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Mini Review

High-throughput screening technologies for drug glucuronidation profiling

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Abstract

A significant number of endogenous and exogenous compounds, including many therapeutic agents, are metabolized in humans via glucuronidation, catalysed by uridine diphosphoglucuronosyltransferases (UGTs). The study of the UGTs is a growing field of research, with constantly accumulated and updated information regarding UGT structure, purification, substrate specificity and inhibition, including clinically relevant drug interactions. Development of reliable UGT assays for the assessment of individual isoform substrate specificity and for the discovery of novel isoform-specific substrates and inhibitors is crucial for understanding the function and regulation of the UGT enzyme family and its clinical and pharmacological relevance. High-throughput screening (HTS) is a powerful technology used to search for novel substrates and inhibitors for a wide variety of targets. However, application of HTS in the context of UGTs is complicated because of the poor stability, low levels of expression, low affinity and broad substrate specificity of the enzymes, combined with difficulties in obtaining individual UGT isoforms in purified format, and insufficient information regarding isoform-specific substrates and inhibitors. This review examines the current status of HTS assays used in the search for novel UGT substrates and inhibitors, emphasizing advancements and challenges in HTS technologies for drug glucuronidation profiling, and discusses possible avenues for future advancement of the field.

Introduction

The detoxification of endogenous and exogenous substances in humans is catalysed by several classes of drug metabolizing enzymes involved in oxidative (phase I) and conjugative (phase II) biotransformation processes. The oxidative phase of drug metabolism includes reactions of catalytic oxidation, reduction and hydrolytic biotransformation. The cytochrome P450s (CYP450s) are the major group of enzymes involved in the oxidative phase. The conjugative phase of drug metabolism includes glucuronidation, sulfation, methylation and acetylation reactions and may follow the oxidative phase or be independent of it. Drug glucuronidation is one of the most important reactions of the conjugative phase (Williams et al 2004) and is catalysed by the uridine diphosphoglucuronosyltransferases (UGTs), a superfamily of membrane-bound enzymes that catalyse conjugation of endobiotics (e.g. bilirubin and steroids) and xenobiotics (e.g. drugs) with uridine diphosphoglucuronic acid (UDPGA, also called UDPGA co-substrate) resulting in the formation of a glucuronide product (metabolite). The resulting glucuronide is more hydrophilic than the parent compound, which facilitates elimination of the metabolite through excretion. Changes in the glucuronidation rate of a compound can have important pharmacological consequences for other drugs that are eliminated primarily via glucuronidation, leading to toxicity and clinically significant drug interactions (Kiang et al 2005).

Successful development of high-throughput screening (HTS) assays for CYP450s has had a significant impact on modern drug discovery, empowering assessment of P450-related toxicity and drug interactions for drug candidates in development. It has also enabled the construction of predictive structure–activity relationship (SAR) models. Such HTS assays for the CYP450s have enabled identification of molecules with undesirable metabolic profiles, and have guided medicinal chemists in producing better clinical candidates (Marks et al 2004). The UGT subfamily is the next most important target for screening diverse compound libraries to identify promising novel UGT substrates and/or inhibitors.

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The fact that the UGTs as a group play a central role in conjugative drug metabolism makes understanding of these enzymes, and particularly prediction of substrate specificity, an important issue for modern drug discovery (Williams et al 2005). The success of this approach relies on advancement of technologies amenable to the rapid profiling of drug glucuronidation, preferably in HTS format (Burchell et al 1995; Kiang et al 2005). However, the development of such technology for the UGTs presents significant challenges in light of the specific characteristics of UGTs and the inherent challenges of working with UGT enzyme membrane preparations. One of the major challenges in human UGT research is to understand the factors that determine the very complex substrate specificity of UGTs, including their regio- and stereo-selectivity. This knowledge is critical to both the search for novel UGT substrates and inhibitors, including isozyme-specific substrates with high affinity and selectivity, and for predictive modelling studies (Miners et al 2006). In practical terms, a quantitative assessment of UGT substrate specificity may enable us to assess the metabolism rates and pharmacokinetics parameters of new lead compounds, and to predict, and possibly prevent, some of the UGT-related drug interactions. This overview focuses on advancements and challenges facing novel HTS technologies for drug glucuronidation profiling and discusses progress and future directions of the field.

General characteristics and substrate specificity of UGTs

The UGTs are membrane-bound proteins of the endoplasmic reticulum that play a critical role in the metabolism and detoxification of a variety of endobiotics and xenobiotics via glucuronidation (Burchell et al 1995; Tukey & Strassburg 2000; Bock 2003; Wells et al 2004; Trubetskoy et al 2007). UGTs catalyse the transfer of glucuronic acid from UDPGA to many different aglycone acceptors. The aglycones are mostly small lipophilic molecules; conjugation with glucuronic acid increases their water solubility, thereby promoting excretion from the body through the bile or urine (Radominska-Pandya et al 1999; Tukey & Strassburg 2001; Ouzzine et al 2003). The aglycone substrates of the UGTs are endogenous molecules such as bilirubin and steroids, and xenobiotics, such as environmental carcinogens, food additives and drugs (Table 1). Glucuronidation reactions represent more than 35% of conjugation reactions in human drug metabolism. Whilst glucuronidation generally leads to detoxification, in some cases it can result in bioactivation of certain drug and compounds (Evans & Relling 1999).

There are 19 human UGTs, which are divided into two families – UGT1 and UGT2 – and three subfamilies (UGT1A, UGT2A and UGT2B), based on sequence homology and gene structure (Tukey & Strassburg 2001). In the UGT1A family, the first exon with distinct 5´ proximal promoter elements is unique for each isoform, whereas exons 2–5 are common to all the family members (Tukey & Strasburg 2001; Mackenzie et al 2005). In the UGT2 family, each gene contains all the exons (with the exception of UGTs 2A1 and 2A2). The expression of the UGTs is tissue specific, and the expression level of individual isozymes in different tissues varies significantly. Moreover, the expression level of many UGT isoforms can be altered during disease or at certain developmental stages. Levels also show marked interindividual variability because of induction of the particular UGT isoforms by nutritional and environmental compounds. Most of the human UGTs are expressed in the liver, including members from both the UGT1A and UGT2B subfamilies. Several UGTs, including UGT1A7, 1A8 and 1A10 isozymes, are predominantly expressed in extra-hepatic tissues, particularly in the gastrointestinal epithelium (Fisher et al 2001). Gastrointestinal UGTs play a major role in the first-pass metabolism of orally administered drugs and in overall oral bioavailability (Fisher et al 2001). In addition to expression in the liver and intestine, UGTs are also found in other organs such as the kidney, gut, lung, skin, brain, adipose, thymus, prostate, breast and placenta (Albert et al 1999; Collier et al 2002). Multiple UGT isoforms are expressed in many of these organs, including both hepatic and extra-hepatic UGT isozymes. In addition to a very broad spectrum of tissue distribution, several members of the UGT family possess significant genetic variability and reveal a polymorphic expression pattern (Fisher et al 2001). Given both genetic polymorphism and high variability of UGT expression levels in-vivo, it is particularly difficult to study substrate specificity of the individual UGT isozymes in a specific tissue. Therefore, the majority of experiments focusing on UGT substrate specificity now rely on preparations of recombinant UGT isozymes in various expression systems. However, recombinant UGT preparations often exhibit significant variability in the rate and specificity of the glucuronidation reaction. Use of recombinant UGTs in experiments to assess the substrate specificity of the individual UGT isoform is further complicated by the occurrence of atypical glucuronidation kinetics and by limited availability of reliable UGT phenotyping probes, including isozyme-specific UGT substrates and inhibitors (Miners et al 2006). Thus, assessment of the variability in substrate specificity of the individual UGT isoforms presents a complicated task.

The substrate specificity of the UGTs is very complex, and most human UGTs can glucuronidate several, sometimes many, different aglycone substrates that vary significantly in

Table 1 Metabolic activity of selected UGT isozymes. More comprehensive data can be found in Kiang et al (2005) and Hakala et al (2005)

UGT isoform	Endogenous substrate	Exogenous substrate	Inhibitor
1A1	Billirubin		Ketoconazole
1A3	Estrone	Norbuprenorphine	
1A4	Androstanediol	Impiramine	
1A6	Serotonin	Acetaminophen	
1A7		Mycophenolic acid	Probenecid
1A9	Estrone	Propofol	Valproic acid
2B4	Estriol	Carvedilol	
2B7	4-hydroxyestrone	Morphine	Valproic acid
2B15	Dihydrotestosterone	Hydroxytamoxifen	Valproic acid

their chemical structure (Court 2005). This characteristic results in partial overlap of substrate specificity between UGTs (Hakala et al 2005). Furthermore, recent kinetic studies have suggested that the two substrates of the UGTs bind in a sequential order, the co-substrate UDPGA binding first (Court 2005). This finding may indicate that the enzyme undergoes a conformational change with UDPGA binding and the affinity of the activated or transition-state protein for the aglycone substrate is significantly higher than the affinity of the resting enzyme. As well as substrates that are glucuronidated by multiple UGT isoforms, there are also some compounds that are highly specific for a single human UGT, or that are glucuronidated at meaningful rates by only one enzyme, as is the case for bilirubin (glucuronidated by UGT1A1), serotonin (UGT1A6) and morphine (glucuronidated at the 6OH position by UGT2B7) (Court 2005) (Table 1).

With a significant overlap in the substrate specificities for the majority of human UGT isoforms, little is known about the structural elements that determine substrate binding to UGTs. A particular interesting aspect of UGT substrate specificity is their regioselectivity, namely the preference for a particular functional group out of the few that potentially could be glucuronidated in a given substrate (Hakala et al 2005). Moreover, several UGTs exhibit clear stereoselectivity, being able to distinguish between enantiomers of drugs that are administered as racemic mixtures (Court et al 2002). This stereoselectivity serves as a good example to highlight the difficulty of trying to predict the substrate specificity of the many human UGTs using quantitative SAR methods (Sorich et al 2002), particularly when the goal is to predict the kinetic parameters of the specific glucuronidation reaction, such as V_{max} and K_m, for a specific substrate rather than the possible formation of at least a small amount of a given glucuronide by a certain UGT isozyme under optimized in-vitro conditions. These challenges are even more pronounced when attempts are made to predict in-vivo parameters of the glucuronidation reaction from in-vitro models (Miners et al 2006).

UGT assays: current approaches and technical challenges

The process of glucuronidation catalysed by UGTs involves cleavage of the UDPGA co-substrate followed by transfer of the glucuronic acid moiety to an aglycone acceptor. Traditionally, analytical procedures for measuring UGT activity involve the separation of the glucuronide conjugation product(s) from the parent aglycone, followed by analytical quantification by fluorimetric, spectrophotometric, radiometric or mass-spectrometric detection. Although simple assays and conditions to identify the individual UGTs participating in the glucuronidation of a test compound are published regularly, most of them are substrate specific (Green et al 1994; Pacifici et al 1996) and new methods are required for new compounds. The method described by Bansal and Gessner (1980) is one of the earliest analytical methods based on an application of ^{14}C-radiolabelled UDPGA, a cofactor common to all glucuronidation reactions, followed by resolution by TLC. Among the clear advantages of this method is its sensitivity and universality in application to a broad range of aglycone substrates without the requirement for synthesizing an individual glucuronide standard for quantification purposes. However, the method is time-consuming, laborious and applicable to only a narrow range of UDPGA concentrations, often below the physiological concentration of the UDPGA cofactor. Additional problems may present with aglycone substrates with multiple glucuronidation sites, where the formation of mono-, di- and multi-glucuronides is possible. Other methods for quantitative analysis of glucuronide formation that are based on radiolabelled UDPGA include assays for carboxylic acids using C18 cartridges (Pritchard et al 1993) and assays for bilirubin and steroids based on one-step extraction with organic solvent (Matern et al 1994). Alhough these methods are more efficient then TLC, it remains to be seen whether they are applicable for detection of the glucuronidation of drugs beyond the initial scope of the selected aglycons, or if readjustment of conditions will be necessary to enable assay of new chemical structures.

A different approach to a universal assay is based on optimization of a rapid-gradient HPLC method coupled with radioactivity detection and is applicable to a variety of structurally diverse substrates (Ethell et al 1998). Furthermore, the development of a universal UGT assay as a 96-well radiometric assay with compounds containing carboxyl, phenol or hydroxyl moieties, followed by separation of reaction products on 96-well plates, has been reported (Di Marco et al 2005). Recent progress in the field has also resulted in the development of a non-radioactive UGT assay that uses HPLC separation and fluorescence detection using the fluorescent substrate 4-trifluoromethylumbelliferyl (TFMU). Although this fluorescence-based UGT assay is non-homogenous and is thus not suitable for HTS applications, TFMU is a valuable substrate that can be used to test numerous compounds with several UGT isozymes during drug discovery (Baranczewski et al 2004). However, the necessity of a separation step precludes the development of most of these technologies for robust HTS applications. An alternative is to focus on the development of a UGT assay in a homogenous format that is amenable for HTS development.

Development of HTS assays for UGTs

Several attempts to develop a homogenous UGT assay have been described recently. One of them uses a homogenous fluorescence assay based on the detection of the products of glucuronidation reactions acting as competitors of a fluorescent reporter reaction involving β-glucuronidase cleavage of 4-methylumbelliferyl β-D-glucuronide (Trubetskoy & Shaw 1999). Although this assay is homogeneous and therefore amenable to HTS applications, in its current format it is limited by low sensitivity, which could be improved by developing a more sensitive method of detection, such as luminescence. Another homogeneous assay uses NMR spectroscopy to detect the binding properties of substrates to the fusion UGT protein (Coffman et al 2001). One limitation of this type of study in terms of understanding the substrate specificity of the UGTs is that these assays fail to detect UGTs that bind the molecule but without glucuronidating it efficiently. This information is important both for trying to

prevent inhibition of important UGTs by drugs that are not glucuronidated by them, as well as for better understanding of substrate binding by these enzymes. There is therefore a clear need to develop methods for efficient HTS of the interactions of many compounds with different UGTs. These methods should be able to detect glucuronidation of a library of compounds, but must also discriminate between unproductive binding and catalysis. In some cases, these goals can be accomplished using surface plasmon resonance (Karlsson & Lofas 2002); unfortunately, however, the difficulties of preparing purified UGTs that retain the activity and substrate affinity of the membrane-bound enzyme have not yet been solved. For instance, an HTS approach that involves quantification of UDP as a subproduct of glucuronidation reactions works fairly well with soluble bacterial glycosyltransferases, but this same method has limited application for membrane-containing fractions of recombinantly expressed human UGTs because of light scattering effects and interference of membrane lipids with substrate absorbance and fluorescent properties (Lowery & Kleman-Leyer 2006).

UGTs are membrane proteins, residing in the endoplasmic reticulum and nuclear membrane of cells. They contain a transmembrane domain that spans the cellular membrane. In general, designing HTS assays for membrane protein targets presents a significant challenge (Walian et al 2004). Application of HTS technology to the members of the UGT superfamily is further complicated. Current technologies for use with membrane proteins in HTS assays use unstabilized membrane fractions or purified protein preparations suspended in solutions of low-molecular-weight detergents (Hormaeche et al 2004). Many membrane-bound enzymes are stable when stored at high concentrations as stock solutions, but rapidly lose activity when diluted for use in an HTS assay (Karlsson & Lofas 2002). Additionally, it is currently very difficult to purify individual recombinant human UGTs as fully functional enzymes. The relative success with UGT1A9 provided an interesting example, not least because purification of this enzyme in an active form was a major advance in the field (Hormaeche et al 2004). However, that study, and a subsequent study, showed that the activity of the purified enzyme was significantly different from that of the membrane-bound enzyme, a change that occurred at an early stage of membrane solubilization (Kurkela et al 2004). In addition, there are several indications that the UGTs may be oligomeric enzymes within the native membrane, including hetero-oligomers that are composed of different UGT isoforms (Kurkela et al 2004). It remains to be clarified whether the different potential partners within such hetero-oligomers affect the activity and substrate specificity of the UGTs.

Another challenge arises from the luminal localization of the UGTs, which restricts access of the substrate molecules to the enzyme active site, leading to the latency effect in microsomal UGT preparations. Application of detergents or pore-making peptides such as alamethicin may lead to membrane disruption and to an increase in activity of UGT-containing human liver microsomes (Fisher et al 2000); however, in the case of preparations of recombinant UGT, this can result in inhibition of UGT activity or in altered substrate specificity. For instance, it has been reported that the latency effect was removed with increasing concentrations of alamethicin but was eventually replaced by an inhibitory effect (Vashishtha et al 2001). Thus, as with detergents, the appropriate concentration of the pore-forming peptides should be determined for each UGT substrate in the incubation mixture employed (Vashishtha et al 2001).

Many fluorescence-based HTS assays are markedly sensitive to the presence of light-scattering particles that often arise from microsomal preparations. Depending on the optics of the plate reader, the presence of light scattering particles may increase or decrease the fluorescence signal, thereby leading to false positives or false negatives. Finally, one of the biggest challenges is high rates of non-specific binding of lipophilic compounds to the microsomal fractions, making it difficult to discriminate between specific and non-specific interactions. The presence of phospholipids in membrane fractions positively affects enzyme stability, but also increases the risk of test compounds binding non-specifically to the phospholipids. Taken together, all these factors negatively affect the quality of HTS assays, resulting in low signal-to-background ratios, high variability, low Z´ factors (Zhang et al 1999) and limited dynamic range. One possible solution has been described recently, namely the generation of UGT-containing membrane particles of reduced size, combined with the addition of chemically inert polymeric micelles to block non-specific interactions (Trubetskoy et al 2007). The application of such 'nanoblockers' has resulted in a robust and efficient HTS assay compatible with robotic liquid-handling equipment that allowed screening of a compound library and selection of a set of the most potent modulators of UGT activity in a competitive displacement setting (Trubetskoy et al 2007). However, one shortcoming of this type of application is the inability to discriminate between substrate and inhibitor in a competitive displacement setting; thus, additional analyses to determine the mechanisms of competition may be necessary (Trubetskoy et al 2007). Despite the limitations, successful screening of large compound libraries for isoform-specific UGT substrates and inhibitors in HTS format may facilitate identification of novel UGT-selective probes and help to predict the pharmacokinetics of new chemical entities and potential drug interactions. Data obtained from such screening will also be useful for building predictive pharmocophore models, including prediction of drug metabolism in-silico.

Lessons from HTS assays for CYP450s

The CYP450s are another important superfamily of membrane-bound drug metabolizing enzymes that express broad, often overlapping, substrate specificity among its different members (Guengerich 2007). Over the years, the development of HTS assays for CYP450s has focused on the early identification of potent inhibitors of major CYP450 isoforms that are likely to cause clinically significant drug interactions and adverse drug reactions later in the drug development process, as a part of the pharmaceutical industry's strategy to reduce the attrition rate in bringing new drugs to market (Kariv et al 2002). Further progress with the CYP450s HTS platform has led to the development of automated ultra-HTS assays (Trubetskoy et al 2005a) to screen multi-thousand libraries of diverse

compounds, and to the development of cell-based assays to screen for induction of CYP450s (Trubetskoy et al 2005b). Results obtained with such assays made a significant impact by accelerating the drug discovery process and reducing the cost, and by contributing to the building of predictive CYP450 QSAR models and in-silico screening (Schuster et al 2006). Several factors contributed to the significant progress made in the CYP450 HTS field, including a few important milestones, such as the discovery of isoform-specific substrates, inhibitors and inhibitory monoclonal antibodies (Gelboin & Krausz 2006), the development of novel sensitive fluorescent substrates to monitor CYP450 metabolism and inhibition (Cohen et al 2003; Trubetskoy et al 2005a), and continuous developments in procedures for successful expression, cloning, purification and reconstitution procedures, allowing the production of large quantities of recombinantly produced active P450 enzymes for protein crystallography and modelling studies (Kjellander et al 2007).

Despite the successes in the CYP450 HTS field and many parallel developments between these CYP450s and UGTs superfamilies of drug metabolizing enzymes, the progress of the UGT-HTS field is still falling behind because of some unresolved issues. These issues include insufficient information related to isoform-specific UGT substrates and inhibitors, an incomplete understanding of UGT-related drug interactions, and a lack of knowledge about the significance of UGT genetic polymorphisms, including the role of environmental factors in the induction of UGTs. Many of these gaps in knowledge are directly related to the absence of advanced methods for UGT purification and crystallography. Given the multiplicity and polymorphisms of UGTs in the liver and other organs, and the fact that the majority of mammalian tissues express these enzymes, current efforts have now shifted almost fully to studies of recombinant proteins. The UGTs have been cloned and expressed in many different systems (for a complete review see Radominska-Pandya et al (2005)), but there are few, if any, reports on the purification of recombinant UGTs as fully active enzymes. The recombinant human UGTs are sensitive to detergent solubilization (Walian et al 2004). With most, if not all, the expression systems tried so far, recombinant enzymes lose activity when the detergent concentration is raised above its critical micellar concentration, a prerequisite for the purification of integral membrane proteins such as the UGTs. Since most of the efforts to purify recombinant UGTs have ended without success, it is difficult at present to provide a suitable reference to this topic. In addition, other factors can contribute to the altered enzymatic activity of a recombinantly expressed UGT isoform in-vitro compared with a native form, including heterologous interactions between two UGT isoforms in the biological membrane (Fujiwara et al 2007; Nakajima et al 2007), or the level of expression of other modulators of UGT activity, such as UDPGA transporters (Kobayashi et al 2006). Moreover, in-vitro assays for UGT activity can be influenced by the presence of other membrane-modifying agents such as the pore-making peptide alamethicin. Assays also may be enhanced by the effects of mediators such as fatty acids and albumin (Uchaipichat et al 2006; Rowland et al 2007), making it difficult to compare data obtained from different studies with in-vivo conditions.

Another obstacle is the lack of sufficient information relating to isozyme-specific substrates and potent inhibitors of UGTs. Until very recently, because of the absence of potent UGT inhibitors, most of the screening for UGT activity, including screening in HTS format, was directed at searching for novel isozyme-selective substrates of high affinity and specificity (Trubetskoy et al 2007). However, this situation may be changing with the recent discovery of potent inhibitors, such as the isoform-specific molecules recently discovered for UGT2B7 (Bichlmaier et al 2007). These inhibitors may be applicable for studies in human tissues to elucidate drug interactions for a specific isoform and for the identification of UGT polymorphisms in-vivo (Grancharov et al 2001). Moreover, the discovery of such inhibitors may have a significant impact on the design of a future strategy for an overall UGT-HTS process. As for the CYP450s, one of the possible strategies for UGT screening may now include first-tier binding assays to determine the potent modulators of UGT binding activity. This may be combined with second-tier functional assays to discriminate whether a modulator is a substrate or inhibitor of a specific isoform, and in addition to predict potential UGT-related drug interactions.

Finally, availability of an active UGT isoform in purified format may facilitate the progress of other methods for detection of UGT activity. For example, a screening methodology based on the quantification of adenine dinucleotides has been introduced for detection of the activity of soluble enzymes, such as kinases and non-membrane-bound soluble bacterial glycosyl transferases (Lowery & Kleman-Leyer 2006). Application of this screening technology in combination with fluorescent probes designed to reduce light scattering effect, such as FarRed probes (Vedvik et al 2004), was successful in assays using purified soluble-protein preparations. However, these technologies are much more difficult to apply to lipid-containing preparations of membrane proteins such as UGTs, because of the possibility of a compound partitioning into the hydrophobic membrane fraction, thereby causing unforeseen changes in fluorescent properties.

Conclusions

Overall, an in-depth understanding of substrate specificity, metabolic liabilities and involvement in clinically relevant drug interactions is critical both for an understanding of the UGTs superfamily of drug metabolizing enzymes, and for the progress of modern drug discovery in general. The current state of technology for the screening of drug glucuronidation in HTS format is not only facing significant challenges, but is also leading to some important innovations as the field continues to evolve. Recently obtained data in UGT HTS applications for the screening of diverse chemical libraries are expected to facilitate further progress of the field, including advancements in UGT modelling studies, the identification of novel isozyme-selective UGT substrates and inhibitors, and delineation of the role and significance that particular UGT isozymes may play in clinically relevant drug interactions.

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