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Commentary

In vitro–in vivo correlation for drugs and other compounds eliminated by glucuronidation in humans: Pitfalls and promises

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ABSTRACT

Enzymes of the UDP-glucuronosyltransferase (UGT) superfamily are responsible for the metabolism of many drugs, environmental chemicals and endogenous compounds. Identification of the UGT(s) involved in the metabolism of a given compound ('reaction phenotyping') currently relies on multiple confirmatory approaches, which may be confounded by the dependence of UGT activity on enzyme source, incubation conditions, and the occurrence of atypical glucuronidation kinetics. However, the increasing availability of substrate and inhibitor 'probes' for the individual UGTs provides the prospect for reliable phenotyping of glucuronidation reactions using human liver microsomes or hepatocytes, thereby providing data directly relevant to drug metabolism in humans. While the feasibility of computational prediction of UGT substrate selectivity has been demonstrated, the development of easily interpretable and generalisable models requires further improvement in the datasets available for analysis. Quantitative prediction of the hepatic clearance of glucuronidated drugs and the magnitude of inhibitory interactions based on in vitro kinetic data is more problematic. Intrinsic clearance (CL_{int}) values generated using human liver microsomes under-predict in vivo hepatic clearance, typically by an order of magnitude. In vivo clearances of glucuronidated drugs are also generally under-predicted by CL_{int} values from human hepatocytes, but to a lesser extent than observed with the microsomal model. While it is anticipated that systematic analysis of the potential causes of under-prediction may provide more reliable in vitro–in vivo scaling strategies, mechanistic interpretation of in vitro–in vivo correlation more broadly awaits further advances in our understanding of the structural and cellular determinants of UGT activity.

The development and use of in vitro approaches to predict aspects of human drug metabolism and pharmacokinetics in vivo ('in vitro–in vivo correlation') has attracted intense interest over the last decade or so, not only to assist in the selection of new drug candidates but also for the rationalisation and optimisation of dosage regimens for established drugs. The principal drug metabolising enzymes, cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), exist as enzyme 'superfamilies'. The individual enzyme forms tend to differ in terms of substrate and inhibitor selectivities, occurrence and frequency of genetic polymorphism and gene regulation, leading to wide interindividual variability in

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metabolic clearance. However, once the drug interaction profile and regulation of the various enzymes are characterised, identification of the enzyme(s) responsible for metabolism of a compound (commonly referred to as 'reaction phenotyping') allows prediction, at least in a qualitative manner, of factors likely to alter hepatic clearance (CL_H) and response in particular patients or population groups [1–3]. At the quantitative level, the intrinsic clearance (CL_{int}) for a metabolic pathway, most commonly determined using either hepatic microsomes or hepatocytes as the enzyme source, may be used to estimate CL_H (and extraction ratio, E_H) using appropriate scaling factors and a mathematical expression derived from one of the several models of hepatic elimination [4–6]. Similarly, the magnitude of an inhibitory drug interaction in vivo can be estimated from the experimentally derived inhibition constant (K_i), using equations that take into account the mechanism of inhibition and the pharmacokinetics and route of administration of the interacting drugs [6–8].

Although such approaches have proved useful for drugs eliminated by CYP—catalysed biotransformation, their application to drugs metabolised by UGT may be problematic. Why should this be so?

1. The reaction phenotyping of glucuronidated compounds

Multiple approaches have been adopted for the reaction phenotyping of CYP substrates, most frequently using human liver microsomes as the enzyme source, including [1–3]: (i) impairment of metabolism by enzyme-selective chemical and antibody inhibitors; (ii) competitive inhibition of the metabolism of enzyme-selective substrates by the compound, with K_m matching K_i; (iii) a significant correlation between rates of metabolism of the compound and immunoreactive enzyme contents or enzyme-selective activities in microsomes from a 'panel' of human livers; (iv) comparative metabolism by recombinant human enzymes. Taken together, these procedures allow identification of the CYP enzymes(s) responsible for the metabolism of a drug with a high degree of certainty, although inhibition by selective inhibitors alone is often considered diagnostic providing experimental conditions for inhibitor selectivity are well established.

Can the strategies employed for the reaction phenotyping of CYP substrates also be applied to glucuronidated compounds? We shall consider the approaches in two categories; those requiring form selective substrates and inhibitors (i.e. (i)–(iii)) and the use of recombinant enzymes.

1.1. *Isoform selective substrates and inhibitors*

Seventeen human UGT proteins have been identified to date: UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28 [9]. Most, but not all, of these enzymes are expressed in liver. Notably, UGT1A7, 1A8 and 1A10 are expressed only in the gastrointestinal tract, and UGT2A1 is believed to be expressed primarily in the nasal epithelium where it is involved in odorant signal termination [10]. In addition, UGT1A5, 2B4, 2B10, 2B11, 2B17 and 2B28 all appear to exhibit low or negligible activity towards drugs and other xenobiotics. Thus, UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 are considered to be the isoforms of greatest importance in hepatic drug elimination [11].

As noted above, the availability of isoform selective substrates and inhibitors underpins the reaction phenotyping of CYP substrates. Importantly, the availability of selective 'probes' permits the use of either human liver microsomes or hepatocytes as enzyme sources. These systems express the full complement of drug metabolising enzymes associated with the endoplasmic reticulum (ER) and relevant accessory proteins in the ER membrane environment. Hence, data are considered directly relevant to drug metabolism in humans. Historically, reaction phenotyping of drug glucuronidation has attracted less attention than reactions catalysed by CYP, possibly due to the relatively lower incidence of drug interactions between glucuronidated compounds [12]. In contrast to CYP, only a limited number of isoform-selective UGT substrates have been identified to date (Table 1), and just two selective inhibitors have been characterised; hecogenin and fluconazole, which inhibit UGT1A4 and UGT2B7, respectively [25,26]. Although apparently isoform selective substrates have been used as inhibitors in some studies, further confirmation of inhibition selectivity is advisable given previous experience with other enzyme systems (e.g. potent inhibition of CYP2D6 by the CYP3A4 substrate quinidine). Indeed, there is evidence that bilirubin, a specific UGT1A1 substrate, may inhibit UGT1A4 [27].

Similarly, the authors are unaware of the availability of isoform-selective, inhibitory antibodies. An antibody to UGT2B7 has been used for immuno-quantification, and then subsequently to explore the relationship between the 'relative' UGT2B7 content of human liver microsomes and the rates of metabolism of a range of glucuronidated compounds [20]. Studies (unpublished) in the authors' laboratory indicate, however, that the commercial antibody used in this work also

Table 1 – Selective substrates for human hepatic UDP-glucuronosyltransferases

Enzyme	Substrate	Reference
UGT1A1	Bilirubin	[13]
	Estradiol 3-glucuronidation[a]	[14]
UGT1A3	Hexafluoro-1α,25-dihydroxyvitamin D_3	[15]
UGT1A4	Trifluoperazine	[16]
UGT1A6	Serotonin	[17]
	1-Naphthol[b]	[18]
UGT1A9	Propofol	[19][c]
UGT2B7	Zidovudine	[20,21]
	Morphine[d]	[20,22]
UGT2B15	S-Oxazepam	[23]

[a] Probably partially selective, with a contribution from UGT1A3. Additionally a substrate for the extrahepatic enzymes UGT1A8 and UGT1A10.
[b] Substrate for other UGTs, but highest CL_{int} observed with UGT1A6.
[c] Subsequent studies have excluded propofol glucuronidation by UGT1A3, 1A10 and 2B15, although propofol has been demonstrated to be a substrate for UGT1A8 (extrahepatic) [24].
[d] Morphine 6-glucuronidation catalyzed only by UGT2B7. Other enzymes catalyze morphine 3-glucuronidation, but highest activity is observed for UGT2B7.

recognises UGT2B4 and UGT2B10. While a significant correlation between enzyme content and an activity measure may provide valuable confirmatory evidence for the involvement of that enzyme in metabolite formation, antibody selectivity is an important consideration. Moreover, immuno-quantification measures total protein rather than active protein content.

Comparison of the rate of metabolism of the test substrate with the activities of a 'battery' of selective substrates in microsomes from a panel of livers provides an alternative approach to the use of antibodies. In addition, mutual competitive inhibition between a compound and a selective substrate may be taken as evidence of involvement of a common enzyme in their metabolism. However, the rate of glucuronidation of a compound by human liver microsomes is highly dependent on incubation conditions [28,29]. In particular, rates of glucuronidation vary with the presence of activators, co-factor concentration, and buffer type, pH and ionic strength. Thus, comparison of activities is not meaningful unless they are measured under identical conditions, generally with fully activated microsomes. It is doubtful, however, that truly 'standardised' incubation conditions for all glucuronidation reactions are feasible. For example, acyl glucuronides hydrolyse at the usual incubation pH of 7.4, and mildly acidic conditions are required to prevent product degradation [30].

Kinetic considerations are also important. Careful selection of substrate concentration is required in activity comparison experiments when multiple UGTs are involved in the glucuronidation of a compound by human liver microsomes, such that the concentration employed reflects only the contribution of the isoform of interest. As discussed later in this Commentary, there is increasing evidence that the glucuronidation of many compounds is best described by 'atypical' or non-Michaelis–Menten kinetic models (for example [18,29]). Thus, kinetic data must be analysed using an appropriate model if meaningful results are to be obtained. Characterisation of glucuronidation kinetics and inhibition mechanism is particularly important in studies of the mutual competitive inhibition of the test substrate and an isoform selective substrate probe. For example, a compound which exhibits glucuronidation kinetics characteristic of autoactivation may variably activate or inhibit the glucuronidation of another substrate, depending on the concentration of each [31].

1.2. *Comparative metabolism of a substrate by recombinant human UGTs*

As noted previously, available evidence indicates that UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 are the isoforms of greatest importance in hepatic drug elimination, although pre-hepatic glucuronidation by UGT1A7, 1A8 and 1A10 may additionally influence bioavailability. Human UGTs have been cloned in a number of laboratories (including the authors'), and many are also available from commercial sources. It is therefore possible to investigate the glucuronidation of any given compound by the individual UGTs, for example [15–23,27,32–34]. But how valid is it to compare the activities of the recombinant enzymes? Unlike CYP, which can be quantified spectrophotometrically, there is currently no universal method that allows measurement of the UGT content of an enzyme preparation, and this precludes the use of approaches such as the relative activity factor [3] for assessing the contribution of individual UGTs to the glucuronidation of a substrate by human liver microsomes. An antibody that recognises UGT1A proteins (presumably via an epitope in the common carboxyl terminus) and a number of antibodies that recognise UGT2B subfamily proteins are commercially available. Although the UGT1A antibody may be used to compare the relative amounts of UGT1A proteins from expression systems, and hence 'normalise' enzyme activities (for example [18]), comparison of the activities of UGT1A and UGT2B enzymes is not possible. An alternative approach is to compare the K_m values derived for a glucuronidation reaction by recombinant enzymes and human liver microsomes (for example [34]), but experience with CYP indicates that differences in this parameter may occur both between enzymes obtained using different expression systems and recombinant and human liver microsomal enzymes. What should we expect with UGTs?

UGTs are integral proteins of the endoplasmic reticulum and are dependent on lipid for catalytic activity. Early studies also demonstrated that membrane lipid composition is a critical determinant of glucuronidation kinetics. For example, an alteration in the unsaturated:saturated fatty acid ratio of guinea pig liver membranes by dietary manipulation increased membrane viscosity in conjunction with an increase in the Hill coefficient of UGT for UDP glucuronic acid [35]. As the lipid composition varies between membranes from different sources and is a determinant of membrane fluidity, thickness, shape, surface curvature and the ability to form lipid rafts, it is conceivable that the kinetic properties of a single recombinant UGT may differ depending on whether it is synthesised in bacteria, yeast, insect or mammalian cells.

Accumulating evidence indicates that enzymatic activity may further be dependent on the capacity of UGTs to form homo- or heterodimers with other UGTs and to exist in functional complexes with other proteins (e.g. CYP, epoxide hydrolase and transporters) [36,37]. It is possible that the kinetic properties of a UGT are dependent on the protein partners with which it interacts and the dynamic nature of this interaction. For example, the ratio of morphine-3-glucuronide to morphine-6-glucuronide formation by UGT2B7 is altered by the interaction of UGT2B7 with CYP3A4 [38]. It is almost certain that the complement of potential UGT interacting partners will not be identical in each of the cell lines used to express recombinant UGT. The extent to which such interactions influence the activity and kinetic properties of a recombinant enzyme synthesised by different cell lines awaits characterisation. Similar considerations apply to the use of human liver microsomes and hepatocytes for activity studies in vitro (discussed later). The metabolic and structural 'connectivity' that occurs in vivo may not be emulated with in vitro systems.

Recent studies indicate that post-translational modifications of the UGT protein are necessary for catalytic activity. Inhibition of phosphorylation of UGT1A1 by calphostin C, a specific Protein kinase C inhibitor, reduced its activity 5–10-fold and altered its pH optimum for the glucuronidation of eugenol, but not bilirubin and some other substrates [39].

N-Glycosylation may alter the catalytic capacity of some UGTs, such as UGT2B15 and UGT2B20 [40] but not others, including UGT2B1 and UGT2B2 [41]. Whether these post-translational modifications are present in the recombinant UGTs synthesised in the various cell expression systems also remains to be established, but clearly this is not so in bacteria, which lack several eukaryotic protein processing pathways.

1.3. *Computational modelling*

Computational (or 'in silico') modelling potentially provides an alternative to laboratory-based procedures for reaction phenotyping. Two dimensional (2D)-, quantum mechanical- and pharmacophore-models that permit the classification of UGT substrates have recently been developed in this laboratory using a database of all known substrates and non-substrates of the individual human UGTs [11,42–46]. The classification models based on 2D- and quantum mechanical-descriptors provided good predictability of metabolism by several isoforms [44,46], but are not readily 'interpretable'. Models based on multiple pharmacophores were more intuitive, but less predictive than those based on the 2D- and quantum mechanical-descriptors [45]. While these approaches demonstrate that computational prediction of UGT isoform substrate selectivity is possible, the development of models that are both generalisable and interpretable requires the availability of datasets of increased size and structural diversity (see below) [47].

1.4. *Do we really understand UGT substrate selectivity?*

It is widely believed that UGTs exhibit overlapping substrate selectivities and that these enzymes possess 'promiscuous' active sites. However, in generating the in silico models referred to above, it was apparent that the chemical 'space' used to define substrate selectivity was frequently very limited. Chemicals screened as substrates most commonly included structurally similar groups of aliphatic alcohols, phenols, carboxylic acids and amines. Compounds used in investigations of UGT substrate selectivity showed less structural diversity than a dataset of marketed drugs and the NCI diversity set (Sorich MJ, Smith PA, McKinnon RA, Miners JO, unpublished results). This chemical diversity 'bias' undoubtedly influences current concepts of isoform substrate and inhibitor selectivities. For example, common features pharmacophores generated for the substrates of UGT1A1, 1A4 and 1A9 suggest that metabolism by these enzymes requires only the site of glucuronidation, two hydrophobic domains, and possibly a hydrogen-bond acceptor [11,47]. It was clear, however, that high affinity substrates of UGT1A1 and 1A4 benefited from further hydrogen-bonding interactions. Thus, additional selective substrates and inhibitors may be identified as chemicals of greater structural complexity and diversity are screened for metabolism by UGTs.

Another factor that has influenced our concept of UGT selectivity is the reaction conditions used to identify substrates of the enzymes encoded by newly isolated cDNAs. A radiometric thin layer chromatographic technique has generally been used for this purpose. The method utilises radiolabelled cofactor (UDP-glucuronic acid; UDPGA), frequently at rate-limiting concentrations, for incubation times ranging from a few hours to 16 h. Thus, compounds which are glucuronidated at rates ranging from several hundred pmol/mg min to <1 pmol/mg 16 h will all be classified as 'substrates' of the enzyme. The adoption of more rigorous approaches for reaction phenotyping is now recommended if our understanding of UGT substrate selectivity is to progress.

2. The quantitative prediction of drug glucuronidation kinetics in vivo

Two approaches may be adopted for the calculation of in vitro CL_{int}, which may subsequently be extrapolated to in vivo CL_H and E_H [4]. Measurement of product formation (e.g. a drug glucuronide) under initial rate conditions over an appropriate substrate concentration range allows calculation of K_m and V_{max} for that pathway (in the absence of atypical kinetics; see below), and then CL_{int} (as V_{max}/K_m). The second approach measures the rate of substrate depletion with time (at a low substrate concentration, presumed to be below K_m) and calculates CL_{int} as the elimination rate constant divided by cell density (cells/ml). Scaling factors (microsome recovery or hepatocellularity, and liver weight) are then used to estimate a 'whole liver' CL_{int}, which may be substituted in the expression for a mathematical model of hepatic clearance, most commonly the well-stirred model:

$$CL_H = \frac{Q_H\, fu_b\, CL_{int}}{Q_H + fu_b\, CL_{int}}$$

where Q_H is liver blood flow, CL_{int} the scaled whole liver intrinsic clearance, and fu_b is the fraction unbound in blood. Studies with human hepatocytes as the enzyme source generally utilise the substrate depletion approach to assess CL_{int}.

There is currently intense debate regarding the relative predictivity offered by human liver microsomes and human hepatocytes. How do they compare in the prediction of clearance by glucuronidation?

2.1. *Prediction of CL_H based on human liver microsomal kinetic data*

Since human hepatocytes are less readily available than human liver microsomes (given the requirement for fresh, good quality tissue), the latter are generally favoured for studies of human drug metabolism. Although predictivity using human liver microsomes as the enzyme source is good for some drugs metabolised by CYP, there is a general trend to under-prediction of CL_H using 'physiologically' based scaling factors [48–50]. In terms of metabolism by UGT, Mistry and Houston initially observed that microsomal CL_{int} values for three glucuronidated opioids (morphine, naloxone and buprenorphine) were consistently lower (18–33-fold) than their in vivo counterparts in the rat [51]. Recent analyses of in vitro–in vivo correlation for two series of glucuronidated drugs demonstrated that in vitro CL_{int} under-predicted human glucuronidation CL_H in vivo by approximately an order of magnitude, irrespective of the mathematical model (well-stirred, parallel-tube or dispersion) used for extrapolation [11,28,52]. Interestingly, a highly significant linear relationship

was observed between the in vivo and predicted CL_H, especially when two outliers were removed from a dataset of 14 drugs [11], suggesting that an empirical scaling factor may be of use in compensating for the under-prediction of CL_{int} for microsomal glucuronidation as proposed by Ito and Houston for drugs eliminated by CYP [48].

2.2. Reasons for under-prediction of CL_H from human liver microsomal kinetic data

Numerous factors influence the kinetic parameters for drug glucuronidation by human liver microsomes. In particular, K_m and V_{max} values can vary with buffer type, pH and ionic strength, and the presence of activators (e.g. alamethicin, detergents, Mg^{2+}) or other treatments (e.g. sonication) which release the 'latency' of microsomal UGT [28,29,53]. Although rates of zidovudine glucuronidation by human liver microsomes have been shown to be approximately doubled in the presence of carbonate buffer (compared to incubations performed in phosphate buffer), due primarily to a lowering of K_m [53], activation by the pore forming agent alamethicin and by non-ionic detergents (e.g. Brij58) generally results in greatest enhancement of microsomal UGT activity (via an effect on V_{max}) [28,29]. Of these, alamethicin is preferred [28,29,54]; activation by detergents follows a bell-shaped relationship and effects on drug glucuronidation are not reproducible between substrates [55]. To illustrate the effects of reaction conditions on glucuronidation kinetic parameters in vitro, CL_{int} values for zidovudine glucuronidation by human liver microsomes were shown to vary six-fold depending on the composition of the incubation medium [28]. However, even under maximally 'activated' conditions in vivo CL_H was still under-estimated three- or four-fold [28,53].

Other confounders of in vitro–in vivo correlation based on human liver microsomal CL_{int} may include differences in physiological scaling factors, non-specific and protein binding, inappropriate kinetic modelling in vitro, metabolism by extrahepatic tissues, and the potential contribution of uptake transporters. Although mean microsome yields used by different investigators for scaling the microsomal CL_{int} to the whole liver CL_{int} vary from 33 to 52.5 mg/g [6,56], this difference clearly does not explain the discrepancy in predicted CL_H for glucuronidated drugs. The use of fraction unbound in plasma rather than blood in the equations for hepatic clearance can also lead to errors in the calculation of CL_H, and it is also important to take into account the fraction of hepatic clearance due to the metabolic pathway of interest in the in vitro–in vivo correlation [57]. Non-specific binding to incubation components (e.g. the microsomal membrane or albumin added to hepatocyte suspensions) results in over-estimation of K_m and hence underestimation of CL_{int} [58], and correction for this parameter is critical for accurate in vitro–in vivo correlation. It is sometimes assumed that non-specific binding in vitro and the protein binding in vivo 'cancel out', and hence both terms are not uncommonly ignored in the calculation of in vivo CL_H (for example [49,59]). However, the two parameters are not related in a linear manner [48] and inclusion of both improves predictivity [49]. There is also increasing awareness that many compounds exhibit 'atypical' drug glucuronidation kinetics in vitro not consistent with the hyperbolic Michaelis–Menten model (for example [18,29,34]). Co-operativity, particularly the sigmoidal kinetics characteristic of autoactivation, has been described for a number of glucuronidation reactions. Atypical kinetic behaviour poses problems for in vitro–in vivo extrapolation since the in vivo correlate of non-hyperbolic kinetics in vitro is unknown [60]. It is noteworthy, however, that in vitro data still under-predict in vivo glucuronidation CL_H in the absence of atypical kinetics and when other factors discussed above are accounted for [28].

As noted previously, UGTs are expressed in tissue other than liver. In particular, specific activities measured in kidney and liver have been shown to be comparable for several drugs (for example [52,61,62]). Activities in gastrointestinal tissue tend to be lower [52,61]. However, prediction of the contribution of the kidney to metabolic clearance by glucuronidation in vivo differs greatly depending on scaling factors employed, particularly microsome yield [52,61]. Interestingly, recent data indicate that the renal extraction ratio of propofol is high and hence this organ may contribute significantly to clearance in vivo [63]. Propofol is both glucuronidated (see Table 1) and hydroxylated by CYP2B6. The relative contribution of these enzymes to renal metabolic clearance is unknown.

It is now recognised that the concentration of some drugs within the hepatocyte may be influenced by hepatocellular uptake and efflux transporters [64,65], the involvement of which potentially influences the accuracy of in vitro–in vivo correlation based on human liver microsomal kinetic data. Human hepatocytes have thus been proposed as providing a superior experimental model for in vivo clearance prediction of glucuronidated drugs since they provide an 'integrated' system containing the full complement of enzymes and transporters involved in hepatic drug disposition [52,53,66]. However, how well do human hepatocytes predict hepatic glucuronidation clearance?

2.3. Prediction of CL_H based on human hepatocyte data

There have been several publications in recent years [50,52,66–68] that have investigated the use of cryopreserved human hepatocytes to generate clearance predictions. A number of these studies included UGT substrates. In all cases parent drug depletion was monitored with time rather than direct measurement of metabolite formation. Thus, for drugs which are subject to other metabolic pathways in addition to glucuronidation it is difficult to evaluate the prediction of this particular metabolic pathway in isolation. However, there are certain trends which can be delineated.

Fig. 1 shows the predicted intrinsic clearance from cryopreserved human hepatocytes for 62 substrates, 19 of which rely on UGT for a varying proportion of their clearance ranging from 10% to almost 100% (as denoted by the different symbols). Firstly, it is apparent that the predictions underestimate the value observed in vivo, although the extent of this under-prediction would appear to be less than that observed in microsomes. Secondly, as there is no clear distinction between the groups of symbols it would appear that predictivity for drugs cleared by UGT does not differ significantly from other enzymes (notably P450s). Thus, the bias for UGT metabolism is similar to CYP metabolism for

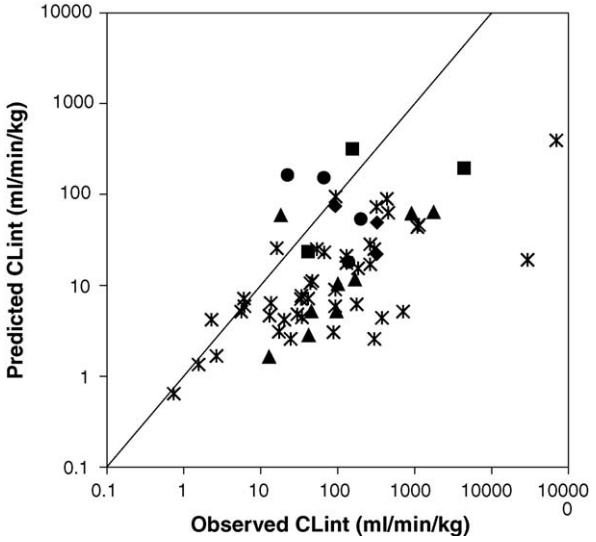

Fig. 1 – Prediction of hepatic intrinsic clearances for 62 drugs using cryopreserved human hepatocytes. Symbols denote the extent of glucuronidation of each drug: 0.75–1 (n = 9, ▲), 0.5–0.75 (n = 3, ■), 0.25–0.5 (n = 4, ●), 0.25–0.1 (n = 3, ◆) and 0 (n = 43, ×).

cryopreserved hepatocytes. The number of studies involving fresh human hepatocytes is relatively small, but consideration of these data would indicate there is no major difference between fresh and cryopreserved hepatocytes either from human or animal origin [52,66,68].

Other aspects of human hepatocyte CL_{int} data warrant comment. Attention has been drawn to the substantial variability in CYP activities between hepatocytes prepared from different donors [50]. Soars et al. [52] similarly reported large variability in the hepatocyte CL_{int} values for codeine, morphine and naloxone. In particular, hepatocytes prepared from one liver were up to 28-fold higher (with codeine as substrate) compared to hepatocytes from three other livers. Furthermore, metabolism of furosemide, ketoprofen and naproxen was not detected in the majority of hepatocyte preparations investigated.

The kinetics of zidovudine glucuronidation by human liver microsomes and hepatocytes have been compared recently [53]. The CL_{int} value for zidovudine glucuronide formation was approximately 30-fold higher (due to a lower apparent K_m and higher V_{max}) with hepatocytes. The hepatocyte CL_{int}, but not the microsomal CL_{int}, predicted in vivo glucuronidation clearance of zidovudine well. However, incubations performed with human liver microsomes in a more physiologically 'relevant' carbonate buffer provided a K_m value for zidovudine glucuronidation that more closely resembled the hepatocyte data. It was speculated that matrix differences, rather than transporters, may be responsible for differences in drug glucuronidation kinetics between microsomes and hepatocytes.

2.4. *Prediction of inhibitory interactions involving glucuronidated drugs from in vitro kinetic data*

Human liver microsomes and recombinant UGTs have been used to assess potential inhibitory interactions between glucuronidated compounds (for example [18,26,30,69], reviewed in [70]). However, few studies have explored whether in vitro approaches predict the magnitude of an inhibitory interaction between glucuronidated compounds in vivo. The magnitude of an inhibitory interaction, as the ratio of the area under the plasma concentration–time curve (AUC) in the presence and absence of the inhibitor, may be determined as [71,72]:

$$\text{AUC ratio} = \frac{1}{\frac{f_m}{1+[I]/K_i} + (1 - f_m)}$$

where K_i is the inhibitor constant (obtained from in vitro experiments), f_m the fraction metabolised via the pathway of interest, and [I] is the inhibitor concentration at the enzyme active site. There is no consensus regarding which value of [I] should be used in the above expression, although it has been demonstrated recently that incorporation of the hepatic input concentration provided best predictivity of inhibitory interactions involving drugs eliminated by CYP [72,73].

In characterising the fluconazole–zidovudine interaction in vitro, we found that the K_i values generated using human liver microsomes and recombinant UGT2B7, the enzyme responsible for zidovudine glucuronidation, under-predicted the known AUC ratio reported for this interaction in vivo by 5–10-fold [26]. Interestingly, however, the addition of bovine serum albumin (BSA, 2%) to incubations decreased the K_i value for fluconazole by approximately 85% with both human liver microsomes and recombinant UGT2B7 as the enzyme sources, resulting in good prediction of the in vivo interaction when the hepatic inlet concentration of fluconazole was used in the expression for the AUC ratio. Addition of BSA also decreased the K_m for zidovudine glucuronidation by human liver microsomes by 90% (without changing V_{max}), although the derived CL_{int} still under-predicted in vivo CL_H by a factor of three. The mechanism of the effect of BSA, which is independent of drug–protein binding, remains unknown. However, similar observations have also been reported for human liver microsomal cytochromes P450, particularly CYP2C9 (for example [74,75]). The lamotrigine–valproic acid interaction was also well-predicted using a K_i value determined in the presence of BSA, and human serum albumin also reproduced the effect of BSA (Rowland et al., submitted for publication). Despite the 'unphysiological' nature of the incubations, it appears that K_i values determined under certain experimental conditions may quantitatively predict inhibition of UGT catalysed drug glucuronidation in vivo.

Inhibition of glucuronidation also needs to be considered more broadly to encompass interactions between xenobiotics and endogenous compounds due to the involvement of common UGT enzymes. For example, inhibition of bilirubin glucuronidation by indinavir predisposes to hyperbilirubinemia in patients with low activity UGT1A1 alleles [76,77]. Recent studies have also demonstrated that C16:1–C20:5 fatty acids are potent inhibitors of xenobiotic glucuronidation by human kidney microsomes, UGT1A9 and UGT2B7 [62]. In particular, potent inhibition was observed with linoleic acid (K_i 6.3 μM) and arachidonic acid (K_i 0.15 μM).

3. Summary and conclusions

The reaction phenotyping of drugs and other chemicals metabolised by glucuronidation is currently feasible, but generally requires the application of multiple experimental approaches. Even so, problems with the interpretation of data may arise due to the dependence of UGT activity in vitro on incubation conditions, enzyme source and the occurrence of atypical glucuronidation kinetics, emphasising the need for careful experimental design. However, UGT reaction phenotyping with human liver microsomes, and possibly hepatocytes, as the enzyme source will undoubtedly improve as increasing numbers of isoform-selective substrates and inhibitors become available from the screening of compounds of greater structural complexity and diversity. Reliable databases which include larger numbers of structurally diverse substrates and inhibitors will similarly improve the computational prediction of UGT isoform selectivity.

Quantitative in vitro–in vivo correlation to predict the CL_H values and the magnitude of inhibitory interactions of glucuronidated drugs in vivo is more problematic. CL_{int} values generated using human liver microsomes consistently under-predict CL_H via glucuronidation in vivo. Similarly, inhibitory interactions may not always be predicted using a K_i value obtained with human liver microsomes. Predictions may be improved, at least for the 'model' glucuronidated drug zidovudine, by the inclusion of albumin or carbonate buffer in the incubation mixture. It is also possible that an empirical scaling factor may be of use in compensating for the under-prediction of CL_{int} for microsomal glucuronidation, but this requires further validation. It has been proposed that human hepatocytes provide a superior experimental model for the in vivo clearance prediction of glucuronidated drugs. However, available data indicate that predictions based on CL_{int} values determined with hepatocytes also tend to under-predict in vivo CL_{int} and, although the extent of under-prediction appears to be less than that observed with the extrapolation of microsomal kinetic data, the precision is poorer. Thus, neither enzyme source can be considered truly predictive for all glucuronidated drugs. It is anticipated that systematic analysis of the potential causes of under-prediction (detailed elsewhere in this Commentary) by the microsomal and hepatocyte models may result in the development of consistently reliable scaling strategies. Additionally, however, there are 'fundamental' questions relating to drug glucuronidation that may influence in vitro–in vivo correlation more broadly, particularly the implications of dimerisation and the dependence of UGT activity on accessory proteins and the local membrane and cellular environment. Mechanistic interpretation of drug glucuronidation in vitro awaits the further characterisation of this enigmatic enzyme.

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