and kidney also host UGT activity (Tukey and Strassburg, 2000). UGT enzymes are integrally associated with the membranes of the endoplasmic reticulum, possibly with most of the protein oriented toward the luminal side, and may form homo- and hetero-oligomeric structures (Bock and Köhle, 2009).

The formation of glucuronide metabolites may pose toxicological problems to patients via increased drug activity on glucuronidation (Coller et al., 2009) or cause dosage complications as a result of the uncontrolled rerelease of parent aglycones by systemic or enteric β-glucuronidase activity (Prueksaritanont et al., 2006). In addition, acyl glucuronides can exhibit chemical reactivity and can readily form covalently bound adducts to proteins. Such protein adducts have been discussed as a cause for idiosyncratic adverse drug reactions (Bailey and Dickinson, 2003). The identification and structure elucidation of drug glucuronides can be accomplished with milligram amounts, but safety testing may demand gram quantities. Because classic chemical synthesis of glucuronide metabolites can be cumbersome, various biological techniques have been developed to this aim, which include metabolite isolation from animal body fluids, the use of liver homogenates or liver microscale cultures, and

C.-A.D. and D.Bu. contributed equally to this work.

This work is part of the following patent application: Dragan C-A, Bureik M, and Buchheit D (2008) Drug metabolism. European patent application EP 08 164 826.3.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.109.030965.

ABBREVIATIONS: P450, cytochrome P450; UGT, UDP glycosyltransferase; UDP-GA, UDP glucuronic acid; 4MU, 4-methylumbelliferone; T, testosterone; TG, testosterone glucuronide; 4MUG, 4-methylumbelliferone-β-D-glucuronide; HPLC, high-performance liquid chromatography; UGDH, UDP glucose-6-dehydrogenase; EMM, Edinburgh minimal medium; LC/MS, liquid chromatography/mass spectrometry; FTMS, Fourier transform mass spectrometry; EIC, extracted ion currents.

enzyme preparations obtained after recombinant expression of metabolizing enzymes in cell culture or microbial systems (Radominska-Pandya et al., 2005; Khetani and Bhatia, 2008). However, each of these methods has its specific drawbacks. For example, human liver microsomes contain native UGTs, but their use may be hampered by their relative scarce availability, batch-to-batch variations, and the absence of UGTs that are only expressed in other tissues (Tukey and Strassburg, 2000; Jia and Liu, 2007). Heterologous expression of human UGTs in various mammalian or insect cell lines was very helpful for the determination of basic kinetic parameters of the enzymes, but it suffers from low expression levels, low activity normalized to biomass, and stability problems (Radominska-Pandya et al., 2005; Trubetskoy et al., 2008). Although functional UGT expression in baker's yeast has been shown per se, glucuronide production could only be achieved after preparation of yeast microsomes and addition of UDP-GA. The use of the costly cofactor UDP-GA at considerable concentrations in existing preparative methods seems to be necessary because of the endoplasmic membrane barrier, which limits the entry of UDP-GA in the lumen. In addition, the use of microsomal preparations implies the presence of many UGT isoforms and of other systems that may reduce the yield of the desired glucuronide product by cofactor competition.

Whole-cell biotransformations with recombinant microbes offer many advantages with respect to scalable metabolite production, and corresponding expression systems for human P450s have thus been established in bacteria and yeasts (Ghisalba and Kittelmann, 2007; Pscheidt and Glieder, 2008). In recent years, we showed the usefulness of recombinant strains of the fission yeast *Schizosaccharomyces pombe* that express human P450s for the production of P450 metabolites of illicit drugs (Peters et al., 2009) and doping substances (Zöllner et al., in press). Because no glucuronidation by whole-cell biotransformation with a unicellular organism that recombinantly expresses human UGTs was yet reported, it was the aim of this study to develop such a system using *S. pombe*.

Materials and Methods

Fine Chemicals. 4-Methylumbelliferone (4MU), testosterone (T), and testosterone glucuronide (TG) potassium salt were purchased from Sigma-Aldrich (Hamburg, Germany); 4-methylumbelliferone-β-D-glucuronide (4MUG) dihydrate was from Carl Roth (Karlsruhe, Germany); and $^{13}C_6$-glucose was from Euriso-Top (Saint-Aubin, France). Deuterated T was synthesized by Toroma Organics Ltd. (Saarbrücken, Germany). All the other chemicals used were either from Carl Roth or Sigma-Aldrich. Methanol [high-performance liquid chromatography (HPLC) grade] was from Thermo Fisher Scientific (Waltham, MA).

Coding DNA Sequences. The cDNAs of human UGT1A1, UGT1A9, UGT2A1, UDP glucose-6-dehydrogenase (UGDH), and/or rat UGT1A7 were synthesized by Entelechon GmbH (Regensburg, Germany); cDNAs of human UGT1A6, UGT1A7, UGT1A8, UGT1A10, UGT2B15, and UGT2B17 were synthesized by GENEART GmbH (Regensburg, Germany).

Media and General Techniques. We used general DNA-manipulating methods, media, and genetic methods for fission yeast as described previously (Drăgan et al., 2005). In addition, we used Edinburgh minimal medium (EMM) containing 100 g/l glucose for biotransformation assays and EMM containing 20 g/l $^{13}C_6$-glucose for the synthesis of isotope-labeled glucuronides.

Construction of Fission Yeast Strains. UGT cDNAs were cloned via NdeI and BamHI into the integrative vector pCAD1 (Drăgan et al., 2005) that integrates into the *leu1* gene of the fission yeast genome, thereby compensating an *ura4* defect. UGDH cDNA was cloned into the expression vector pREP1 (Maundrell, 1993) using NdeI and BamHI yielding pREP1-UGDH. The correctness of all the constructs was verified by automatic sequencing (MWG-Biotech, Ebersberg, Germany). The construction of fission yeast strains expressing functional UGTs was done in two steps. pCAD1-UGT constructs were prepared, before transformation, as reported previously (Drăgan et al., 2005), and used to transform yeast strain NCYC 2036 (*h*$^-$*ura4-D18*). Transformation was done, using competent cells prepared as described elsewhere (Suga and Hatakeyama, 2005), and yielded strains CAD200, DB1, DB3, DB5, DB23, DB24, DB25, DB26, DB32, and DB33. Correct integration into the *leu1* locus was verified by selection of leucine auxotrophs on EMM dishes containing 5 μM thiamine but no leucine. Subsequently, strains containing an integrated UGT expression cassette were transformed with pREP1-UGDH as described previously (Okazaki et al., 1990) to yield strains CAD203, DB11, DB13, DB15, DB43, DB44, DB45, DB46, DB52, and DB53. Both fission yeast expression vectors used in this study contain the strong endogenous *nmt1* promoter (Maundrell, 1990, 1993) that permits expression regulation via the presence or absence of thiamine in the medium. Therefore, transformed cells were selected by plating on EMM dishes with 5 μM thiamine to allow better growth under repressed conditions. All the yeast strains used in this study are available from PomBioTech GmbH (Saarbrücken, Germany).

Biomass Production. All the cultures were set up in absence of thiamine to induce expression by the *nmt1* promoter. Incubation was carried out at 30°C and 150 rpm. Ten milliliters of EMM containing the appropriate supplements and lacking thiamine was inoculated with cells grown on a dish for 3 days and incubated to stationary phase; these cells were then used to inoculate 100 ml main cultures. Main cultures were incubated for 1 to 2 days for the parental strains NCYC2036, CAD200, DB1, DB3, DB5, DB23, DB24, DB25, DB26, DB32, and DB33 and 2 to 5 days for the coexpressing strains CAD203, DB11, DB13, DB15, DB43, DB44, DB45, DB46, DB52, and DB53.

Whole-Cell Biotransformation Assay. The biomass was centrifuged (3000*g*, 5 min, room temperature) and resuspended in 12 ml of EMM with 100 g/l glucose and supplements as required. Substrate was added to a final concentration of 500 μM by adding 600 μl of 10 mM stock solutions in ethanol. The biotransformations were carried out in 250-ml wide-neck Erlenmeyer flasks for 72 h at 30°C and 150 rpm. Sample volumes of 2 ml were taken at 0 and 72 h and centrifuged (10,000*g*, 5 min, room temperature). The cell pellets were used to determine the biomass dry weight, whereas the supernatants were centrifuged again and then used for HPLC and liquid chromatography/mass spectrometry (LC/MS) analyses. All the results shown were obtained in at least three independent experiments.

Synthesis of Isotope-Labeled Glucuronide Metabolites. Because of the high cost of the labeled compounds, these experiments were done at 1-ml scale. For the comparison of the biosynthesis of labeled 4MUG with that of unlabeled 4MUG, the cultivation of the cells was done in EMM containing either $^{13}C_6$-glucose or unlabeled glucose at a concentration of 20 g/l. Five milliliters of medium without thiamine was inoculated with cells of strain DB13 and incubated for 1 day at 30°C and 150 rpm. One milliliter of this culture was then used to set up a 10-ml main culture, which in turn was incubated for 3 days under the same conditions. The biomass was harvested by centrifugation (3000*g*, 5 min, room temperature), and cells were resuspended in 1 ml of EMM containing a final concentration of 500 μM 4MU (50 μl of an ethanolic 10 mM 4MU stock solution). The assay was carried out in a 96-deep-well plate for 24 h at 30°C and 750 rpm in triplicate. For the synthesis of stable isotope-labeled TG, the biotransformation assay was carried out with strain DB53 as described before using T doubly deuterated at C-2, deuterated in β and α positions at C-4 and C-6, respectively, and deuterated at the C-17's hydroxyl group as substrate. In the acidic fission yeast medium, rapid exchange of the D atom in the OD group at C-17 leads to the 4-fold labeled substrate. All the samples were prepared as described above and analyzed by HPLC and LC/MS.

HPLC Analysis. HPLC was performed using a Series II 1090 system (Hewlett Packard, Palo Alto, CA) equipped with a Lichrospher 100 column (125 × 4.6 mm, RP-18, 5 μM; Merck, Darmstadt, Germany) and a diode array detector. The flow rate was 1 ml/min, and the column temperature was 40°C. For the simultaneous detection of 4MU and 4MUG, the initial mobile phase composition was 85% acetic acid (0.1%) (A) and 15% methanol (B). B was linearly increased to 55% from 5 to 10 min, maintained at 55% for further 2 min, and then immediately returned to 15% until the end of the run at 15 min. The eluents were monitored at 320 nm. The retention times of 4MU and 4MUG were 12.4 and 8.4 min, respectively. Quantification of 4MUG was done using an external 4MUG standard, prepared in the initial mobile phase at a concentration of 200 μM.

For the simultaneous detection of T and TG, the initial mobile phase composition was 85% acetic acid (0.1%) (A) and 15% methanol (B). B was

TABLE 1

Fission yeast strains used in this study

Expressed Protein(s)	Species Type	Strain Name	Parent Strain	Genotype	Reference
		NCYC2036		*h⁻ ura4-D18*	(Losson and Lacroute, 1983)
UGT1A1	Human	DB1	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A1*	This study
UGT1A6	Human	DB23	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A6*	This study
UGT1A7	Human	DB24	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A7*	This study
UGT1A8	Human	DB25	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A8*	This study
UGT1A9	Human	CAD200	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A9*	This study
UGT1A10	Human	DB26	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT1A10*	This study
UGT2A1	Human	DB3	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2A1*	This study
UGT2B15	Human	DB32	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2B15*	This study
UGT2B17	Human	DB33	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-UGT2B17*	This study
rUGT1A7	Rat	DB5	NCYC2036	*h⁻ ura4-D18 leu1::pCAD1-rUGT1A7*	This study
UGT1A1, UGDH	Human	DB11	DB1	*h⁻ ura4-D18 leu1::pCAD1-UGT1A1/pREP1-UGDH*	This study
UGT1A6, UGDH	Human	DB43	DB23	*h⁻ ura4-D18 leu1::pCAD1-UGT1A6/pREP1-UGDH*	This study
UGT1A7, UGDH	Human	DB44	DB24	*h⁻ ura4-D18 leu1::pCAD1-UGT1A7/pREP1-UGDH*	This study
UGT1A8, UGDH	Human	DB45	DB25	*h⁻ ura4-D18 leu1::pCAD1-UGT1A8/pREP1-UGDH*	This study
UGT1A9, UGDH	Human	CAD203	CAD200	*h⁻ ura4-D18 leu1::pCAD1-UGT1A9/pREP1-UGDH*	This study
UGT1A10, UGDH	Human	DB46	DB26	*h⁻ ura4-D18 leu1::pCAD1-UGT1A10/pREP1-UGDH*	This study
UGT2A1, UGDH	Human	DB13	DB3	*h⁻ ura4-D18 leu1::pCAD1-UGT2A1/pREP1-UGDH*	This study
UGT2B15, UGDH	Human	DB52	DB32	*h⁻ ura4-D18 leu1::pCAD1-UGT2B15/pREP1-UGDH*	This study
UGT2B17, UGDH	Human	DB53	DB33	*h⁻ ura4-D18 leu1::pCAD1-UGT2B17/pREP1-UGDH*	This study
rUGT1A7, UGDH	Rat, human	DB15	DB5	*h⁻ ura4-D18 leu1::pCAD1-rUGT1A7/pREP1-UGDH*	This study

linearly increased to 90% from 2 min to 12 min and then immediately returned to 15% B until the end of the run at 17 min. The eluents were monitored at 240 nm. The retention times of T and TG were 13.3 and 11.5 min, respectively. TG concentrations were quantified using an external T standard at a concentration of 200 μM.

LC/MS. Samples were analyzed by nanospray ionization high-resolution Fourier transform mass spectrometry (FTMS) in the positive ion mode using a linear ion trap/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a TriVersa NanoMate nanospray ion source (Advion, Ithaca, NY). The instrument was coupled to the postcolumn flow of an Agilent Technologies (Santa Clara, CA) 1100 HPLC detection system equipped with a YMC ODS-AQ column (150 × 4 mm, 5 μm; YMC America, Inc., Allentown, PA). The flow rate was 800 μl/min, and the column temperature was 40°C. A linear gradient from 10 to 90% acetonitrile versus water containing 50 mM formic acid was applied from 0 to 14 min, maintained at 90% for further 3 min, and then immediately returned to the initial conditions for 3 min.

The bulk of the LC flow (700 μl/min) was discarded, and approximately 100 μl/min was passed to the nanospray source. The source voltage and capillary voltage were set to 1.5 kV and 22 V, respectively. Nitrogen was used as bath gas to slow down the motion of the ion population in the C-trap. Collision-induced dissociation experiments ($FTMS^n$) were performed in the linear ion trap with collision energies of 26% normalized collision energy at activation Q values of 0.250 with helium as collision gas. The isolation width for collision-induced dissociation experiment in the linear ion trap was set to ±1 atomic mass unit. The FTMS mass analyzer was operated at a resolution of approximately 60,000 full width at half maximum at m/z 400 in the full-scan MS mode and approximately 15,000 full width at half maximum at m/z 400 in the full-scan MS^n mode. High-resolution mass spectra were acquired in the range of m/z 50 to 800. Exact mass measurements in the FTMS- and FT-MS^n mode were performed after external calibration using the manufacturer's calibration mixture from previous LC/$FTMS^n$ investigations.

Results

Synthetic cDNA sequences coding for nine wild-type human UGTs or rat UGT1A7, respectively, were each cloned into the integrative fission yeast expression vector pCAD1 (Drăgan et al., 2005). Transformation of fission yeast strain NCYC2036 with the resulting constructs yielded the first set of strains, each of which expresses a single mammalian UGT isoform (see Table 1; Fig. 1). The newly created strains grew normally and did not display a visible phenotype (data not shown). As mentioned above, UGT1 and UGT2 isoenzymes require UDP-GA as a cofactor, which is synthesized from UDP glucose by UGDH in mammals. Although the formation of UDP glucose in yeasts is known, wild-type baker's yeast does not produce UDP-GA (Oka and Jigami, 2006), and the fission yeast genome contains no open reading frame that shows significant homology to mammalian UGDH enzymes (Wood et al., 2002). Still, the presence of an endogenous UGDH activity as a result of a nonhomologous fission yeast protein could not a priori be ruled out. However, incubation of human UGT-expressing strains (Table 1) with either 4MU (Uchaipichat et al., 2004) or T (Sten et al., 2009) as standard substrate did not lead to detectable glucuronide formation (data not shown). Therefore, for the development of a whole-cell biotransformation process, an endogenous source of UDP-GA had to be provided by coexpression of UGDH (see Fig. 2). A synthetic cDNA coding for human UGDH was cloned into the autosomally replicating expression vector pREP1 (Maundrell, 1993) to yield the new plasmid pREP1-UGDH. Transformation of all the UGT-expressing strains with this plasmid yielded a new set of 10 UGT- and UGDH-coexpressing strains (Table 1; Fig. 1). These new strains showed significantly reduced growth under induced expression conditions and yielded biomass concentrations between 1 and 2 g/l after 2 to 5 days in contrast to biomass yields between 2 and 3 g/l for strains that only express UGTs (data not shown). Whole-cell biotransformation assays showed that all the coexpressing strains were able to catalyze the glucuronidation of either 4MU or T (Table 2). The identity of the products 4MUG and TG was in each case confirmed by LC/MS analysis (data not shown). As could be expected, the biotransformation activities of the different UGT-expressing strains toward 4MU varied from approximately 1 μM/day for UGT1A1 and UGT1A6, respectively, up to 150 μM/day in the case of UGT1A9. In terms of specific production rates (i.e., normalized to the biomass dry weight), the highest values of almost 20 μmol/day/g were achieved by the UGT1A9- and UGT2A1-expressing strains, respectively, whereas the strains expressing UGT1A1, UGT1A6, and rat UGT1A7, respectively, yielded only values around or even less than 0.1 μmol/day/g. Strain DB53 expressing human UGT2B17 produced TG with a maximum space-time yield of 32.6 μM/day and a maximum specific production rate of 3.1 μmol/day/g. To the best of our knowledge, these data show for the first time a successful whole-cell biotransformation using recombinant human UGTs expressed in a unicellular organism.

The availability of stable isotope-labeled glucuronides is a prerequisite for the sensitive quantification of glucuronide metabolites in biological matrices by LC/tandem MS, e.g., for the toxicokinetic

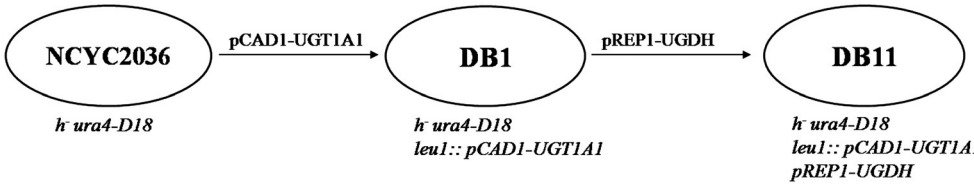

FIG. 1. Fission yeast strain construction scheme. The procedure is exemplarily shown for UGT1A1. The uracil-deficient parental strain NCYC2036 was transformed using the integrative plasmid pCAD1-UGT1A1. The resulting leucine-deficient strain DB1 was in turn transformed with the autosomal plasmid pREP1-UGDH yielding strain DB11. For all the other UGT isoforms, the cloning procedure was done accordingly.

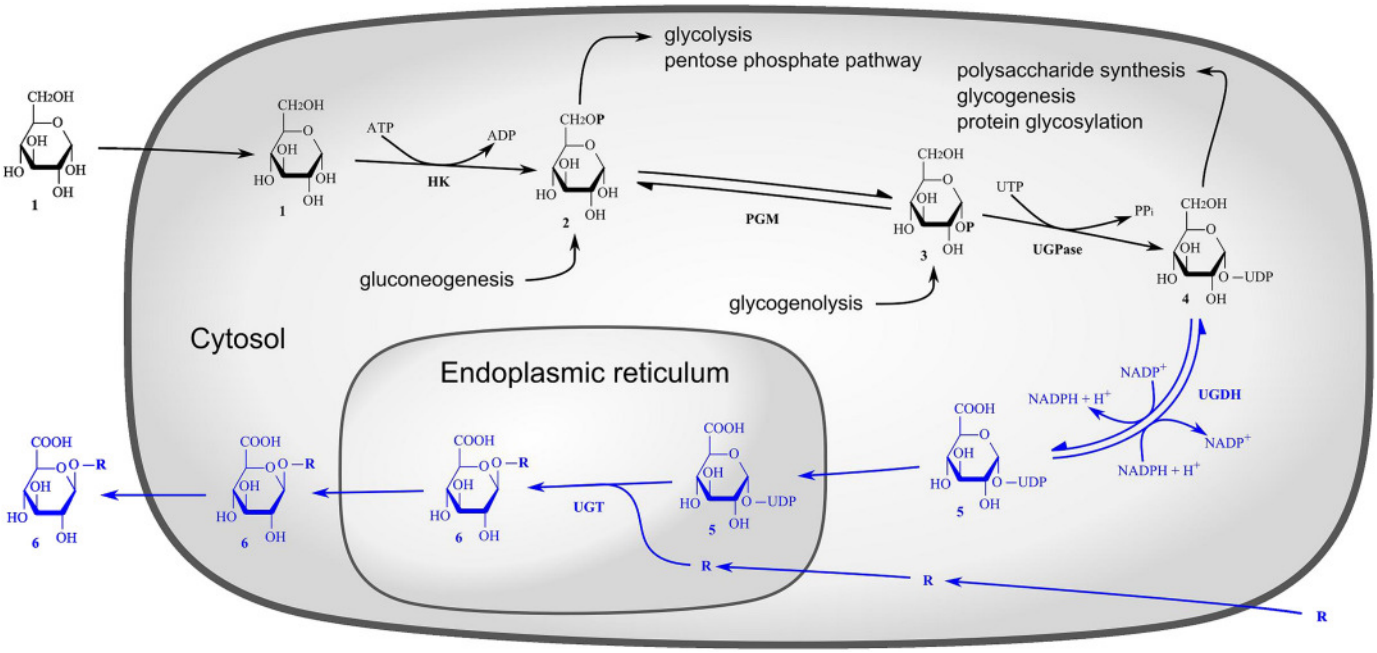

FIG. 2. Schematic representation of the engineered glucuronide biosynthetic pathway in recombinant fission yeast strains. The scheme shows in black the endogenous pathway that leads to the formation of UDP glucose with branching points to several pathways of the central carbon metabolism; reactions depicted in blue indicate the reaction steps introduced into the host by the heterologous coexpression of human UGDH and a mammalian UGT. Glucose (1) enters the cell and is converted to glucose 6-phosphate (2) by hexokinase (HK), which can be interconverted to glucose-1-phosphate (3) by phosphoglucomutase (PGM). The latter is conjugated with UDP by UDP glucose pyrophosphorylase (UGPase) to yield UDP glucose (4). Human UGDH oxidizes 4 to UDP-GA (5), which then enters the lumen of the endoplasmic reticulum. Eventually, the heterologously expressed mammalian UGT performs the conjugation of 5 to an aglycon (R) that passes both plasma and endoplasmic membrane to yield a glucuronide metabolite (6). The glucuronide metabolite in turn leaves both the endoplasmic reticulum and the cell.

TABLE 2

Glucuronide metabolite formation rates determined in whole-cell biotransformations of fission yeast strains coexpressing human UGDH and mammalian UGT enzymes

Strain	Expressed Protein(s)	Substrate	Product	Space-Time Yield		Specific Production Rate	
				Min	Max	Min	Max
				μM/day	*μM/day*	*μmol/day/g biomass dry wt.*	*μmol/day/g biomass dry wt.*
DB11	UGT1A1, UGDH	4MU	4MUG	0.5	1.8	0.04	0.14
DB43	UGT1A6, UGDH	4MU	4MUG	0.6	3.5	0.04	0.22
DB44	UGT1A7, UGDH	4MU	4MUG	8.2	18.2	0.85	2.10
DB45	UGT1A8, UGDH	4MU	4MUG	5.9	23.0	0.43	2.50
CAD203	UGT1A9, UGDH	4MU	4MUG	64.2	151.5	12.10	19.70
DB46	UGT1A10, UGDH	4MU	4MUG	7.0	18.2	0.56	1.50
DB13	UGT2A1, UGDH	4MU	4MUG	22.8	114.5	4.00	19.50
DB52	UGT2B15, UGDH	4MU	4MUG	3.3	7.9	0.24	0.63
DB53	UGT2B17, UGDH	4MU	4MUG	0.0	0.0	0.00	0.00
DB53	UGT2B17, UGDH	T	TG	15.5	32.6	1.20	3.10
DB15	rUGT1A7, UGDH	4MU	4MUG	2.3	2.3	0.16	0.19

monitoring of glucuronides during nonclinical safety studies. Therefore, having established a functional in vivo system for the production of glucuronides, we investigated the possibility to produce isotope-labeled glucuronide metabolites by applying two different strategies. First, either nonlabeled T or 4-fold deuterated T (D_4-T) were subjected to whole-cell biotransformation with strain DB53 that coexpresses UGT2B17 and UGDH (Fig. 3a). The comparison of the LC/MS analysis of the extracted ion currents (EICs) of nonlabeled and 4-fold labeled TG, as well as the respective fragmentation spectra, unambiguously shows the successful formation of D_4-TG (Fig. 3,

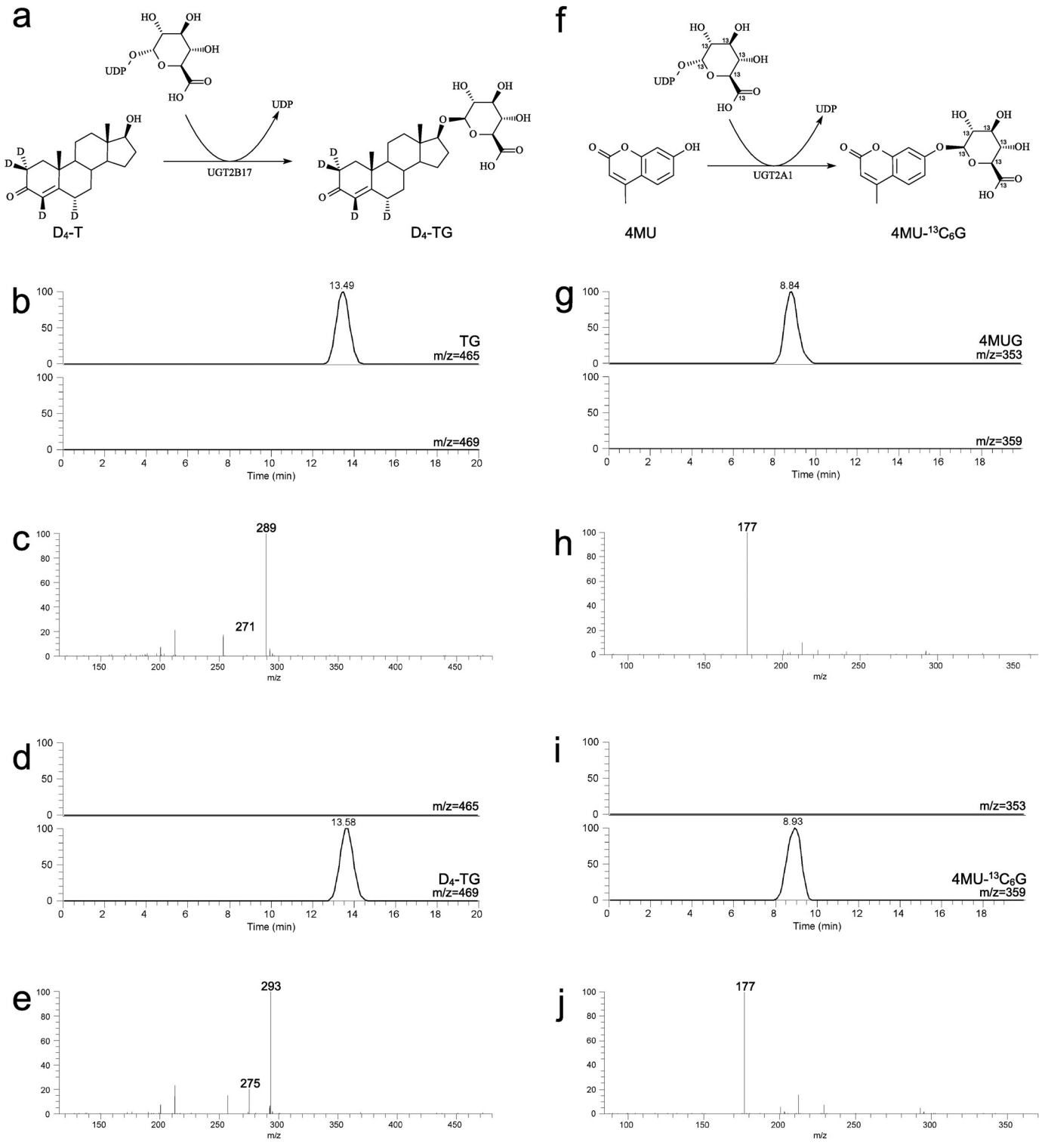

FIG. 3. Production of stable isotope-labeled glucuronides using either a labeled aglycon (a–e) or a labeled sugar moiety (f–j). Scheme of the glucuronidation of D_4-testosterone (D_4-T) by UGT2B17 (a). Comparison of the LC/MS analysis of the EIC of nonlabeled (b) and 4-fold labeled (d) TG and fragmentation spectra of TG (TG → T, m/z_{theo} = 289.21621, Δ = −0.28 ppm; c) and D_4-testosterone glucuronide (D_4-TG → D_4-T, m/z_{theo} = 293.24131, Δ = 0.17 ppm; e). Scheme of the glucuronidation of 4MU by UGT2A1 with UDP-$^{13}C_6$-GA (f), which is produced from $^{13}C_6$-glucose according to Fig. 2. Comparison of the LC/MS analysis of nonlabeled (g) and 6-fold labeled (i) 4MUG and fragmentation spectra of 4MUG (4MUG → 4MU, m/z_{theo} = 177.05462, Δ = −1.35 ppm; h) and 4MU $^{13}C_6$-glucuronide (4MU-$^{13}C_6$G → 4MU, m/z_{theo} = 177.05462, Δ = −0.57 ppm; j).

b–e). Second, we intended to show a more general labeling technique by using ^{13}C isotope-labeled glucuronic acid as substrate and strain DB13 (expressing UGT2A1 and UGDH). Based on the biosynthesis scheme outlined above (Fig. 2), which leads to the endogenous formation of UDP-GA in UGDH-expressing fission yeast strains, we used $^{13}C_6$-glucose as metabolic precursor of 6-fold labeled UDP-GA (Fig. 3f). For this purpose, $^{13}C_6$-glucose was added throughout all the culturing periods before performing the biotransformation to deplete

^{12}C-compounds in the central carbon metabolism of the cells as much as possible. Again, the comparison of the LC/MS analysis of the EICs of 4MUG and 4MU-$^{13}C_6$G proves the successful formation of the 6-fold labeled product (Fig. 3, g and i). In this case, the main signal of the fragmentation spectra is not changed as it corresponds to the unlabeled aglycon (Fig. 3, h and j). These results show that the generation of isotope-labeled glucuronide metabolites can be conveniently achieved in vivo using the fission yeast system presented in this study, and that according to requirements, either the aglycon or the sugar moiety may be labeled.

Discussion

In this study, 10 fission yeast strains were cloned that express either one of nine human UGTs or rat UGT1A7, respectively (Table 1). As fission yeast does not have an endogenous UGDH enzyme (Wood et al., 2002), neither of these strains displayed glucuronidation activity toward 4MU or T because of a lack of UDP-GA (data not shown). Therefore, a second set of strains was created that coexpress the UGTs mentioned above together with human UGDH. In addition to the intracellular production of UDP-GA as such, its subcellular localization also had to be considered: in human cells, UDP-GA is formed by UGDH in the cytoplasm and subsequently transported by nucleotide sugar transporters into the lumen of the endoplasmic reticulum, where the UGTs are located (Kobayashi et al., 2006). If after expression in fission yeast the subcellular localization of both UGTs and UGDH corresponds to their targeting in mammalian cells, then for biotransformation to occur at least one of the endogenous nucleotide sugar transporters (such as *vrg4* or *hut1*) (Nakanishi et al., 2001) must be able to transport UDP-GA into the endoplasmic reticulum lumen. This seems to be the case, because all the coexpressing strains were able to catalyze the glucuronidation of either 4MU or T (Table 2). The time-space yield of the different UGT-expressing strains toward 4MU varied by roughly 2 orders of magnitude, from approximately 1 μM/day up to 150 μM/day. With respect to the relative activity of some of the UGT isoforms (e.g., UGT1A6 versus UGT1A9), these results partially vary from earlier results obtained with enzymes purified from transfected human embryonic kidney cells (Uchaipichat et al., 2004). However, the very different experimental settings are likely to account for some of these variations. Strain DB53 expressing human UGT2B17 produced TG with a maximum space-time yield of 32.6 μM/day and a maximum specific production rate of 3.1 μmol/day/g. Thus, a successful whole-cell biotransformation using recombinant human UGTs expressed in a unicellular organism could be established for the first time. Compared with the alternatives mentioned previously, a significant benefit of this system is its self-sufficiency with respect to the expensive cofactor UDP-GA. A further advantage is its cost-effective scalability as a result of the endogenous generation of all the reaction constituents except for the substrate. Although fission yeast is at present not a widely used organism in biotechnology, its widely tolerable pH range makes adaptation to substrate and product requirements easily achievable; for example, the production of (notoriously unstable) acyl glucuronides may be done under acidic conditions to avoid rapid degradation (Ebner et al., 1999). In addition to up-scaling, scaling down could lead to a simple and efficient high-throughput screening method for large-scale UGT profiling or inhibition studies using living cells.

Because the availability of stable isotope-labeled glucuronides is desirable for the LC/tandem MS analysis of glucuronide metabolites in biological matrices, we established two different methods for their preparation. In the first approach, a labeled aglycon served as a substrate, as in a recent study where pooled human liver microsomes were used for a similar biotransformation (Turfus et al., 2009). In this case, 4-fold deuterated T was successfully glucuronidated by strain DB53 (Fig. 3, a–e). Although this approach for the production of stable isotope-labeled glucuronides is very straightforward, it depends on the availability of a sufficiently labeled aglycon, which may not always be at hand. Moreover, in some instances, unfavorable isotope effects may occur. Therefore, a second strategy was developed that uses $^{13}C_6$-glucose as a metabolic precursor, which is converted within the UGDH-expressing fission yeast strains to $^{13}C_6$-labeled UDP-GA (Fig. 2). Exemplarily, strain DB13 (expressing UGT2A1 and UGDH) was successfully used for the production of 6-fold labeled 4MUG (4MU-$^{13}C_6$G) by this method (Fig. 3, f–j). In conclusion, in this study we show the functional expression of human UGDH with nine human and one rat UGT enzymes in fission yeast that can be conveniently used for the synthesis of either labeled or unlabeled glucuronide metabolites by whole-cell biotransformation.

Acknowledgments. We thank Maria Widjaja for plasmid construction and Ellen Schmitt for experimental support.

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Buchheit D, Drăgan C-A, Schmitt EI and Bureik M: Production of Ibuprofen Acyl Glucosides by Human UGT2B7. *Drug Metab Dispos*. 2011 Dec; 39(12): 2174-81. Epub 2011 Aug 23.

0090-9556/11/3912-2174–2181$25.00
DRUG METABOLISM AND DISPOSITION
Vol. 39, No. 12
41640/3727406
DMD 39:2174–2181, 2011
Printed in U.S.A.

Production of Ibuprofen Acyl Glucosides by Human UGT2B7

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Received July 6, 2011; accepted August 23, 2011

ABSTRACT:

UDP-glycosyltransferases (UGTs) are an important group of enzymes that participate in phase II metabolism of xenobiotics and use the cofactor UDP-glucuronic acid for the production of glucuronides. When acting on molecules bearing a carboxylic acid they can form acyl glucuronides, a group of metabolites that has gained significant interest in recent years because of concerns about their potential role in drug toxicity. In contrast, reports about the production of drug acyl glucosides (which might also display high reactivity) have been scarce. In this study, we discovered the formation of acyl glycoside metabolites of *R*- and *S*-ibuprofen (Ibu) by human liver microsomes supplied with the cofactor UDP-glucose. Subsequently, human UGT2B7*1 and UGT2B7*2 recombinantly expressed in fission yeast *Schizosaccharomyces pombe* could be shown to catalyze these reactions. Moreover, we could enhance the glucoside production rate in fission yeast by overexpressing the fission yeast gene SPCC1322.04, a potential UDP-glucose pyrophosphorylase (UGPase), but not by overexpression of SPCC794.10, and therefore suggest to name this gene *fyu1* for fission yeast UGPase1. It was interesting to note that pronounced differences between the two polymorphic UGT2B7 variants were observed with respect to acyl glucoside production. Finally, using the metabolic precursor [$^{13}C_6$]glucose, we demonstrated the production of stable isotope-labeled reference standards of Ibu acyl glucoside and Ibu acyl glucuronide by whole-cell biotransformation in fission yeast.

Introduction

The UDP-glycosyltransferases (UGTs) are a superfamily of enzymes that catalyze the addition of glycosyl residues to small molecular-weight lipophilic chemicals and in this way play an important role in the elimination of different endogenous and exogenous compounds from the human body (Mackenzie et al., 2005). Humans have 22 UGTs that belong to four families: UGT1, UGT2 (with the subfamilies 2A and 2B), UGT3, and UGT8. The 19 human UGT isoforms in the UGT1 and UGT2 families have an often overlapping but sometimes very distinct substrate selectivity and are thought to typically prefer UDP-glucuronic acid (UDP-GA) as a sugar donor. UGTs may convert carboxylic-acid-containing drugs into acyl glucuronides (AGs), which because of their special properties and risks are of considerable pharmacological interest. It was noted decades ago that AGs are potentially reactive electrophiles that can interact with and covalently bind to nucleophilic targets (Faed, 1984). Accordingly, it was suggested that glucuronidation is not always a harmless detoxication reaction but that in some instances it can be a bioactivation pathway leading to potential toxicity (Spahn-Langguth and Benet, 1992). However, the overall toxicological significance of AGs is still a subject of debate (Boelsterli, 2011; Stachulski, 2011).

The nonsteroidal anti-inflammatory drug ibuprofen (Ibu) acts as an cyclooxygenase inhibitor and is predominantly orally applied as a racemic mixture; in the body, much of the *R*-enantiomer is converted to the active *S*-form (Lee et al., 1985). Ibu metabolism comprises a complex interaction of different phase I and phase II enzymes including several cytochrome P450 enzymes, dehydrogenases, and UGTs (Adams, 1992; Spraul et al., 1993; Kepp et al., 1997; McGinnity et al., 2000; Hao et al., 2005; Chang et al., 2008). To the best of our knowledge, all UGT metabolites of Ibu described so far are glucuronides, with the glucuronic acid being either attached to the acyl moiety of the parent compound (yielding Ibu AGs) or to hydroxy groups newly created in phase I metabolism.

In this study, we describe new acyl glycoside metabolites of Ibu. In vitro studies with human liver microsomes (HLMs) and the substrates (*R*)-(−)-ibuprofen (*R*-Ibu), (*S*)-(+)-ibuprofen (*S*-Ibu), and racemic ibuprofen (*rac*-Ibu) demonstrated that such metabolites are only produced in the presence of the cofactor UDP-glucose (UDP-Glc), but not UDP-galactose (UDP-Gal), thus confirming their identity to be either the *R*- or *S*-enantiomers of Ibu acyl glucoside (Ibu-Glc). UGT2B7 is one of the most important human UGT isoforms with respect to drug clearance in general (Williams et al., 2004) and significantly contributes to Ibu metabolism (Sakaguchi et al., 2004; Kuehl et al., 2005). There are two important polymorphic variants of this enzyme, UGT2B7*1 (268His) and UGT2B7*2 (268Tyr), with an allelic distribution of approximately 1:1 in white persons and 3:1 in Japanese individuals (Bhasker et al., 2000). Several studies demonstrated notable differences in the catalytic activity of these two forms, although this effect seems to be limited to certain substrates (Bernard et al., 2006; Thibaudeau et al., 2006; Belanger et al., 2009). Like some other UGT isoforms, UGT2B7 has been shown to use other UDP-

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.111.041640.

ABBREVIATIONS: UGT, UDP-glycosyltransferase; AG, acyl glucuronide; *fyu1*, fission yeast UDP-glucose pyrophosphorylase 1; HLMs, human liver microsomes; Ibu, ibuprofen; *R*-Ibu, (*R*)-(−)-ibuprofen; *S*-Ibu, (*S*)-(+)-ibuprofen; *rac*-Ibu, racemic ibuprofen; Ibu-AG, ibuprofen acyl glucuronide; Ibu-Glc, ibuprofen acyl glucoside; UDP-GA, UDP-α-D-glucuronic acid; UDP-Gal, UDP-α-D-galactose; UDP-Glc, UDP-α-D-glucose; UGDH, UDP-glucose-6-dehydrogenase; UGPase, UDP-glucose pyrophosphorylase; LC-MS, liquid chromatography-mass spectrometry; DAD, diode array detector; EMM, Edinburgh minimal medium.

sugars in addition to UDP-GA, such as UDP-Glc and UDP-Gal, and at least in some cases this property also seems to be substrate dependent (Mackenzie et al., 2003; Tang et al., 2003; Tang and Ma, 2005). We therefore investigated the ability of recombinantly expressed UGT2B7*1 and UGT2B7*2 to produce Ibu-Glc using our previously described fission yeast system (Dragan et al., 2010). Whole-cell biotransformations with UGT2B7-expressing fission yeasts demonstrated that the rate of metabolization of Ibu to Ibu-Glc depends on the Ibu enantiomer and the enzyme variant. Moreover, overexpression of an endogenous UDP-glucose pyrophosphorylase (UGPase) significantly enhanced Ibu-Glc production by UGT2B7*1, but not by UGT2B7*2, thus indicating a differential effect of cofactor availability. We suggest to name this UGPase, which is described in this study for the first time, *fyu1*, for fission yeast UGPase 1.

Materials and Methods

Chemicals. *R*-Ibu was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY), *S*-Ibu was purchased from Thermo Fisher Scientific (Waltham, MA), and *rac*-Ibu disodium salt was purchased from Sigma-Aldrich (St. Louis, MO). UDP-GA trisodium salt was purchased from Sigma-Aldrich, UDP-Glc disodium salt was purchased from Merck (Darmstadt, Germany), and UDP-Gal disodium salt was purchased from VWR (West Chester, PA). [$^{13}C_6$]glucose was purchased from Euriso-Top (Saint-Aubin, France). Methanol (high-performance liquid chromatography grade) was purchased from VWR. All other chemicals used were either from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich.

In Vitro Metabolism of Ibu Using HLMs. Pooled HLMs (BD Biosciences, San Jose, CA) were incubated using UDP-GA, UDP-Glc, or UDP-Gal as cofactor as indicated. In addition, incubations without a UDP-sugar cofactor were done as a negative control. The total reaction volume was 100 μl. Final concentrations of in vitro incubations were 0.5 mg $\cdot$ ml^{-1} protein, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, and 200 μM ibuprofen (*S*-Ibu, *R*-Ibu, or *rac*-Ibu). Reactions were started by the addition of 2 mM UDP-GA, UDP-Glc, or UDP-Gal, and samples were incubated for 18 h at 37°C and then mixed with an equal volume of acetonitrile. All experiments were done in triplicate.

Coding DNA Sequences. The cDNA for human UGT2B7*2 optimized for expression in *S. pombe* was synthesized by Entelechon GmbH (Regensburg, Germany). The cDNA of human UGT2B7*1 was generated by site-directed mutagenesis using UGT2B7*2 as a template. The cDNAs of SPCC794.10 and SPCC1322.04 (*fyu1*) were amplified by polymerase chain reaction using cells of the *S. pombe* strain NCYC2036 (Losson and Lacroute, 1983) as a template. For amplification and introduction of 5′-NdeI- and 3′-BamHI sites, the following oligonucleotides were used: SPCC794.10, 5′-TAG TTT CAT ATG TTG CAT CGT CGA ATT C-3′ and 5′-TTT GGA TCC TCA ACA CTC CAT TAT TTT AC-3′; and SPCC1322.04, 5′-TAG ATA CAT ATG GAT TTG GCA CC-3′ and 5′-TTG GAT CCT TAG TGC TCC AAG ATA TTG-3′.

Media and General Techniques. General DNA manipulating methods as well as media and genetic methods for fission yeast have been described (Sambrook and Rusell, 2001; Forsburg and Rhind, 2006). In addition, Edinburgh minimal medium (EMM) containing 100 g $\cdot$ l^{-1} glucose was used for biotransformation assays and EMM with 20 g $\cdot$ l^{-1} [$^{13}C_6$]glucose was used for the synthesis of isotope-labeled glucuronides.

Construction of Fission Yeast Strains. Fission yeast strain construction was in principle done as described previously (Dragan et al., 2010). UGT2B7*1 and UGT2B7*2 cDNAs were cloned via NdeI and BamHI into the integrative vector pCAD1 (Dragan et al., 2005), which disrupts the leu1 locus of *S. pombe* and contains an ura4 marker gene. The construction of the uridine diphospho-glucose-6-dehydrogenase (UGDH) expression plasmid pREP1-UGDH has been described previously (Dragan et al., 2005). UGT2B7*1, UGT2B7*2, SPCC794.10, and SPCC1322.04 (= *fyu1*) cDNAs were cloned into the autosomal expression vector pREP1 using NdeI and BamHI, respectively. The correctness of all constructs was verified by automatic sequencing (Eurofins MWG Operon, Huntsville, AL or Seq-it GmbH, Kaiserslautern, Germany). pCAD1 and pREP1 contain the strong endogenous nmt1 promoter that permits regulation of expression via the presence or absence of thiamine in the media. Therefore, transformed cells were grown on EMM dishes with 5 μM thiamine to allow for better growth under repressed conditions.

All strains used in this study are listed in Table 1. Plasmids pCAD1-UGT2B7*1 and pCAD1-UGT2B7*2 were prepared as described previously (Dragan et al., 2005) and used for the transformation of the parental *S. pombe* strain NCYC2036 (Losson and Lacroute, 1983) to yield strains DB7 and DB4. Correct integration into the leu1 locus was verified by testing growth of clones on EMM dishes without leucine. Subsequently, strains containing an integrated UGT expression cassette were each transformed with pREP1-UGDH, pREP1-SPCC794.10, and pREP1-SPCC1322.04, respectively, to yield strains DB14, DB64, and DB65 from the parental strain DB4, and DB17, DB66, and DB67 from the parental strain, DB7, respectively. In addition, strains containing two expression cassettes for UGT2B7*1 or UGT2B7*2 were generated by transforming DB4 with pREP1-UGT2B7*2 and DB7 with pREP1-UGT2B7*1 to yield strains DB62 and DB63, respectively.

Biomass Production. All liquid cultures lacked thiamine to induce expression by the nmt1 promoter. Incubations were performed at 30°C and 150 rpm. Ten milliliters of EMM precultures containing the appropriate supplements were inoculated with cells grown on a dish for 2 to 3 days and incubated to stationary phase; these cells were then used to inoculate 100-ml main cultures. Main cultures were incubated for 1 day for the strain NCYC2036; for 2 days for the strains DB4, DB7, DB62, DB63, DB64, DB65, DB66, and DB67; and for 5 days for the strains DB14 and DB17.

Whole-Cell Biotransformation Assay. Whole-cell biotransformation assays were done as described previously (Dragan et al., 2010) using *S*-Ibu, *R*-Ibu, or *rac*-Ibu as substrate (from a 10 mM stock solution in 50% ethanol) with a final concentration of 500 μM. Afterward, culture samples were frozen until sample preparation and liquid chromatography-mass spectrometry (LC-MS) analysis. All experiments were done in triplicate.

Synthesis of Isotope-Labeled Glucuronide Metabolites. For the synthesis of $^{13}C_6$-labeled glucuronides, whole-cell biotransformation assays were done in 200-μl scale. A main culture (1.6 ml) was centrifuged (5 min, 3000*g*, and room temperature), washed with 1 ml of water to remove a maximum of remaining unlabeled glucose, and resuspended in EMM containing 20 g $\cdot$ l^{-1} [$^{13}C_6$]glucose at 200 μl. Ten microliters of a 10 mM substrate stock solution (in 50% ethanol) were added to reach a substrate concentration of 500 μM. Samples were horizontally shaken in a 2-ml reaction tube with a pinhole for 72 h at 30°C and 150 rpm. The biomass dry weight was determined from the initial main culture. All experiments were done in triplicate.

TABLE 1

Fission yeast strains used in this study

Strain	Parental Strain	Expressed Protein(s)	Genotype	Reference
NCYC2036			*h*$^-$ *ura4-D18*	(Losson and Lacroute, 1983)
DB4	NCYC2036	UGT2B7*2	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*2*	This study
DB7	NCYC2036	UGT2B7*1	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*1*	This study
DB14	DB4	UGT2B7*2, UGDH	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*2/pREP1-UGDH*	This study
DB17	DB7	UGT2B7*1, UGDH	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*1/pREP1-UGDH*	This study
DB62	DB4	UGT2B7*2	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*2/pREP1-UGT2B7*2*	This study
DB63	DB7	UGT2B7*1	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*1/pREP1-UGT2B7*1*	This study
DB64	DB4	UGT2B7*2, SPCC794.10	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*2/pREP1-* SPCC794.10	This study
DB65	DB4	UGT2B7*2, SPCC1322.04	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*2/pREP1-* SPCC1322.04	This study
DB66	DB7	UGT2B7*1, SPCC794.10	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*1/pREP1-* SPCC794.10	This study
DB67	DB7	UGT2B7*1, SPCC1322.04	*h*$^-$ *ura4-D18 leu1::pCAD1-UGT2B7*1/pREP1-* SPCC1322.04	This study

Sample Preparation. Cell suspensions from whole-cell biotransformations were thawed if frozen and diluted 1:1 with acetonitrile. All samples (from in vitro incubations and whole-cell biotransformations) were centrifuged (5 min, 15,000*g*, and room temperature), the supernatant was centrifuged again (10 min, 15,000*g*, and room temperature), and the resulting supernatants were analyzed by LC-MS.

Liquid Chromatography-Mass Spectrometry. Samples were analyzed on an Agilent/HP 1100 series high-performance liquid chromatograph equipped with a G1315B diode array detector (DAD) and a G1946A single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Components were separated on a Lichrospher RP 18 column (particle size 5 μm, column inner diameter 4 mm, and length 125 mm; WICOM, Heppenheim, Germany) using water (mobile phase A) and methanol containing 0.1% formic acid (mobile phase B). The flow rate was 0.8 ml/min, and the column temperature was 40°C. LC-MS settings were as follows: flow profile 0 to 5 min, 10% B; 5 to 20 min, 10 to 90% B; 20 to 25 min, 90% B; 25 to 30 min, 10% B; run time 30 min; injection volume 10 μl; detector 1 G1315B DAD; DAD range 200 to 600 nm, slit width 2 nm; detector 2 G1946A mass selective detector, ionization electrospray ionization (−), and mode scan; and N_2 flow rate 12 $l \cdot min^{-1}$, nebulizer pressure 45 psig (0.07 bar), nebulizer temperature 350°C, capillary voltage 3500 V, and fragmentor voltage 50 V. The quantification of metabolites was done using an external *S*-Ibu straight calibration line prepared in a 1:1 mixture of mobile-phase components (concentrations from 10 to 250 μM *S*-Ibu). Labeled metabolites were quantified using an external *S*-Ibu standard of 100 μM prepared in a 1:1 mixture of mobile-phase components.

Results

Human Liver Microsomes Produce Ibuprofen Acyl Glucosides but Not Galactosides. HLMs were incubated with all possible combinations of one of the substrates (*S*-Ibu, *R*-Ibu, or *rac*-Ibu) and one of the cofactors (UDP-GA, UDP-Glc, or UDP-Gal). As expected, subsequent LC-MS analysis revealed that the presence of UDP-GA led to the generation of Ibu-AGs, which were identified by a *m/z* value of 381 for $[M-H]^-$ in negative ion mode (Fig. 1). With all three substrates, samples with UDP-Glc displayed an UV peak containing the *m/z* values 403 and 413, which correspond to chlorine $[M+Cl]^-$ or formic acid $[M+FA-H]^-$ adducts of Ibu acyl glycosides. Abundance values of the extracted ion currents indicate that in samples with both cofactors (i.e., UDP-GA and UDP-Glc), metabolism of *S*-Ibu was faster than that of *R*-Ibu. It was interesting to note that Ibu-AG was also present in a very low amount in samples with UDP-Glc (data not shown), which indicates the presence of a (albeit low) UGDH activity in the microsome preparation. In samples with UDP-Gal and in control reactions (without a cofactor), essentially no Ibu metabolites were detected. A scheme of the glucuronidation and glucosidation reactions of both Ibu isomers is shown in Fig. 2.

Production of Ibu-AG and Ibu-Glc by Recombinant Fission Yeast Strains That Overexpress Different Combinations of UGT2B7 and UGDH. We previously showed that the fission yeast *S. pombe* is a suitable organism for the functional expression of human UGTs (Dragan et al., 2010). Because human UGT2B7 exhibits high activity toward Ibu (Sakaguchi et al., 2004; Kuehl et al., 2005) and, moreover, is able to use UDP-Glc as a cofactor for the production of a glucoside (Tang et al., 2003), we cloned UGT2B7-expressing fission yeast strains in the study presented here to examine their ability for Ibu glucosidation. Because the two polymorphic variants UGT2B7*1 and UGT2B7*2 have been shown to possess varying activities against some substrates (Thibaudeau et al., 2006; Belanger et al., 2009), we included them both in this work. Fission yeast strain NCYC2036 was transformed with the expression plasmids pCAD1-UGT2B7*1 and pCAD1-UGT2B7*2 to yield the new strains DB7 and DB4, respectively (all strains are listed in Table 1). Because (in contrast to UDP-Glc and UDP-Gal) there is no endogenous UDP-GA production in *S. pombe* (Dragan et al., 2010), these two strains lack the potential of glucuronide formation. For control purposes, both strains were then transformed with the plasmid pREP1-UGDH (Dragan et al., 2010) to yield DB17 (from DB7) and DB14 (from DB4). Because UGDH catalyzes the production of UDP-GA from UDP-Glc, these latter strains are capable of self-sufficient glucuronide production. In addi-

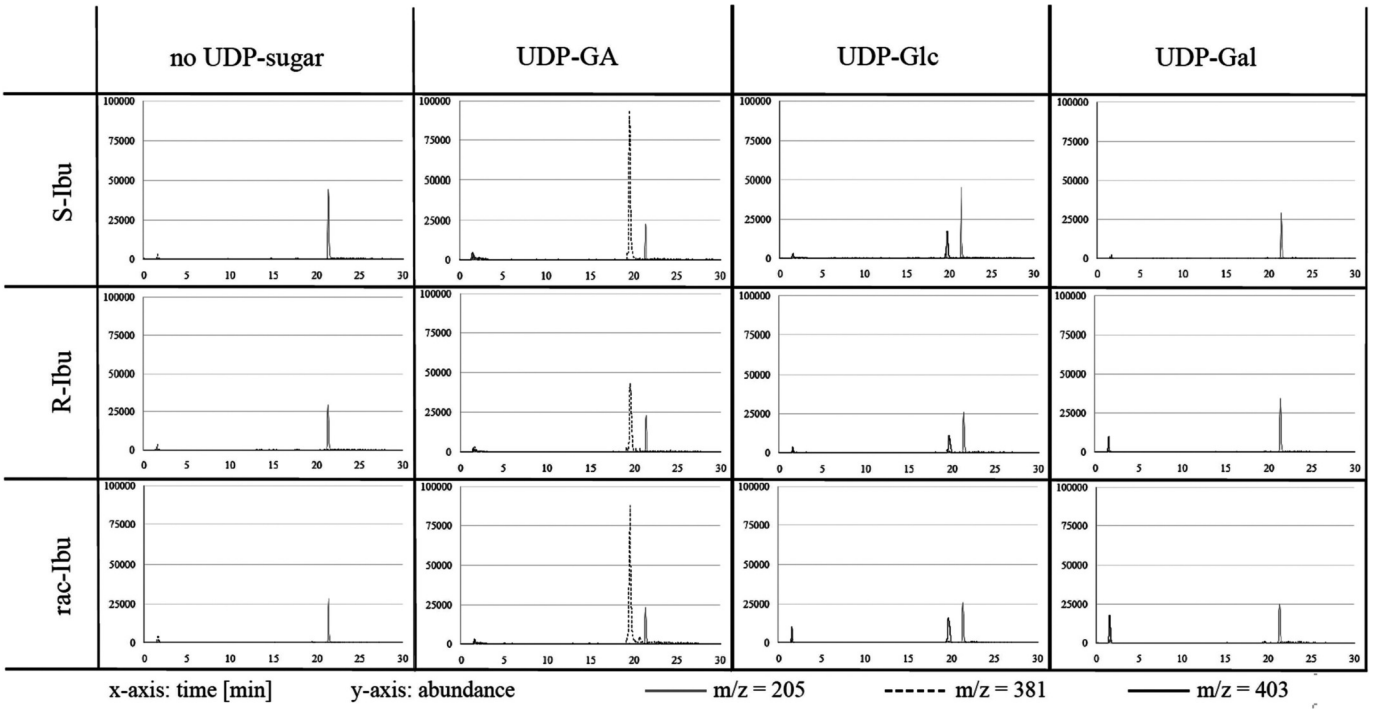

FIG. 1. Extracted ion currents derived from incubations of HLMs with *S*-Ibu, *R*-Ibu, and *rac*-Ibu with either no UDP-sugar, UDP-GA, UDP-Glc, or UDP-Gal as indicated. Ibu, $[M-H]^- \rightarrow m/z = 205$; Ibu-AG, $[M-H]^- \rightarrow m/z = 381$; and Ibu-Glc, $[M+Cl]^- \rightarrow m/z = 403$. All experiments were done in triplicate, and data from one experiment each are shown exemplarily.

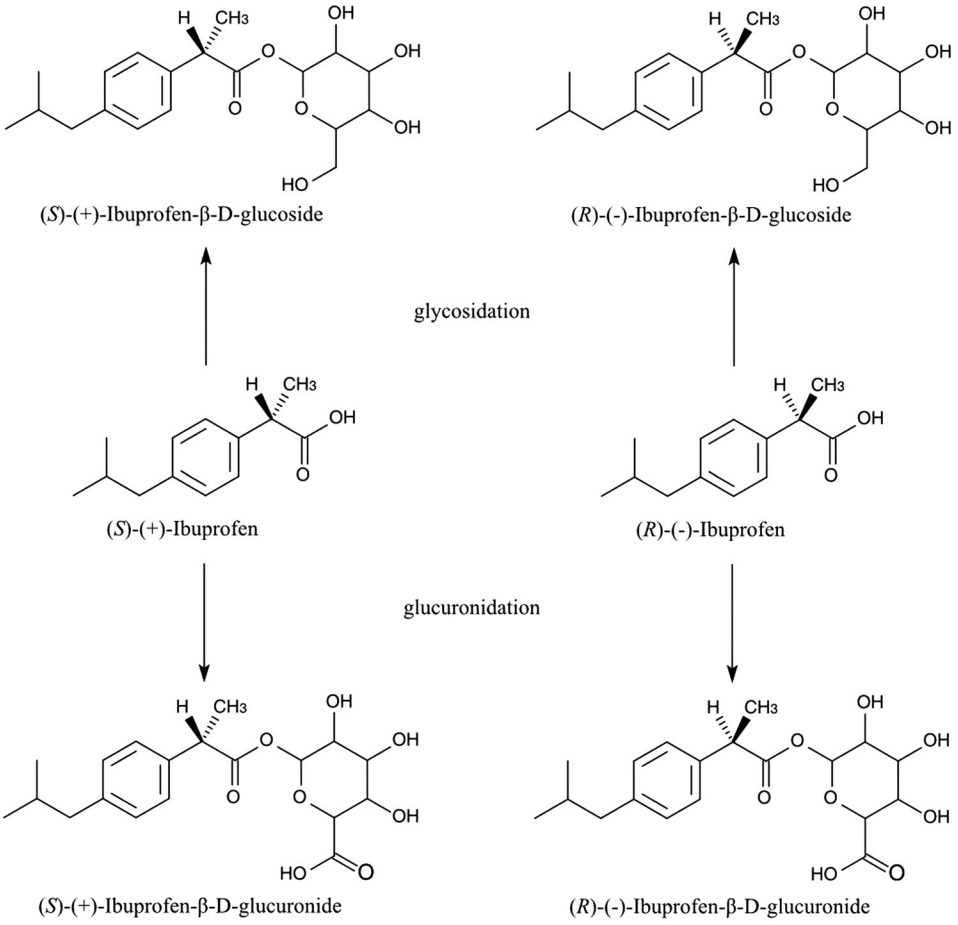

FIG. 2. Scheme of the glucuronidation and glucosidation reactions of *S*-Ibu and *R*-Ibu, respectively.

tion, UGT double-expressing strains were generated by transformation of strain DB7 with the plasmid pREP1-UGT2B7*1 and of DB4 with the plasmid pREP1-UGT2B7*2, respectively, to yield strains DB63 and DB62. All newly created strains were used for whole-cell biotransformation experiments with the substrates *S*-Ibu, *R*-Ibu, or *rac*-Ibu (Table 2). As expected, the parental strain NCYC2036 did not produce any Ibu metabolites and the single expressor strains DB4 and DB7 (containing UGT2B7*2 and UGT2B7*1, respectively) produced Ibu-Glc but not Ibu-AG because of a lack of UDP-GA. It was interesting to note that the Ibu-Glc production rates for the different substrate enantiomers differed significantly, with *S*-Ibu again being much more readily converted than *R*-Ibu. Furthermore, in almost all cases the two UGT2B7*1 expressing strains showed an almost doubled biotransformation rate as compared with the UGT2B7*2 expressors. In addition, the UGT double-expressing strains DB62 and DB63 did not display enhanced reaction rates in comparison to the parental single expressing strains DB4 and DB7 (data not shown), indicating that the intracellular UGT levels were not rate-limiting under these conditions. In line with expectations, the UGDH coexpressing strains DB14 and DB17 were able to produce Ibu-AG and Ibu-Glc, and biotransformation of *S*-Ibu was again preferred over *R*-Ibu. However, there was no consistent picture with respect to the relative formation of Ibu-AG versus Ibu-Glc, with glucuronide production being preferred over glucoside production in some instances but not in others.

Identification of the Fission Yeast UGPase *fyu1*. We speculated that an increase in intracellular UDP-Glc levels would enhance UGT-dependent glucoside production by whole-cell biotransformation in fission yeast. UDP-Glc production depends on UGPases, a family of enzymes that catalyze the balanced reaction of the formation of UDP-Glc and pyrophosphate from UTP and glucose-1-phosphate and that were identified in many organisms (Thoden and Holden, 2007). In the genome of fission yeast, there are two putative UGPase homologs with the systematic designations SPCC794.10 and SPCC1322.04, respectively (Wood et al., 2002). We amplified the coding sequences of both genes from strain NCYC2036 and cloned each of them into the expression plasmid pREP1. Strain DB4 was then transformed with pREP1-SPCC794.10 to obtain strain DB64 and with pREP1-SPCC1322.04 for strain DB65. In the same way, DB7 was transformed with pREP1-SPCC794.10 for strain DB66 and with pREP1-SPCC1322.04 for strain DB67. Thus, these strains each co-express a human UGT2B7 isoform and one of the two fission yeast UGPase homologs. Whole-cell biotransformations of these strains with Ibu (Fig. 3) showed that overexpression of SPCC794.10 did not lead to an enhanced Ibu-Glc production rate for any of the substrates in strains DB64 and DB66 as compared with their parental strains DB4 and DB7, respectively. Thus, these data do not support the notion that SPCC794.10 is an UGPase. In contrast, overexpression of SPCC1322.04 led to a significant increase in the production of Ibu-Glc from *S*-Ibu by strain DB67 in comparison to the parental strain DB7, whereas for strain DB65 no enhanced Ibu-Glc production could be detected (probably because of the weaker activity of UGT2B7*2 for this reaction). These data suggest that only one of the two putative fission yeast UGPase homologs—SPCC1322.04—indeed displays this activity. We therefore suggest to name it *fyu1* for *f*ission *y*east UGPase 1.

Production of Stable Isotope-Labeled Ibu-AG and Ibu-Glc. The availability of stable isotope-labeled reference standards is expedient for the sensitive quantification of metabolites in biological matrices

TABLE 2

Ibu-AG and Ibu-Glc formation rates in whole-cell biotransformations using the substrates R-Ibu, S-Ibu, or rac-Ibu and fission yeast strains expressing enzymes as indicated

S. pombe Strain	UGT	Cofactor Generating Enzyme	*R*-Ibu		*S*-Ibu		*rac*-Ibu	
			Ibu-AG	Ibu-Glc	Ibu-AG	Ibu-Glc	Ibu-AG	Ibu-Glc
			nmol/(g_{dw} · d)	*nmol/(g_{dw} · d)*	*nmol/(g_{dw} · d)*	*nmol/(g_{dw} · d)*	*nmol/(g_{dw} · d)*	*nmol/(g_{dw} · d)*
NCYC 2036			0	0	0	0	0	0
DB4	UGT2B7*2		0	156.7 ± 12.3	0	295.5 ± 20.8	0	226.8 ± 60.8
DB7	UGT2B7*1		0	346.8 ± 28.0	0	559.8 ± 49.6	0	429.6 ± 143.5
DB14	UGT2B7*2	UGDH	77.1 ± 3.0	109.3 ± 42.7	368.1±142.4	233.2 ± 65.2	437.7 ± 79.9	141.4 ± 41.0
DB17	UGT2B7*1	UGDH	163.2 ± 46.4	222.2 ± 82.4	679.7±393.2	262.9 ± 182.1	389.6 ± 200.6	328.6 ± 62.3

by LC-MS and thus facilitates the toxicokinetic monitoring of metabolites during nonclinical safety studies. We have previously demonstrated that cultivation of UGT-expressing fission yeast strains in media containing [$^{13}C_6$]glucose is a convenient method of producing $^{13}C_6$-labeled glucuronides (Dragan et al., 2010). In this study, whole-cell biotransformations were done using the substrate *S*-Ibu and strains DB14 and DB17 for the production of *S*-Ibu-[$^{13}C_6$]AG and strain DB67 for the production of *S*-Ibu-[$^{13}C_6$]Glc, respectively (Fig. 4). The comparison of the LC-MS analysis of the extracted ion currents of nonlabeled (Fig. 4, a and d) and 6-fold labeled metabolites (Fig. 4, b and e), as well as the respective fragmentation spectra (Fig. 4, c and f), unambiguously shows the successful formation of *S*-Ibu-[$^{13}C_6$]AG and *S*-Ibu-[$^{13}C_6$]Glc, respectively.

Discussion

Although glucuronide formation is a major reaction in mammalian phase II metabolism, a significant number of glucoside metabolites has also been identified, including the *O*-acyl glucoside of an endothelin ET_A antagonist (Tang et al., 2003) and the *N*-glucoside of bromfenac (Kirkman et al., 1998). In addition, the formation of bile acid *O*-glucosides by human liver microsomes and their occurrence in human urine has been known for a long time, whereas acyl galactosides of cholic acid and deoxycholic acid were described more recently (Goto et al., 2005). Thus, it was the aim of this study to investigate the possible formation of acyl glucoside metabolites of Ibu. Biotransformation experiments with HLMs demonstrated the production of Ibu-Glc in the presence of the cofactor UDP-Glc (Fig. 1). As expected, Ibu-AG was produced in the presence of UDP-GA, whereas traces of it could also be detected in all incubations with UDP-Glc, in two incubations with UDP-Gal and one incubation without cofactor (out of nine incubations each), respectively. The detection of Ibu-AG in incubations with UDP-Glc is most likely caused by remaining UGDH activity in the microsome preparation, whereas in samples with UDP-Gal detection of Ibu-AG correlated with that of Ibu-Glc, indicating that in those two samples two further reactions took place. First, UDP-Gal was converted by UDP-Gal-4-epimerase to UDP-Glc and subsequently yielded Ibu-Glc; second, part of the UDP-Glc was converted by UGDH to UDP-GA and allowed the production of Ibu-AG. Unfortunately, quantitative analysis of the Ibu-Glc formation was not possible because of further compounds in the reaction mixture that contributed to the UV signals of the targets to an unknown extent (data not shown). Still, these data unambiguously demonstrate the conversion of *R*-Ibu and *S*-Ibu to their respective acyl glucosides by human enzymes.

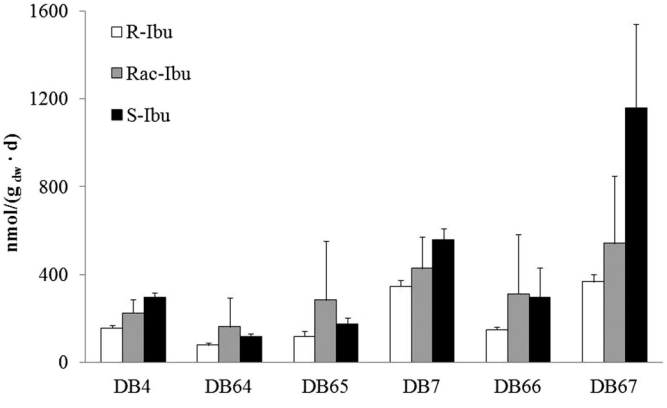

FIG. 3. Comparison of the Ibu glucosidation activities of fission yeast strains DB64, DB65, DB66, and DB67 with their parental strains. Data shown were calculated from at least three independent experiments for each combination of strain and substrate and S.D. are indicated.

Because it was known that human UGT2B7 can metabolize Ibu (Sakaguchi et al., 2004; Kuehl et al., 2005) and produce glucosides from other xenobiotics (Tang et al., 2003), it was reasonable to assume a participation of this enzyme in Ibu-Glc production. To verify this assumption, we adapted our previously established UGT expression system (Dragan et al., 2010) to the functional expression of the two polymorphic variants UGT2B7*1 and UGT2B7*2. Recombinant fission yeast strains that express one of these variants were found to be capable of producing Ibu-Glcs, whereas upon coexpression of human UGDH production of Ibu-Glcs and Ibu-AGs was observed (Table 2). The data clearly show a preference of both UGT2B7 isoforms for the isomer *S*-Ibu as a substrate for glucosidation and glucuronidation reactions, which is in agreement with previous studies in which a preferred glucuronidation of *S*-Ibu was shown in vivo (Lee et al., 1985; Tan et al., 2002) and for immobilized HLM proteins in vitro (el Mouelhi et al., 1987). However, to complicate matters, it is known that a conversion of *R*-Ibu to *S*-Ibu is accomplished by 2-arylpropionyl-CoA epimerase (Shieh and Chen, 1993; Reichel et al., 1997). A bioinformatic search did not reveal homologs of this enzyme in the genome of *S. pombe* (data not shown), but nevertheless it cannot be excluded that such a reaction might also occur in fission yeast. In fact, such an effect might contribute to the rather large S.D. that we observed in some of the biotransformations. In addition, an endogenous UDP-Gal-4-epimerase homolog has been described (Suzuki et al., 2010), and thus, a production of ibuprofen acyl galactoside can also not completely be ruled out, but it appears to be unlikely in view of the HLM results described above. It is interesting to note that a comparison of the activities of strains DB14 and DB17 with their parental strains DB4 and DB7 shows that in most cases the presence of UDP-GA does not significantly inhibit the Ibu-Glc production rate, indicating no strong preference of the enzymes for this cofactor. The one exception to this observation is the production of *S*-Ibu-Glc by UGT2B7*1, which upon UGDH coexpression is reduced to less than half of its former value. In this connection it is interesting to note that another study even observed a preference for UDP-Glc as a cofactor for a UGT2B7-dependent glucosidation (Tang et al., 2003). It will be

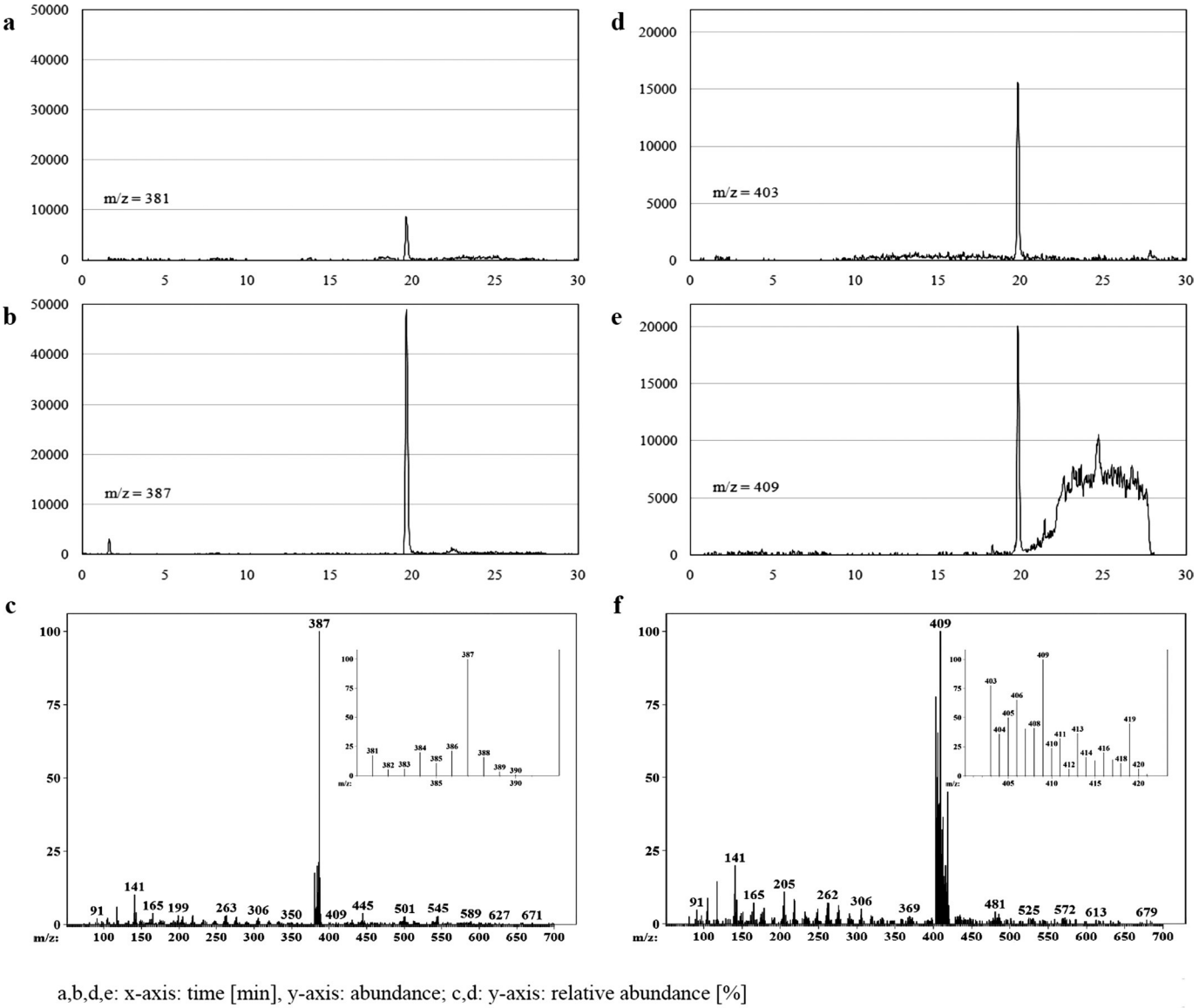

FIG. 4. Production of isotope-labeled *S*-Ibu-AG (a–c) using strain DB14 and stable isotope-labeled *S*-Ibu-Glc (d–f). Extracted ion currents of LC-MS analysis of a whole-cell biotransformation with strain DB14 and the substrate *S*-Ibu show the pseudomolecular ion of unlabeled *S*-Ibu-AG (a) and of *S*-Ibu-[$^{13}C_6$] AG (b). The mass spectrum at retention time t = 19.611 min is given in (c). Extracted ion currents of LC-MS analysis of a whole-cell biotransformation with strain DB67 and the substrate *S*-Ibu show the chlorine adduct $[M+Cl]^-$ of unlabeled *S*-Ibu-Glc (d) and of *S*-Ibu-[$^{13}C_6$]Glc (e). The mass spectrum at retention time t = 19.817 min is given in f.

interesting to see whether more examples of human UGTs preferring to convert some substrates with other UDP-sugars than UDP-GA will be presented in the future.

The findings of this study naturally raise the question of the physiological significance of UGT2B7-dependent acyl glucoside production. Although it is, according to current knowledge, less common than AG formation, acyl glucoside production as such appears to be relevant for endogenous substances as well as drug molecules (Stachulski, 2011). Furthermore, other groups have demonstrated before that human UGT2B7 may use UDP-Glc as a cofactor for the in vitro production of various glucosides (Mackenzie et al., 2003; Tang et al., 2003; Toide et al., 2004). Microarray data show that there are many human tissues that express UGDH to various extents (with strong signals in liver and colon), whereas UGT2B7 is predominantly expressed in liver and kidney (Yanai et al., 2005; Dezso et al., 2008). Thus, it is conceivable to envision a metabolic situation in a certain tissue (e.g., kidney) where there is strong UGT2B7 activity together with a significant level of UDP-Glc but low availability of UDP-GA. Such a situation would be expected to favor UGT2B7-dependent glucoside production. Finally, the example of the N-glucosidation of the aldose reductase inhibitor (*R*)-(−)-2-(4-bromo-2-fluorobenzyl)-1,2,3,4-tetrahydropyrrolo[1,2-α]pyrazine-4-spiro-3′-pyrrolidine-1,2′,3,5′-tetrone (AS-3201) demonstrated that UGT-dependent glucosidation might even take place in the presence of UDP-GA because some UGT isoenzymes (including UGT2B7) only catalyzed the glucosidation but not the glucuronidation reaction of this substrate (Toide et al., 2004). Taken together, all of this evidence may be interpreted in a way that allows for UGT2B7-dependent acyl glucoside production as a rather normal physiological process. Whether these metabolites are readily detected in blood or urine samples is another matter; in contrast to AGs, acyl glycosides do not per se contain an anion moiety and it is reasonable to assume that the dynamics of these conjugates differ from those of the respective glucuronides because in general they should differ in their ligand properties toward organic anion-transporting polypeptides or multidrug resistance-associated proteins.

With respect to Ibu metabolism, the pharmacological potency of the newly described Ibu-Glcs remains obscure, and it is conceivable that at least some of the phase I metabolites of Ibu give rise to additional acyl glucoside metabolites. A prediction of the properties of these molecules is difficult because it has been recently shown that apparently small changes in the aglycon structure may have unexpected consequences (Iddon et al., 2011). Therefore, further studies are needed to investigate the acyl migration, the hydrolysis rate, and the protein binding capacities of all of these metabolites.

From a biotechnological point of view, efficient metabolite production systems are desired to allow for the convenient production of those metabolites that are tedious to synthesize by chemical means (Zöllner et al., 2010). For the purpose of enhancing UGT2B7 biotransformation activity in our whole-cell system, we first constructed fission yeast strains that harbor two UGT expression units; however, the use of these strains did not lead to an increase in product formation as compared with the parental single-expressing strains DB4 and DB7 (data not shown). However, it stood to reason that an increase in intracellular UDP-Glc levels would facilitate UGT-dependent glucoside production. In principle, this aim might be pursued either by coexpression of a foreign (e.g., human) UGPase or by overexpression of an endogenous UGPase. In this study it was decided to attempt the latter because two putative UGPase homologs of fission yeast had already been identified by the *S. pombe* genome sequencing project (Wood et al., 2002). Expression plasmids containing either one of the two sequences were cloned and used to transform strains that recombinantly express UGT2B7*1 or UGT2B7*2, respectively, to yield the new strains DB64 to DB67 (Table 1). It was observed that one of two proteins—which we suggest to name *fyu1*—was able to significantly enhance the Ibu-Glc reaction rate, albeit only in combination with UGT2B7*1 (Fig. 3). This result suggests that, in addition to cofactor availability, UGT2B7 polymorphisms also influence the production rate of glucosides. It would be interesting to purify both proteins and determine their cofactor binding kinetics; however, these experiments were out of the scope of the study presented here. Still, it can be predicted that such an analysis will result in marked differences between the two variants.

Because the availability of stable isotope-labeled metabolites is desirable for the LC-MS analysis of biological matrices, we used our previously established general labeling technique (Dragan et al., 2010) using [$^{13}C_6$]glucose as a metabolic precursor that is efficiently converted within the *fyu1* expressing fission yeast strains to $^{13}C_6$-labeled UDP-Glc, which in turn serves as the cofactor for the UGT2B7-dependent production of *S*-Ibu-[$^{13}C_6$]Glc (Fig. 4). Likewise, when using UGDH-expressing fission yeast strains, [$^{13}C_6$]glucose is converted to $^{13}C_6$-labeled UDP-GA and thus allows for the formation of *S*-Ibu-[$^{13}C_6$]GA. It is expected that the availability of these stable isotope-labeled metabolites will be helpful for further studies in this direction.

Acknowledgments

We thank Eva Johannes for technical assistance.

Authorship Contributions

Participated in research design: Bureik and Buchheit.
Conducted experiments: Buchheit and Schmitt.
Contributed new reagents or analytic tools: Dragan and Buchheit.
Performed data analysis: Buchheit and Dragan.
Wrote or contributed to the writing of the manuscript: Buchheit and Bureik.

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Buchheit D, Schmitt EI, Bischoff D, Ebner T and Bureik M: *S*-Glucuronidation of 7-mercapto-4-methylcoumarin by human UDP glycosyltransferases in genetically engineered fission yeast cells. *Biol Chem.* 2011 Dec; 392(12): 1089-95.

Biol. Chem., Vol. 392, pp. 1089–1095, December 2011 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/BC-2011-194

S-Glucuronidation of 7-mercapto-4-methylcoumarin by human UDP glycosyltransferases in genetically engineered fission yeast cells

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Abstract

Human UDP glycosyltransferases (UGTs) play an important role in xenobiotic detoxification. They increase the solubility of their substrates by adding a sugar moiety (such as glucuronic acid) to different functional entities (such as hydroxyl groups). The aim of this study was to investigate how glucuronidation of a standard substrate is affected by a change of the heteroatom at the conjugation site. For this purpose, we compared the *in vitro* glucuronidation rates of 4-methylumbelliferone and 7-mercapto-4-methylcoumarin, respectively. Human liver microsomes catalyzed the *S*-glucuronidation of 7-mercapto-4-methylcoumarin almost as efficient as the *O*-glucuronidation of 4-methylumbelliferone. When testing isoenzyme specificity by whole cell biotransformation with fission yeast strains that recombinantly express all 19 human members of the UGT1 and UGT2 families, it was found that 13 isoenzymes were able to glucuronidate 7-mercapto-4-methylcoumarin, with five of them being specific for this substrate and the other eight also converting 4-methylumbelliferone under these conditions. The remaining six UGTs did not accept either substrate. Out of the eight isoenzymes that glucuronidated both substrates, four catalyzed both reactions approximately to the same extent, while three displayed higher conversion rates towards 4-methylumbelliferone and one preferred 7-mercapto-4-methylcoumarin. These data suggest that 7-mercapto-4-methylcoumarin is a convenient new standard substrate for monitoring *S*-glucuronidation.

Keywords: coumarin; fission yeast; *S*-glucuronide; thiol; UGT; whole cell biotransformation.

Introduction

Human uridine diphospho glycosyltransferases (UGTs) are a class of enzymes that is best known for catalyzing the transfer of glucuronic acid from uridine diphosphate (UDP) glucuronic acid (UDPGA) to xenobiotics as well as endogenous substrates, thereby increasing their hydrophilic properties and facilitating their elimination from the body (Bock, 2010; Guillemette et al., 2010). This function makes them the main enzymes of phase II metabolism, a role illustrated by the fact that they contributed to the clearance of approximately 10% of the top 200 drugs prescribed in the USA in 2002 (Williams et al., 2004). UGT homologs have been identified in mammals, fish, worm, viruses, yeast, plants, bacteria and insects (Mackenzie et al., 1997; Bock, 2003). In humans, there are four UGT families (UGT1, UGT2 with subfamilies A and B, UGT3, and UGT8, respectively) with a total of 22 single isoforms. The 19 members of the UGT1 and UGT2 families typically prefer UDPGA as cofactor and are therefore often referred to as UDP-glucuronosyltransferases (Bock, 2003; Mackenzie et al., 2005). Human UGT3A1 recently has been shown to perform *N*-acetylglucosaminidations (Mackenzie et al., 2008) and UGT3A2 uses UDP-glucose and UDP-xylose as cofactor for the conjugation of both endogenous and exogenous substrates (Meech and Mackenzie, 2010; Mackenzie et al., 2011). UGT8 uses UDP-galactose to catalyze the synthesis of galactosylceramide (Bosio et al., 1996). The known substrates of the human UGTs that participate in phase II metabolism are structurally very diverse and, moreover, the sugar moieties may be transferred to multiple functional sites, such as amine, carbonyl, carboxyl, hydroxyl, and thiol groups (Tukey et al., 2000); even *C*-glucuronides have been reported, although rarely (Richter et al., 1975; Kerdpin et al., 2006). Although thiol groups are not a very rare occurrence in drug compounds, the number of reports on *S*-glucuronides is limited. Some examples are the metabolites of dalcetrapib (Kuhlmann and Heinig, 2011), HMR1098 (Ethell et al., 2003), AR-C133611XX (Martin et al., 2003), 2-mercaptobenzothiazole (Fukuoka et al., 1995) or malotilate (Nakaoka et al., 1989). A study aimed at the identification of UGT isoforms responsible for a specific *S*-glucuronidation reaction showed that all of the four tested candidates (UGT1A1, 1A9, 1A6, and 2B7, respectively) were able to catalyze it, although at significantly varying rates (Ethell et al., 2003), thus indicating that *S*-glucuronidation is not a specific function performed by a single UGT isoenzyme. However, a systematic approach involving all 19 members of the human UGT1 and UGT2 families had not yet been done. It was the aim of this study to compare the *O*-glucuronidation of the standard substrate 4-methylumbelliferone (4MU) with the *S*-glucuronidation of its thiol analog 7-mercapto-4-methylcoumarin (7M4MC; Figure 1). For this purpose, we used human liver microsomes (HLMs) and recombinant fission yeast

strains that co-express human UDP glucose dehydrogenase (UGDH) and one of the 19 human UGTs, and we demonstrate that the majority of human UGTs are capable of catalyzing the *S*-glucuronidation of 7M4MC.

Results

Glucuronidation of 4MU and 7M4MC by human liver microsomes

For a global comparison, glucuronidation activity of HLMs towards either 4MU or 7M4MC was monitored in the presence of UDPGA. 4MU of course is a standard UGT substrate (Uchaipichat et al., 2004) and its biotransformation to 4MU glucuronide (4MUG) proceeded efficiently with a total conversion of 99.3±0.1% under the test conditions. The thiol analog of 4MU, 7M4MC, was also efficiently glucuronidated; the product gave an absorption peak at 320 nm which contained the mass for the pseudomolecular ion $[M\text{-}H]^-$ of 7M4MC glucuronide (7M4MCG), *m/z*=367 (Figure 2). Using the same conditions, the total turnover was 82.3±1.3%. When using the alternative cofactors UDP glucose or UDP galactose instead of UDPGA, the formation of reaction products was also observed (data not shown), but this line of investigation was not pursued further.

Glucuronidation of 7M4MC using recombinantly expressed human UGTs

Having established that at least some human UGTs are able to glucuronidate 7M4MC, we next sought to identify the relevant isoforms. We recently reported the construction of an efficient UGT expression system using recombinant fission yeast of the species *Schizosaccharomyces pombe*, which is based on the co-expression of human UGDH (which produces the essential cofactor UDPGA not present in wild-type fission yeast) and UGT enzymes (Dragan et al., 2010). So far, we reported the functional expression of ten human and one rat UGT isoform using this system (Dragan et al., 2010; Buchheit et al., 2011). In this study, the remaining nine members of the human UGT1 and UGT2 families (UGT1A3, UGT1A4, UGT1A5, UGT2A2, UGT2A3, UGT2B4, UGT2B10, UGT2B11, and UGT2B28, respectively) were also expressed in fission yeast (all strains are listed in Table 1). The glucuronidation activity of all 19 UGT isoforms towards the substrates 4MU and 7M4MC was determined by whole cell biotransformation at shaking flask scale and subsequent HPLC analysis (Table 2). For strains showing glucuronide formation, product identity was confirmed by LC-MS analysis. In nine cases, 4MUG production rates had already been reported previously as indicated (Dragan et al., 2010). Six out of 19 UGT isoenzymes did not accept either substrate under these conditions, eight UGTs metabolized both and five converted only one substrate, 7M4MC. Thus, a total of 13 *S*-glucuronidation and eight *O*-glucuronidation reactions were observed, respectively. To the best of our knowledge, these data make 7M4MC one of the most widely accepted human UGT substrates described to date. For both substrates, biotransformation rates varied significantly from less than 0.1 to more than 5 µmol/(g dw×d), an observation that correlates well with previous data on 4MU glucuronidation activities of different human UGT isoenzymes (Uchaipichat et al., 2004). Out of the eight isoenzymes that did work on both substrates, three displayed higher conversion rates towards 4MU, while one acted faster on 7M4MC and four catalyzed both reactions approximately to the same extent.

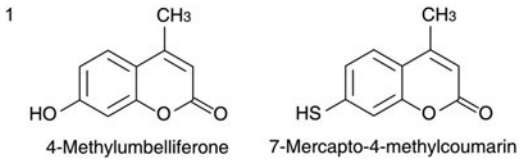

Figure 1 Compounds used in this study.

Generation of stable isotope labeled 7M4MCG

By use of $[^{13}C]_6$-labeled glucose, we previously demonstrated the production of stable isotope labeled *O*-glucuronides, with the sugar moiety being either attached to a hydroxyl

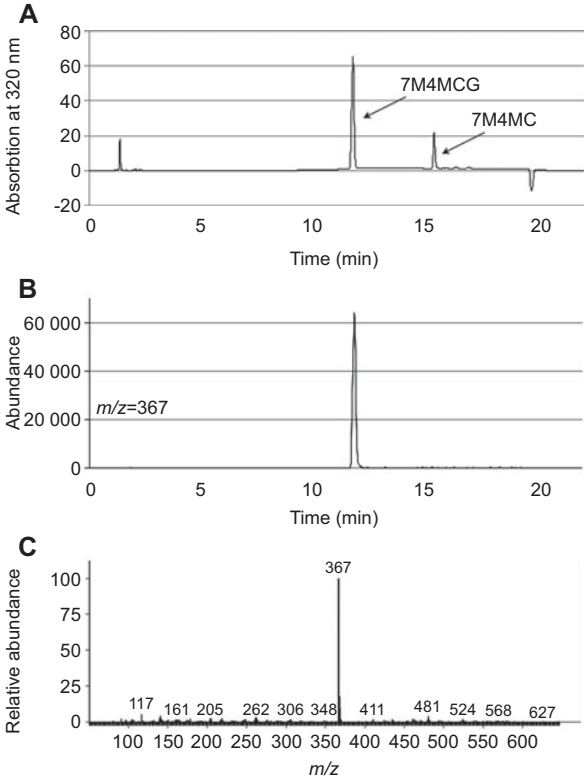

Figure 2 Production of 7M4MCG by HLMs.
(A) HPLC chromatogram of an incubation of HLMs with the substrate 7M4MC and the cofactor UDPGA. (B) Extracted ion current: 7M4MCG $[M\text{-}H]^- \rightarrow m/z$=367 (C) Mass spectrum at t_{Ret}=11.826. The experiment was done in triplicate, data from one experiment are shown exemplarily.

Table 1 Fission yeast strains used in this study.

Strain	Parental strain	Expressed protein(s)	Genotype	Reference
NCYC2036	–	–	*h⁻ ura4-D18*	(Losson and Lacroute, 1983)
DB21	NCYC2036	UGT1A3	*h⁻ ura4-D18 leu1::pCAD1-UGT1A3*	This study
DB2	NCYC2036	UGT1A4	*h⁻ ura4-D18 leu1::pCAD1-UGT1A4*	This study
DB22	NCYC2036	UGT1A5	*h⁻ ura4-D18 leu1::pCAD1-UGT1A5*	This study
DB27	NCYC2036	UGT2A2	*h⁻ ura4-D18 leu1::pCAD1-UGT2A2*	This study
DB28	NCYC2036	UGT2A3	*h⁻ ura4-D18 leu1::pCAD1-UGT2A3*	This study
DB29	NCYC2036	UGT2B4	*h⁻ ura4-D18 leu1::pCAD1-UGT2B4*	This study
DB30	NCYC2036	UGT2B10	*h⁻ ura4-D18 leu1::pCAD1-UGT2B10*	This study
DB31	NCYC2036	UGT2B11	*h⁻ ura4-D18 leu1::pCAD1-UGT2B11*	This study
DB34	NCYC2036	UGT2B28	*h⁻ ura4-D18 leu1::pCAD1-UGT2B28*	This study
DB11	DB1	UGT1A1, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A1/pREP1-UGDH*	(Dragan et al., 2010)
DB41	DB21	UGT1A3, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A3/pREP1-UGDH*	This study
DB12	DB2	UGT1A4, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A4/pREP1-UGDH*	This study
DB42	DB22	UGT1A5, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A5/pREP1-UGDH*	This study
DB43	DB23	UGT1A6, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A6/pREP1-UGDH*	(Dragan et al., 2010)
DB44	DB24	UGT1A7, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A7/pREP1-UGDH*	(Dragan et al., 2010)
DB45	DB25	UGT1A8, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A8/pREP1-UGDH*	(Dragan et al., 2010)
CAD203	CAD200	UGT1A9, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A9/pREP1-UGDH*	(Dragan et al., 2010)
DB46	DB26	UGT1A10, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT1A10/pREP1-UGDH*	(Dragan et al., 2010)
DB13	DB3	UGT2A1, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2A1/pREP1-UGDH*	(Dragan et al., 2010)
DB47	DB27	UGT2A2, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2A2/pREP1-UGDH*	This study
DB48	DB28	UGT2A3, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2A3/pREP1-UGDH*	This study
DB49	DB29	UGT2B4, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B4/pREP1-UGDH*	This study
DB17	DB7	UGT2B7, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B7/pREP1-UGDH*	(Buchheit et al., 2011)
DB50	DB30	UGT2B10, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B10/pREP1-UGDH*	This study
DB51	DB31	UGT2B11, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B11/pREP1-UGDH*	This study
DB52	DB32	UGT2B15, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B15/pREP1-UGDH*	(Dragan et al., 2010)
DB53	DB33	UGT2B17, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B17/pREP1-UGDH*	(Dragan et al., 2010)
DB54	BD34	UGT2B28, UGDH	*h⁻ ura4-D18 leu1::pCAD1-UGT2B28/pREP1-UGDH*	This study

(Dragan et al., 2010) or a carboxyl group (Buchheit et al., 2011). In the present study, we wanted to confirm that a similar strategy can also be used for the production of labeled *S*-glucuronides. For this purpose, fission yeast strain CAD203 was used in a whole cell biotransformation of 7M4MC at miniaturized scale. LC-MS analysis of the extracted ion currents of unlabeled (Figure 3A) and 6-fold labeled metabolites (Figure 3B), as well as the respective fragmentation spectra (Figure 3C), clearly show the successful formation of 7M4MC[^{13}C]$_6$G.

Discussion

Thiols are chemically and biochemically very active functional groups that, among other reactions, easily undergo oxidation in the presence of an electron acceptor to form sulfenic, sulfinic, and finally sulfonic acids or disulfides. Despite these properties, active pharmaceutical compounds that comprise thiol functions are not uncommon: cysteamine, tiopronin, D-penicillamine, captopril, mesna, *N*-acetylcysteine, thyreostats, and thiopurines are some of the most important examples. During phase II metabolism, thiol groups may be converted into *S*-glucuronides by UGT enzymes. Moreover, other sulfur functions may also yield *S*-glucuronides, either after prior phase I reactions or by direct conjugation, as in the case of the thiones AR-C133611XX (Martin et al., 2003) and tanaproget, where the *S*-glucuronide actually is a major metabolite (Keating et al., 2006). In addition, there are tetrahydrothienopyridine antithrombotic prodrugs (such as ticlopidine, clopidogrel, and prasugrel) that are intended to be metabolized *in vivo* into pharmacologically active thiol derivatives (Dansette et al., 2010). Thus, while conjugation reactions that take place at hydroxyl groups will almost certainly continue to receive most attention, the phase II metabolism of thiol groups is also of interest. It was the purpose of this study to compare the UGT-dependent glucuronidation of the well-known standard UGT substrate 4MU with its thiol analog 7M4MC (Figure 1). We first investigated the glucuronidation activity of HLMs towards both substrates and observed that they are metabolized with roughly comparable efficiency (Figure 2). Skellern and coworkers published a somewhat similar study almost 20 years ago, comparing the glucuronidation of 4-nitrophenol and 4-nitrothiophenol by guinea pig liver microsomes and observing that the *S*-glucuronidation reaction was significantly slower than the *O*-glucuronidation (Smith et al., 1992). Both studies show that the exchange of the hydroxyl function by a thiol group

Table 2 Biotransformation of 4MU and 7M4MC by recombinant *Schizosaccharomyces pombe* strains.

Strain	UGT	Product formation	
		4MU	7M4MC
MB163	None	–	–
DB11	UGT1A1	+[a]	++
DB41	UGT1A3	–	+
DB12	UGT1A4	–	–
DB42	UGT1A5	–	–
DB43	UGT1A6	++[a]	+
DB44	UGT1A7	+++[a]	+++
DB45	UGT1A8	+++[a]	+++
CAD203	UGT1A9	++++[a]	+++
DB46	UGT1A10	+++[a]	+++
DB13	UGT2A1	++++[a]	++
DB47	UGT2A2	–	+
DB48	UGT2A3	–	+
DB49	UGT2B4	–	–
DB17	UGT2B7	–	+
DB50	UGT2B10	–	–
DB51	UGT2B11	–	–
DB52	UGT2B15	++[a]	++
DB53	UGT2B17	–[a]	+
DB54	UGT2B28	–	–

[a]Data from Dragan et al. (2010). –, no product detected; +, product formation rate below 0.1 µmol/(g dw×d); ++, product formation rate between 0.1 and 0.5 µmol/(g dw×d); +++, product formation rate between 0.5 and 5 µmol/(g dw×d); ++++, product formation rate above 5 µmol/(g dw×d).

in a given compound does not preclude its glucuronidation but affects its rate of metabolism. Interestingly, in this study additional HLM experiments using the alternative cofactors UDP glucose or UDP galactose instead of UDPGA also indicated product formation with both substrates (data not shown). However, the investigation of glucoside and galactoside formation from 4MU or 7M4MC was out of the scope of the present study and thus no further experiments were done in this direction.

For the identification of the UGT isoforms that participate in 7M4MC glucuronidation, we made use of our recently established fission yeast system (Dragan et al., 2010). The existing set of ten human UGTs functionally expressed in this system was expanded by nine further isoenzymes to now encompass all 19 human members of the UGT1 and UGT2 families (Table 1). Somewhat surprisingly, the determination of the glucuronidation activity of all isoforms towards the substrates 4MU and 7M4MC revealed that the latter coumarin is actually metabolized by more human UGTs than the former (Table 2). More specifically, seven out of nine members of the UGT1 family, all three members of the UGT2A subfamily and three of the seven UGT2B isoforms were found to catalyze this *S*-glucuronidation reaction. In these experiments, not a single human UGT that acted on 4MU did not convert its thiol analog, although three displayed higher conversion rates towards the substrate with the hydroxyl group; one isoform showed the opposite preference and four catalyzed both reactions equally well.

It must be noted that the failure of some of UGT isoforms to glucuronidate 4MU in our system is at variance to earlier studies from other groups (Levesque et al., 2001; Uchaipichat et al., 2004; Barre et al., 2007; Sneitz et al., 2009). However, the data from the literature are also not consistent in all cases, and differences in experimental setup or assay conditions might be critical if very small activities are to be detected.

As expected, our whole cell biotransformation system could also be utilized for the production of a stable isotope-labeled *S*-glucuronide from $[^{13}C]_6$-labeled glucose (Figure 3), as shown previously for similarly labeled *O*-glucuronides and *O*-glucosides, respectively (Dragan et al., 2010; Buchheit et al., 2011). Other stable isotope-labeled *S*-glucuronides of drug compounds or candidates should be producible in the same way, and the availability of such standards should be helpful for the LC-MS analysis of drug metabolites in biological matrices.

As described above, there is no common tendency when comparing the *O*- and *S*-glucuronidation activities of the different UGT isoforms in this study. Considering the UGT reaction mechanism, this observation is somewhat surprising. Because acceptor-β-D-glucuronides are built from UDP-α-D-glucuronic acid, the catalysis mechanism of glucuronidation is assumed to be a S_N2 reaction. It is proposed that a base deprotonates the functional group at the site of glucuronidation, e.g. a -OH or -SH group, which increases the nucleophilicity of the charged heteroatom. This in turn performs a nucleophilic attack on the anomeric carbon of the glucuronic acid from UDPGA, which results in glucuronide formation (Ouzzine et al., 2003). The elements sulfur and oxygen are neighbors in the chalkogen group and therefore have somewhat similar physical properties. However, in general, sulfur has higher nucleophilicity because of its large size, which makes it more polarizable, and its lone pairs of electrons are readily accessible. Therefore, it could be speculated that the glucuronidation of a thiol group may be more likely than that of a hydroxyl group at the same position of a molecule. Even if this reasoning is not correct, one might at least assume that the replacement of one function by the other in a given molecule (as was done here) would affect its UGT-dependent biotransformation by all isoforms in the same way. However this was not observed, the data rather suggest that an extrapolation of the known glucuronidation activity towards a hydroxyl group in a given target molecule to a thiol group at the same position (or vice versa) is not possible.

It would be interesting to expand the approach of the present study by introducing further functional groups in the 7-position of 4-methylcoumarin, such as an amino group. Preliminary experiments in our lab showed that 7-amino-4-methylcoumarin is also metabolized by HLMs to its *N*-glucuronide, although to a significantly lesser extent than 4MU or 7M4MC (data not shown). Lewis and coworkers reported that an exchange of a thione group in the CXCR2 receptor antagonist AR-C133611XX by an amino group completely eliminated

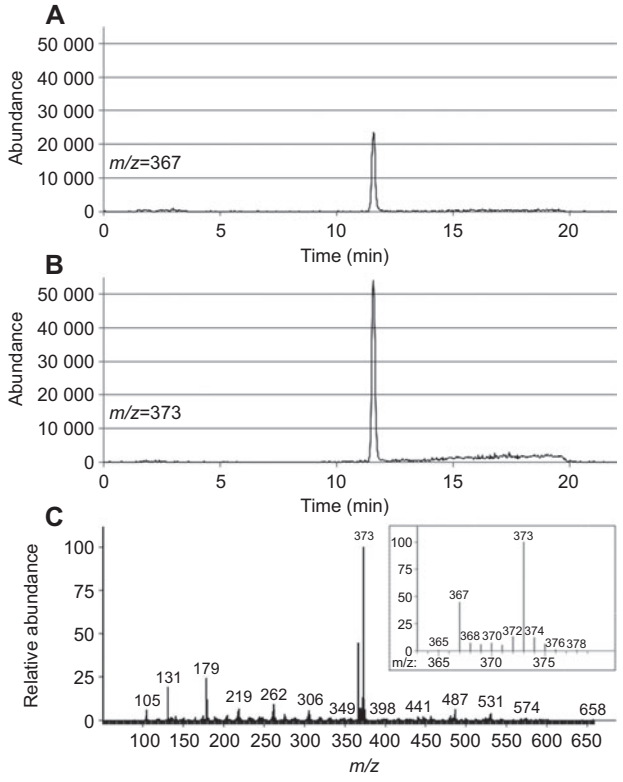

Figure 3 Production of isotope labeled 7M4MC[^{13}C]$_6$G using strain CAD203.
Extracted ion currents of LC-MS analysis show the pseudomolecular ion of unlabeled 7M4MCG (A) and of 7M4MC[^{13}C]$_6$G (B). The mass spectrum at t_{Ret}=11.586 min is given in (C).

the previously efficient glucuronidation reaction (Martin et al., 2003). Therefore, the replacement of a hydroxyl group by an amine function will probably have a more pronounced significant effect on glucuronidation than in the case of a thiol group; however, more investigations in this direction are needed before general conclusions can be drawn.

In summary, we here report the generation of the *S*-glucuronide of 7M4MC using human liver microsomes. We further expanded our whole cell biotransformation system based on UGT-expressing fission yeast strains to comprise all 19 human members of the UGT1 and UGT2 families. This system was shown in the past to be useful for the production of *O*-glucuronides (including acyl glucuronides) and *O*-glucosides, whereas here it was successfully employed for the production of a *S*-glucuronide and for a comparison of all human UGT isoforms in their thiol glucuronidation potential.

Materials and methods

Chemicals

7M4MC and 4MU were from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were either from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich. Methanol (HPLC grade) was from VWR (Leuven, Belgium).

In vitro metabolism of 4MU and 7M4MC using HLMs

Pooled HLMs (BD Biosciences, New York, NY, USA) were incubated using 4MU or 7M4MC as substrate. The total reaction volume was 100 µl. Final concentrations of *in vitro* incubations were: 0.5 mg/ml protein, 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$ and 200 µM substrate. Reactions were started by the addition of 2 mM UDPGA; samples were incubated for 18 h at 37°C and then mixed with an equal volume of acetonitrile. As a negative control incubations without cofactor were done. All experiments were done in triplicates.

Coding DNA sequences

The cDNA of human UGT1A4 was synthesized by Entelechon GmbH (Regensburg, Germany); UGT1A3, UGT1A5, UGT2A2, UGT2A3, UGT2B4, UGT2B10, UGT2B11, and UGT2B28, respectively, were synthesized by GeneArt GmbH (Regensburg, Germany). All cDNAs were optimized for expression in *S. pombe*.

Media and general techniques

We used general DNA manipulating methods as well as media and genetic methods for fission yeast as described previously (Sambrook and Rusell, 2001; Forsburg and Rhind, 2006). In addition, we used EMM containing 100 g/l glucose for biotransformation assays and EMM with 20 g/l [^{13}C]$_6$-glucose for the synthesis of isotope labeled glucuronides.

Construction of expression plasmids and fission yeast strains

Fission yeast strain construction was done following the strategy described previously (Dragan et al., 2010). Briefly, UGT cDNAs were cloned via NdeI and BamH I into the integrative vector pCAD1 (Dragan et al., 2005), which disrupts the *leu1* gene and contains an *ura4* marker. The correctness of all constructs was verified by automatic sequencing (MWG-Biotech, Ebersberg, Germany). The UGDH expression plasmid pREP1-UGDH has been described in the literature (Dragan et al., 2010). The vectors pCAD1 and pREP1 contain the strong endogenous *nmt1* promotor, which is downregulated by the presence of thiamine in the culture media (Maundrell, 1990). Therefore, transformed cells were grown on EMM dishes with 5 µM thiamine to allow better growth under repressed conditions. All strains used in this study are listed in Table 1. The newly created pCAD1-UGT plasmids were used to transform the parental *S. pombe* strain NCYC2036 (Losson and Lacroute, 1983) as described previously (Suga and Hatakeyama, 2005) to yield strains DB2, DB21, DB22, DB27, DB28, DB29, DB30, DB31, and DB34, respectively. Correct integration into the *leu1* locus was verified by selection of leucine auxotrophs on EMM dishes containing 5 µM thiamine without leucine. Subsequently, these new strains containing an integrated UGT expression cassette were each transformed as described previously (Okazaki et al., 1990) with pREP1-UGDH to yield strains DB12, DB41, DB42, DB47, DB48, DB49, DB50, DB51, and DB54, respectively, to yield UGT and UGDH co-expressing strains.

Biomass production

Liquid cultures were prepared as described previously (Dragan et al., 2010). Main cultures were incubated for 1 day in the case of NCYC2036 and for 4–5 days in the case of the UGT and UGDH co-expressing strains.

Whole cell biotransformation assay

Whole cell biotransformation assays were done as described previously (Dragan et al., 2010) using the substrates 4MU or 7M4MC as indicated at a final concentration of 500 µM. After the reaction, culture samples were frozen if necessary until sample preparation and analysis. All experiments were done in triplicates.

Sample preparation

Supernatants from cell suspensions from whole cell biotransformations with 4MU as substrate were thawed if frozen and centrifuged (10 min, 10 000 *g*, RT) and the supernatants were analyzed by HPLC. Cell suspensions from whole cell biotransformations with 7M4MU as substrate were thawed if frozen and diluted with 1:1 acetonitrile. Samples were centrifuged (5 min, 15 000 *g*, RT), the supernatant was centrifuged again (10 min, 15 000 *g*, RT), and the resulting supernatants were analyzed by HPLC and LC-MS.

Synthesis of isotope-labeled glucuronide metabolites

The synthesis of isotope-labeled 7M4MC[^{13}C]$_6$G was done as described in the literature (Buchheit et al., 2011) using the strain CAD203. Experiments were done in triplicates.

HPLC analysis

Analysis of 4MU and 4MUG was done as described in the literature (Dragan et al., 2010). For analysis of 7M4MC and 7M4MCG, a gradient using 0.1% acetic acid (A) and methanol (B) as mobile phases was used as follows. The initial mobile phase composition was 85% A and 15% B. B was increased linearly to 80% from 5 to 10 min, maintained for 4 min until initial conditions were reconstituted at 14 min. Those were maintained again until the end of the run at 18 min. The eluents were monitored at 320 nm. Quantification of 7M4MCG was done using a straight calibration line of 4MUG, prepared in a 1:1 mixture of mobile phase components (concentrations from 10 to 250 µM 4MUG).

Liquid chromatography mass spectrometry

7M4MC(G) samples were analyzed on an Agilent 1100 HPLC equipped with a G1315B diode array detector (DAD) and a G1946A single quadrupole mass spectrometer (MS). Components were separated on a Lichrospher RP 18 column (particle size 5 µm, column inner diameter 4 mm, length 125 mm) using water (mobile phase A) and methanol containing 0.1% formic acid (mobile phase B). The flow rate was 0.8 ml/min and the column temperature was 40°C. LC-MS settings were: flow profile 0 min to 6 min, 10% B, 6 min to 12 min, 10% to 90% B, 12 min to 17 min, 90% B, 17 min to 22 min, 10% B; runtime 22 min; injection volume 10 µl; detector 1 G1315B DAD, DAD range 200 nm to 600 nm, slit width 4 nm; detector 2 G1946A MSD, ionization ESI(-), mode scan; N_2 flow rate 12 l/min, nebulizer pressure 45 psig (0.07 bar), nebulizer temperature 350°C, capillary voltage 3.500 V, fragmentor voltage 50 V for 7M4MC(G). Quantification of labeled 7M4MCG was done using an external 100 µM 4MUG standard prepared in a 1:1 mixture of mobile phase components.

Acknowledgement

We would like to thank Eva Johannes for technical assistance.

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Received August 3, 2011; accepted August 23, 2011

4 Conclusions

UGTs are important enzymes for the detoxification and excretion of foreign compounds. The existence of active or disproportionate metabolites and a demand for reference standards require suitable glucuronide production technologies. Existing methods have their individual restrictions. A new system for the production of glucuronide was developed before the beginning of this work. It is based on fission yeast cells which recombinantly coexpress human UGT1A9 and human UGDH. Intact cells were shown to be able to produce UGT products in whole-cell biotransformations. In this work, for each of the remaining 18 human UGT isoforms as well as rat UGT1A7 a fission yeast expression strain was generated. Following pretests of the prior study using UGT1A9, yeast strains were generated with a single integrated gene copy of the *UGT* gene and multiple copies of UGDH on an autosomal plasmid. Applying this strain construction strategy which had been shown to result in high glucuronide production rates, the parental strain NCYC2036 (Losson and Lacroute, 1983) was at first transformed with one of the different *UGT* genes. Targeted integration of this first expression unit into the *leu1* locus yielded a selection marker for a second transformation of the resulting strains with an expression plasmid carrying the *UGDH* gene. For UGT2B7, the two main polymorphic variants (UGT2B7*1: H268; UGT2B7*2: Y268) were used in the yeast system due to several reasons; (I) UGT2B7 is one of the most important UGTs for drug metabolism (Williams et al., 2004), (II) the two polymorphic variants are distributed almost 50:50 in Caucasians (Bhasker et al., 2000) and (III) the polymorphic variants have been shown in several instances to have discrete glucuronidation rates (Bernard et al., 2006; Thibaudeau et al., 2006; Belanger et al., 2009). Therefore, it was interesting to have both enzymes available in the yeast system for comparison studies. A first publication introduced this new system based on the initial study using UGT1A9 performed by Călin-Aurel Drăgan and described the functional expression of a total of nine human and one rat UGT isoforms in fission yeast and the production of glucuronides by whole-cell biotransformations (Dragan et al., 2010). Until the end of this work, the functional expression of 15 out of 21 isoforms in fission yeast could be shown. The previous assumption that UDP-GA is not present in fission yeast could be confirmed by the fact that no glucuronide production was observed with strains expressing only a UGT isoform and no UGDH. Interestingly, cell growth is significantly inhibited by the expression of UGDH, maybe due to the removal of the

pivotal compound UDP-Glc. β-glucuronidase is a counter-player of UGTs as it catalyzes the cleavage of glucuronides (Bock and Kohle, 2009). Whole-cell biotransformations with the parental strain NCYC2036 and 4-methylumbelliferyl-glucuronide as substrate did not lead to the appearance of 4-methylumbelliferone, indicating that – as expected – no endogenous β-glucuronidase homolog is present in fission yeast (data not shown). The lack of activity in fission yeast of so far six UGTs probably is due to the lack of appropriate substrates tested. However, during these studies, substrates for at least UGT1A4 were tested, which did not lead to a significant glucuronidation by the respective fission yeast strain (traces close the detection limit could be detected). However, some of those substrates were efficiently converted in our lab by cell lysates from UGT1A4 expressing insect cells (expressing the same *UGT1A4* coding sequence; data not shown). These data indicate that the fission yeast system also seems to have its disadvantages. UGT1A4 may be posttranslationally modified in a way which may not be accomplished by fission yeast; or the fission yeast cell wall may be not passable by certain compounds (Tanaka et al., 2001). Interestingly, the substrates used in those experiments carried tertiary amines as functional groups for glucuronidation. One could speculate that the charged quarternary N^+-glucuronide was not able to leave the yeast cell. Still, substantial drawbacks of other biological glucuronide production systems could be bridged by the glucuronide production system with *S. pombe*. One major advantage is the ability of a self sufficient glucuronidation reaction by the endogenous production of the essential cofactor UDP-GA. Thus, experiments using living animals may be avoided. As fission yeast only has two endogenous CYPs, which are expected to act on steroids (Wood, 2002), background metabolism by other DMEs is expected to have only a minor impact, if any, on the production of UGT metabolites. The scale up of fission yeast cells is easy to accomplish and isoform specific metabolites can be generated. In addition, encouraging results were obtained when testing for the production of different glucuronide types. *S. pombe* was shown to be suitable for the production and release of the alkyl *O*-glucuronides of testosterone or 11-α-hydroxyprogesterone, the aryl *O*-glucuronides of 4-methylumbelliferone, umbelliferone or 1-naphtol, the acyl glucuronide and glucoside of ibuprofen, and the *S*-glucuronide of 7-thio-4-methylcoumarin, respectively (Dragan et al., 2010; Buchheit et al., 2011a; Buchheit et al., 2011b). Some of those metabolites were additionally produced as $^{13}C_6$-labelled metabolites (assisted by Ellen Schmitt, under

supervision of the author). In contrast, as explained above, for the production of *N*-glucuronides so far no glucuronide production could be achieved. The testing of primary or secondary amines would be interesting for further studies. Also, the production of an *N*-carbamoyl glucuronide from varenicline was tested (by Ellen Schmitt), unfortunately without success. A special attention should be directed to the ability to produce pH-labile acyl glucuronides. While *in vitro* systems in general are incubated in physiological buffer at pH 7.4 for glucuronide production, *S. pombe* decreases the pH value of its medium to around 3 or lower. Such low pH values have been shown to stabilize acyl glucuronides against degradation (Regan et al., 2010); thus, whole-cell biotransformations with fission yeast should provide a suitable environment allowing for the stability of acyl glucuronides. Furthermore, this new system could be shown to be applicable for the study of UGT enzyme properties, as for the study of UGT substrate preferences or cofactor usage. UGTS are known to accept other UDP sugars like UDP-Glc besides UDP-GA, and UDP-Glc is endogenously present in fission yeast. Fission yeast strains coexpressing UGT2B7*1 or UGT2B7*2 with UGDH were shown to be able to glucuronidate and to glucosidate *S*-(+)-ibuprofen and *R*-(-)-ibuprofen, respectively. Likewise, similar strains without UGDH coexpression were able to perform just the glucosidation reactions (Buchheit et al., 2011a). Within this study, a so far uncharacterized fission yeast gene could be discovered to act as UGPase. A coexpression of this gene, named *fyu1* for *f*ission *y*east *U*GPase *1*, with UGT2B7*1 led to a significant increase of *S*-(+)-ibuprofen glucoside production, which is likely due to an increased intracellular UDP-Glc level. Further studies concerning glucosidation properties of the other UGT isoforms are in progress to date. The applicability of the fission yeast system for studies concerning the acceptance of certain substrates of single UGT isoforms also could be shown. In this study, the ability of each UGT and UGDH coexpressing yeast strain for the production of the *S*-glucuronide of 7-mercapto-4-methylcoumarin and likewise of the *O*-glucuronide of 4-methylumbelliferone was compared (Buchheit et al., 2011b). It could be shown that a change of the functional group at the site of glucuronidation from a hydroxyl group to a thiol led to a lower amount of glucuronide in assays using human liver microsomes. But interestingly, more UGT isoforms were able to catalyze the formation of the *S*-glucuronide than the *O*-glucuronide, albeit with a tendency to lower rates in total. In

summary, this system seems to be well suited not only for the production of UGT metabolites, but also for the convenient study of UGT enzyme properties.

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Danksagung

Ich möchte mich bedanken…

… bei meinem Doktorvater PD Dr. Matthias Bureik dass ich meine Doktorarbeit bei der PomBioTech anfertigen durfte. Vielen Dank Matthias für ein spannendes Projekt, für ganz viel Vertrauen und Unterstützung. Danke dass du mir die Möglichkeit gegeben hast an einer internationalen Konferenz teilzunehmen und vor allem, dass du dich so sehr eingesetzt hast, meine Promotion überhaupt erst möglich zu machen.

… bei Frau Prof. Dr. Alexandra K. Kiemer für die freundliche Übernahme des Zweitgutachtens.

… bei Călin-Aurel Drăgan. Danke dass ich so viel von dir lernen durfte.

… bei meiner Diplomandin und lieben Freundin Ellen Schmitt für die tolle Zuarbeit für die Publikationen; und für die vielen Sauna-Abende, an denen ich wieder Energie auftanken konnte.

… bei meiner Azubine Eva Johannes, die mir durch hervorragende Laborarbeit eine riesige Unterstützung war.

… bei Julia Maria Naumann für das Korrekturlesen meiner Arbeit, für viele aufmunternde Worte und diverse Eiskaffees.

… bei allen weiteren ehemaligen und aktuellen Mitarbeitern der PomBioTech für viele interessante Diskussionen, für sehr viel Hilfsbereitschaft und ein angenehmes und kollegiales Arbeitsklima.

Außerdem möchte ich mich bedanken...

… bei meinem Feund Pascal Becher. Danke Pascal, dass du immer an mich glaubst, mich immer wieder aufbaust und mir immer wieder den Kopf zurecht rückst. Ich bin stolz, dass du mein Freund bist.

… bei meinen Eltern Claudia und Georg und meiner Schwester Sarah. Danke dass ich mich immer immer immer absolut auf euch verlassen kann. Ihr seid die Besten!