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COMMENTARY

USE OF *IN VITRO* HUMAN METABOLISM STUDIES IN DRUG DEVELOPMENT

AN INDUSTRIAL PERSPECTIVE

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As part of the drug development process, pharmaceutical companies are mandated by regulatory agencies to study the metabolism of a given drug candidate. Historically, this has been done as part of preclinical absorption–distribution–metabolism–excretion studies. Such studies are usually performed *in vivo* using animal models such as the rat, dog and monkey. In recent years, however, the use of model systems for studying the *in vitro* metabolism of drug candidates has increased greatly. Studies have centered on the liver, since this is considered to be the major site of metabolism, and have included primary cultures of hepatocytes, precision-cut liver tissue slices, subcellular fractions and heterologously expressed drug-metabolizing enzymes [1–6]. Various methodologies are being used routinely now by researchers to screen for slowly metabolized or potentially highly bioavailable compounds, to predict drug–drug interactions and metabolic profiles in humans. Preclinical predictions can be very useful, since *in vivo* human carbon-14 absorption–distribution–metabolism–excretion and drug–drug interaction studies often occur relatively late in the drug development process.

Interest in these *in vitro* techniques has been fueled largely by the explosion in our knowledge of the various drug-metabolizing enzymes at the molecular level [7, 8]. Another important factor is the increasing availability of liver tissue from human subjects that have undergone biopsy and/or organ donation procedures. Publicity concerning drug interactions with agents such as the antihistamine Seldane® (terfenadine) and toxicological issues surrounding the (H^+,K^+)-ATPase inhibitor omeprazole have also spurred interest, especially when one realizes that these situations could have been predicted with currently available *in vitro* methodologies [9–12].

The following is an account of some of the important issues that are often encountered when using these *in vitro* techniques. Particular emphasis will be placed on their use in determining which form(s) of liver microsomal cytochrome P450 is involved in the metabolism of a given drug, since this seems to be the area of most activity [13–19]. The apparent bias towards the human liver microsome CYP† "superfamily" of proteins results from the fact that our present understanding of this system exceeds that of other drug-metabolizing enzyme systems [20–22].

OBTAINING AND CHARACTERIZING HUMAN TISSUE

It is important to have a steady and reliable supply of material when establishing an in-house "bank" of human tissue. In addition, case histories of the organ donor subjects should be obtained, especially when enzyme-inducing agents such as phenytoin, dexamethasone, phenobarbital and/or rifampicin have been administered prior to removal of the tissue from the donor. At present, this does not seem to be a problem since many of the tissue procurement centers are providing this information. Before embarking on studies with novel test compounds, it is advisable to spend some time characterizing the tissue for metabolic competency, especially when there is large inter-subject variation in enzyme activities [20–22]. This variability is often associated with the age, gender, drug and smoking history of the organ donor subject. Additional factors, such as inflammation and infection, should also be considered; drug-metabolizing capacity may be decreased, due to the effects of cytokines. In many instances, tissue characterization can be easily done using conventionally prepared S-9 and/or

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† Abbreviations: CYP, cytochrome P450; FMO, NADPH-dependent flavin-containing monooxygenase; UDPGT, UDP-glucuronosyltransferase; AO, aldehyde oxidase; HPLRC, high pressure liquid radiochromatography; S-9, 9000 *g* supernatant fraction; CR, carbonyl reductase; ERNDase, erythromycin *N*-demethylase; TOLase, tolbutamide methyl hydroxylase; COHase, coumarin hydroxylase; DEXase, dextromethorphan *O*-demethylase; ERODase, 7-ethoxyresorufin *O*-de-ethylase.

Table 1. Characterization of human liver tissue: measurement of drug-metabolizing enzyme activities in various subcellular fractions

Enzyme(s)*	Substrate	Product†	Assay type	Activity range‡	Reference(s)
UDPGT	1-Naphthol	Naphthyl-β-D-glucuronide	Fluorometric	2.0–12 (1.4–6.6)§	23,24
AO	Benzaldehyde	Reduced $K_3Fe(CN)_6$	Colorimetric	— (11–41)§	—
CR	Benzoylpyridine	NADPH oxidation	Colorimetric	5.5–22.9	25
FMO	*N*,*N*-Dimethylaniline	NADPH oxidation	Colorimetric	1.0–2.0	26
CYP1A2	7-Ethoxyresorufin	Resorufin	Fluorometric	27–182 (32–262)	27
CYP2A6	Coumarin	7-Hydroxycoumarin	Fluorometric	0–2600 (46–1373)	27,28
CYP2D6	[^{14}C]Dextromethorphan	[^{14}C]HCHO	Radiometric	1.0–150 (1.0–220)	29,30
CYP2C9/10	[^{3}H]Tolbutamide	4-Methyl-hydroxytolbutamide	HPLRC	80–340 (90–350)	27
CYP3A	Erythromycin	HCHO	Fluorometric/ Colorimetric	230–1080 (460–1150)	31
CYP2E1	Dimethylnitrosamine	HCHO	Fluorometric/ Colorimetric	180–2990 (270–720)	32,33
CYP2Cmp	(*S*)-[^{14}C]Mephenytoin	4-Hydroxymephenytoin	HPLRC	20–440	34

* All activities were measured using human liver microsomes, except for the AO (9000 *g* supernatant fraction, S-9), FMO (whole homogenate) and CR (cytosol) assays.

† Products that can be measured in order to minimize the use of chromatographic procedures.

‡ Activities are expressed as pmol product formed/min/mg microsomal protein, except for UDPGT (nmol/min/mg microsomal protein), AO (nmol/min/mg S-9 protein), FMO (nmol/min/mg homogenate protein) and CR (nmol/min/mg cytosol protein). Unless otherwise indicated, the data in parentheses were obtained at Abbott Laboratories [35]. The remaining data were obtained from the references cited in the table.

§ Unpublished data.

microsomes, and should cover as many different enzyme systems as possible, e.g. AO (EC 1.2.3.1), FMO (EC 1.14.13.8), CYP (EC 1.14.14.1), and UDPGT (EC 2.4.1.17). Many of the associated assays are sensitive, simple, rapid and do not require the use of chromatographic methods or large amounts of tissue (see examples in Table 1). In those cases where chromatography is required, the assays can be made more rapid with the use of radiolabeled substrates. Therefore, the need for internal standards, standard curves and elaborate extraction procedures can be circumvented. In the case of tissue slices and/or hepatocytes, the concurrent use of commercially available substrates such as 7-ethoxycoumarin or [^{14}C]nicotine as positive controls can be useful, since the metabolism of these compounds involves one or more enzyme systems, e.g. CYP, UDPGT and sulfotransferase (7-ethoxycoumarin), AO, CYP and FMO (nicotine) [36, 37]. In this way, the functionality of the major enzyme systems involved in drug metabolism can be ascertained and the results compared with those of the ever-expanding data base found in the literature.

WHAT IS THE BEST CONCENTRATION OF DRUG TO USE?

While it is important to carry out *in vitro* metabolism studies at "physiologically relevant" drug concentrations, the actual concentrations used will be determined in great part, by the availability of the compound, physico-chemical properties such as its solubility in aqueous-buffered medium, and the sensitivity of the assay used to monitor metabolism. These factors will often apply in the early or "discovery" stages of drug development. In later stages, the use of plasma drug concentrations may be helpful.

However, predicting liver concentrations from plasma data has its difficulties, since many drugs undergo extensive first pass metabolism and/or biliary secretion (e.g. propranolol and terfenadine [38, 39]), and often plasma concentrations do not reflect liver concentrations. The extent of protein binding, either in plasma and/or tissues, should also be considered since the *unbound* fraction will determine the concentration of drug at the sites of metabolism. If the data are available, the concentration of *unbound* drug in human liver can be estimated from the maximal (C_{max}) or steady-state (C_{ss}) plasma concentration, the fraction unbound (f_u) in human plasma, animal tissue distribution data (i.e. tissue/plasma ratio) or *in vitro* human liver tissue/water (homogenate) partition ratio. The estimated concentration of unbound drug in the liver can be converted to units that are routinely used for *in vitro* methods (e.g. μM or mM and not μg/mL or μg/L). When in doubt, it is best to use the widest concentration range possible (e.g. 0.5–1000 μM). One can then use the data to determine *in vitro* steady-state kinetic parameters, such as the Michaelis–Menten constant (K_m) and maximal reaction velocity (V_{max}), and obtain a measure of the intrinsic clearance of the drug (V_{max}/K_m). A wide range of concentrations *in vitro*

increases both the likelihood of encompassing clinically relevant *in vivo* concentrations and the chances of detecting biphasicity in kinetic plots, i.e. so-called "low affinity" (K_m usually >100 μM) and "high affinity" (K_m usually <20 μM) components of metabolism. This high-affinity component often yields the most clinically relevant *in vitro* information, e.g. *S*-warfarin 7-hydroxylation by CYP2C9 ($K_m \leq 4.0$ μM) and dextromethorphan O-demethylation by CYP2D6 (K_m 5–20 μM) [40–42]. Tritiated (high specific activity) compounds may be useful in this instance, since metabolic profiles can be obtained using highly sensitive assay methods.

WHAT IS THE BEST MODEL TO USE?

Selection of the model is largely dependent on the question being asked. However, an "integrated approach" enables one to exploit both the strengths and weaknesses of each model. Such an approach would initially involve an investigation of the metabolism of a drug in an intact cell system (e.g. liver slices and/or cultured hepatocytes), followed by experiments with subcellular fractions. Ideally, these should be conducted with the same tissue sample.

Intact cell models offer several advantages. First, all of the drug-metabolizing enzyme systems are coupled, which can be difficult to achieve with subcellular fractions; second, concerns over possible enzyme instability due to tissue disruption can be minimized; third, cofactor supplementation is not required. These issues may be critical in the early stages of drug development, when the enzymes involved in the metabolism of a drug are largely unknown. Precision-cut liver slices are especially useful, since the procedures involved are not labor-intensive and the technology has improved greatly in recent years [43, 44]. In addition, as techniques for the cryopreservation of human hepatocytes become optimized [45], it may become possible to obtain these preparations from commercial sources. In this way the complete metabolic profile of the drug can be obtained, and any subsequent data attained with subcellular fractions can be seen in perspective. This is important if the data are to be used in support of long-term toxicity testing; the choice of the animal model for such studies is often made on the basis of which species has a metabolic profile that is most similar to that of humans. It is imperative, however, to monitor the levels of the various drug-metabolizing enzymes, since incubations often have to be performed over extended periods of time (2–24 hr) [46, 47].

On the other hand, subcellular fractions can be stored frozen and are thus a convenient source of "concentrated" drug-metabolizing enzymes. There is also the added advantage that cellular uptake of drug is not an issue, so that the rate of parent drug consumption is solely a function of metabolism; the issue of drug uptake may be especially problematic in those instances where carrier-mediated or active (saturable) transport is involved. Subcellular fractions are also useful when identifying which enzyme(s) is involved in the metabolism of a given drug, based on the subcellular localization of metabolism, cofactor requirements and the use of enzyme-selective inhibitors. However, it may be useful to employ S-9 for initial studies, since many drugs undergo both microsome- and cytosol-dependent oxidations. Therefore, microsomal suspensions should be used with care, until the overall metabolic profile of a drug is known or until lack of oxidation by cytosolic enzymes has been verified.

WHAT INFORMATION CAN BE OBTAINED?

Species differences in metabolism and prediction of metabolic profiles in humans

The best models for studying *qualitative* species-related differences in metabolism are liver slices and/or cultured hepatocytes [46–51]. For example, experiments can be carried out *in vitro* with tissue specimens prepared from rat, dog, monkey or rabbit liver and then the drug can be administered to each of these species, so that an *in vitro–in vivo* correlation can be obtained. In doing so, the animal models are validated, which strengthens the predictions made with human tissue slices and/or hepatocytes. However, comparing *rates* of metabolism in this instance should be performed with caution, unless the issue of species-related differences in cellular drug uptake has been addressed.

Species-related differences in metabolism and the prediction of metabolic profiles in humans can also be investigated with subcellular fractions. If the metabolism of a drug candidate is known to be mediated by a given enzyme(s), then the observed species differences in metabolism can be linked to the relative levels of that enzyme within each fraction; these can be determined using marker substrates such as N^1-methylnicotinamide (AO), coumarin hydroxylase (CYP), *N*,*N*-dimethylaniline *N*-oxidase (FMO) and 1-naphthol glucuronidation (UDPGT) (Fig. 1)*. For instance, it is known that liver cytosolic AO activity in dogs is low [52], that AO in humans does not metabolize charged substrates very efficiently [53], and that UDPGT-dependent N-glucuronidation activity tends to be high in human liver microsomes [54]. However, it is important to realize that each of the major drug-metabolizing enzyme systems (e.g. liver microsomal CYP and UDPGT) represent "superfamilies," "families" and/or "subfamilies" of proteins [7, 8]. Even members of the *same* subfamily of enzymes from different species may exhibit distinctly different substrate selectivity, so that a marker substrate for one species may not be suitable for another, e.g. there are CYP forms in monkey, rat, dog and human liver microsomes that immunologically cross-react with antibodies to CYP2A, but exhibit distinctly different patterns of metabolism as in the case of coumarin [55]. It is also important to realize that differences in enzyme levels measured in various subcellular fractions, and hence differences in metabolic patterns, may also be related to differential enzyme stability during tissue processing.

Screening for metabolically "stable" compounds

Screening a series of pharmacologically active

* Data obtained at Abbott Laboratories (previously unpublished).

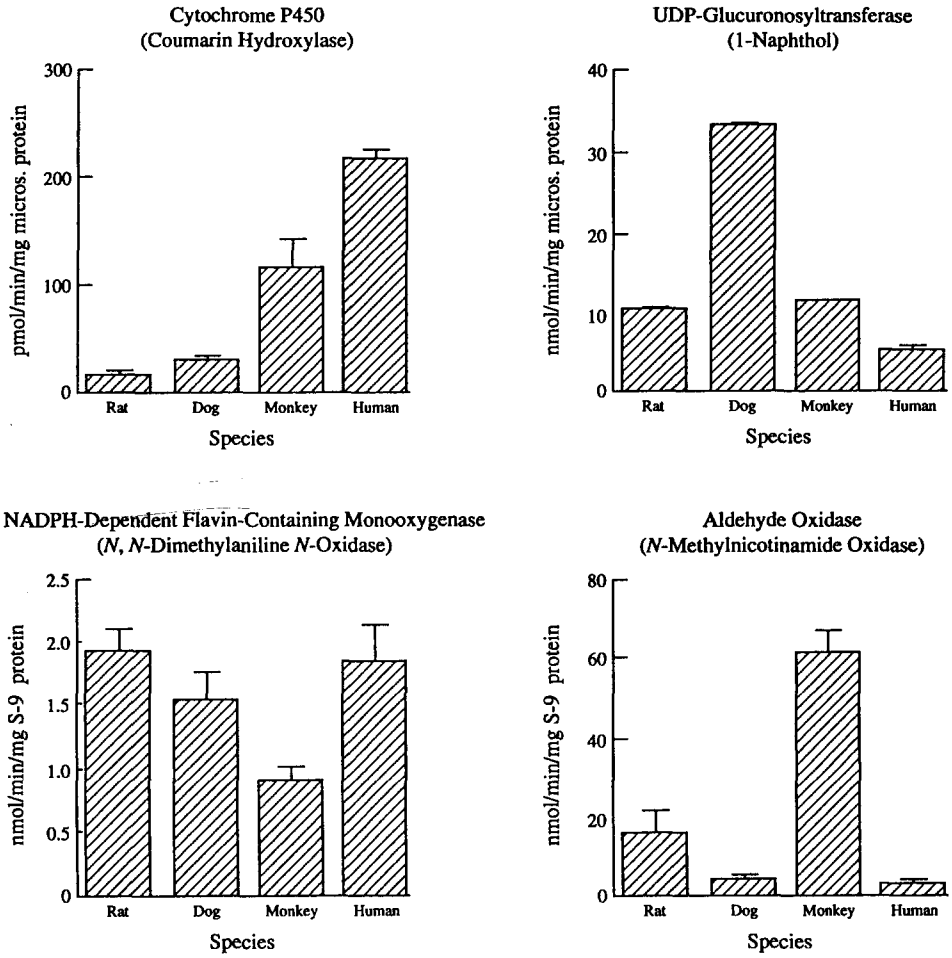

Fig. 1. Species-related differences in the levels of selected hepatic drug-metabolizing enzyme activities. The data represent means ± SD (N = 3) and were determined using either liver microsomes (UDP-glucuronosyltransferase, cytochrome P450) or 9000 *g* supernatant fraction, S-9 (aldehyde oxidase and NADPH-dependent flavin-containing monooxygenase). Subcellular fractions were prepared from Sprague–Dawley rat, beagle dog, cynomolgus monkey and human organ donor liver tissue. Activities were measured in the presence of 1-naphthol (50 μM), *N*,*N*-dimethylaniline (3.0 mM), coumarin (0.2 mM) and N^1-methylnicotinamide (5.0 mM).

compounds occurs in the early or "discovery" stage of drug development, where the emphasis is placed on finding one or more compounds that are "resistant" to oxidative and/or conjugative metabolism. These studies are often carried out at several drug concentrations, and the rate of metabolism is measured based on the disappearance of parent drug, since there is usually no prior knowledge of the metabolic pathways involved. The rationale behind this approach is that compounds resistant to metabolism are likely to exhibit low hepatic intrinsic clearances *in vivo* and hence have potentially higher bioavailability. The assumption is made that hepatic metabolism is the rate-determining step in the "first pass" effect experienced by the drug(s) *in vivo*. Such an extrapolation should be made with caution, since the first pass effect of a given drug may *not* be solely governed by its hepatic intrinsic clearance. Other factors, such as the rate of drug absorption and/or metabolism by intestinal cells and the extent of drug secretion in bile, will also contribute. In addition, the flux of the drug into and out of the circulatory system, and hence the net area under the plasma concentration versus time curve (AUC) after oral dosing, will also be determined by the extent of protein binding and a host of pharmacokinetic parameters (e.g. blood flow to and from the liver). Furthermore, the "lack" of metabolism is often difficult to prove in subcellular fractions and whole cell systems, since this might be the result of either enzyme instability or reduced drug uptake. In this instance, it may be prudent to establish an *in vitro–in vivo* correlation as early as possible, using a select number of compounds of varying bioavailability in a suitable model such as the rat or dog.

Prediction of drug–drug interactions in humans

It is becoming increasingly necessary for phar-

maceutical companies to submit drug applications to regulatory agencies containing some detailed knowledge of the enzyme(s) involved in the metabolism of a given drug. Nowhere is this more prevalent than in the case of the human liver microsomal CYP system. If two or more co-administered drugs are metabolized by or interact with the same CYP form(s), then the likelihood of a drug–drug interaction is increased [14]. In addition, if a given drug is metabolized by polymorphically expressed CYP forms (e.g. CYP2D6 and CYP2Cmp), then the likelihood of exaggerated and/or toxic side-effects in subjects lacking the enzyme(s) may also be elevated [17]. This is of particular importance if the test compound or one of the co-administered drugs is characterized as having a relatively narrow therapeutic index (e.g. theophylline, warfarin, phenytoin and cyclosporin). The emphasis here is placed on determining which form(s) of CYP is metabolizing a given drug *in vitro*. If correctly applied, this information can be indicative of which *in vivo* interaction studies need to be performed, in addition to those involving often-evaluated drugs such as theophylline, phenytoin, warfarin, oral contraceptives and cimetidine, and/or if subject phenotyping is required.

The procedures usually involve the use of correlation analyses, CYP form-selective chemical inhibitors and/or immunoinhibitory antibodies and heterologously expressed CYP proteins [13, 16]. Before embarking on such studies, however, it is imperative to have some idea of the overall metabolic profile of the drug and whether or not CYP is actually involved in its oxidative metabolism, although lack of metabolism by CYP does not preclude an inhibitory interaction.

Correlation analyses. The first of these procedures involves correlating the metabolism of the test compound with CYP selective monooxygenase activities (Table 1) in a panel of liver microsomes from 10 to 20 subjects. The statistical significance of the correlation coefficient is then determined in each case. However, a number of factors should be considered:

(1) Is the K_m of the test compound known? If solubility permits, correlation studies should be carried out at a saturating substrate concentration ($\geq K_m$) so that differences in reaction rates reflect the levels of the CYP enzyme(s) and not substrate depletion effects. However, the use of lower substrate concentrations may be warranted if the drug exhibits biphasic steady-state kinetics and the high- and low-affinity component reflects metabolism by different CYP forms as in the case of warfarin [41, 42].

(2) Beware of "false correlations." A correlation matrix should be constructed with the various CYP marker activities *before* analyzing the test compound. For instance, CYP1A (e.g. 7-ethoxyresorufin *O*-deethylase) activity may be significantly correlated with CYP2D6 activity (e.g. dextromethorphan *O*-demethylase). If the metabolism of the test compound significantly correlates with CYP1A, it cannot be assumed that CYP2D6 is also involved in the metabolism of the drug. Any assumed involvement of CYP2D6 should be verified using selective inhibitors and the appropriate cDNA expressed enzyme.

(3) What is the spread of activities within the panel of microsomes and are the activities clustered? This could weaken the analyses and can be avoided by increasing the number of livers in the panel ($N > 6$).

(4) Are any induced livers included in the matrix? Such samples will have elevated levels of a given CYP, which may dominate a particular correlation. It may be helpful to omit these specimens from the correlation analyses.

(5) In those cases where the correlation appears to be significant, does the correlation line pass through the origin, a factor that many investigators view as being significant [56]? If not, this may indicate that multiple CYP forms are involved in the metabolism of the test compound. In certain instances, however, the lack of a zero interception may be explained, in part, by assays that are inherently insensitive or have high backgrounds, or may be a function of the number of liver samples used in the correlation experiment.

While correlation analyses are very useful, this methodology can be of limited value if a given drug is metabolized by two or more forms of CYP. This is especially evident when one of the major forms of CYP is implicated (e.g. CYP3A forms), which can represent >25% of the CYP pool in human liver microsomes [57]. These forms of CYP will always predominate in correlation analyses and can "mask" the contributions made by minor forms (<10% of the CYP pool), unless the involvement of the two proteins can be unraveled by biphasic steady-state kinetic analyses.

Heterologously expressed enzymes. The potential use of heterologously expressed human drug-metabolizing enzymes has already been reviewed extensively [3]. Presently, only the human CYP forms are commercially available (Gentest Corp., Woburn, MA), but it is only a matter of time before multiple forms of other drug-metabolizing enzymes (e.g. UDPGT and FMO) also become available. The main advantage here is that the various forms of the enzyme are present in a "pure" state. This is ideal for determining which forms of the enzyme are involved in the metabolism of a given drug candidate, and heterologously expressed enzymes are thus an excellent addition to the arsenal of CYP selective inhibitors and substrates. However, there are a number of factors that should be considered when using these. Although the CYP system is discussed, these factors will also apply to any enzyme system:

(1) What is the level of expression of each form of the enzyme? For instance, in commercially available human B-lymphoblastoid cell microsomal preparations, the specific content of the different CYP forms varies from 8.0 to 160 pmol/mg (data available from Gentest Corp.). Therefore, reaction rates with a given test compound should be expressed as nmol/min/nmol CYP and *not* as nmol/min/mg microsomal protein.

(2) If the metabolism of a given drug is specifically mediated by one or two cDNA expressed enzymes, what are the relative levels of these proteins in human liver microsomes? For instance, CYP3A4 ($P450_{NF}$) and CYP2B6 contribute 5–33% and ≤2.0%

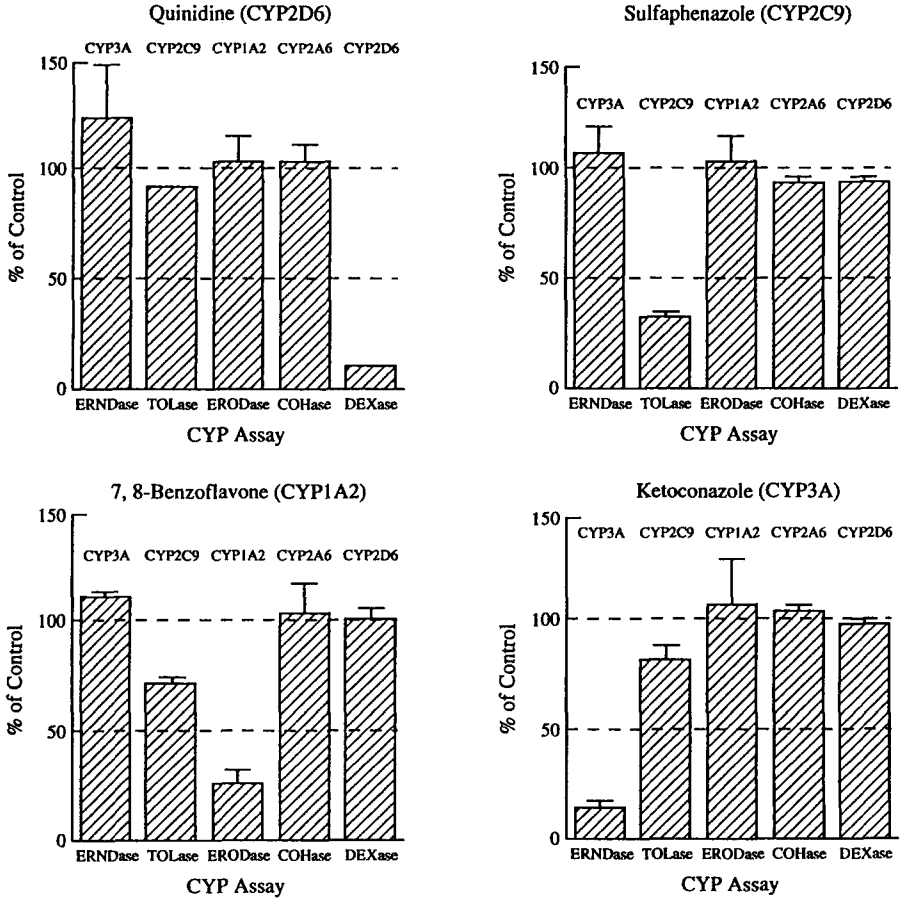

Fig. 2. Inhibition of various cytochrome P450 (CYP) monooxygenase activities in human liver microsomes by some purported CYP form-selective inhibitors. The data represent means ± SD (N = 3) and are expressed relative to control incubations (solvent alone). The final concentrations of quinidine, sulfaphenazole, 7,8-benzoflavone and ketoconazole were 5, 2, 10 and 2 μM, respectively. The CYP form selectivity of each inhibitor (in parentheses) and substrate is indicated. Control activities for the ERNDase, TOLase, ERODase, COHase and DEXase were 1.7 ± 0.3, 0.05 ± 0.01, 0.02 ± 0.01, 0.16 ± 0.01 and 0.1 ± 0.01 nmol/min/mg, respectively. Abbreviations used (final substrate concentration in parentheses): ERNDase, erythromycin *N*-demethylase (0.5 mM); TOLase, tolbutamide methyl hydroxylase (0.1 mM); ERODase, 7-ethoxyresorufin *O*-deethylase (2.5 μM); COHase, coumarin hydroxylase (0.2 mM); and DEXase, dextromethorphan *O*-demethylase (20 μM).

of the total CYP pool in human liver microsomes, respectively [57, 58]. However, the specific content of the cDNA expressed proteins in B-lymphoblastoid cell microsomes is similar (30–55 pmol/mg).

(3) If a given drug is metabolized by more than one CYP isoform, what is the relative K_m of the drug for the different cDNA expressed CYP forms? If the K_m values are similar, then the contribution to metabolism by the more abundant CYP form(s) will predominate in microsomes. Minor forms of CYP (e.g. CYP2A6 and CYP2B6) will only contribute significantly to metabolism if this is mediated with relatively high affinity.

Inhibition studies. While many of the chemical inhibitors are commercially available, the same cannot be said for antibodies to the various human CYP forms. In many cases, investigators are forced to use antibodies to rodent CYP forms. Such an approach warrants caution, since the assumption is made that an antibody raised to CYP forms of one species will cross-react with and immunoinhibit the same CYP subfamily members in a second species [59]. At present, commercially available antibodies have been developed primarily for immunoquantitation of CYP proteins, and their ability to immunoinhibit various monooxygenase activities cannot be assumed.

The various CYP form selective chemical inhibitors should be fully characterized in-house, despite literature claims. Many of these are now commercially available and include both reversible and mechanism-based (quasi-reversible) inhibitors (Table 2). Their selectivity should be pre-determined by incubation with CYP selective substrates (Fig. 2)*. It is important to determine the "window of selectivity"

* Data obtained at Abbott Laboratories (previously unpublished).

Table 2. Cytochrome P450 (CYP) form selective inhibitors

CYP form	Inhibitor	Working concentration (μM)*	Reference(s)
CYP1A2	Furafylline†	20–40	60,61
	7,8-Benzoflavone	1–10	62,63
CYP2A6	Coumarin‡	2–200	
	Diethyldithiocarbamate (DDC)†	15–30	64,65
CYP2D6	Quinidine	1.0–10	66
CYP2C9/10	Sulfaphenazole	1.0–5.0	67,68
CYP2E1	4-Methylpyrazole	15–30	65
	Diethyldithiocarbamate (DDC)†	15–30	64,65
CYP3A	Troleandomycin (TAO)†	50–200	69
	Ketoconazole	≤2.0	70
	Gestodene†	50–100	69

* Concentrations where a number of monooxygenase activities catalyzed by the indicated CYP form are inhibited (≥70%) in human liver microsomes.

† "Mechanism-based" inhibitor of cytochrome P450, requiring NADPH-dependent metabolism to form an inhibitory metabolite–cytochrome P450 (quasi-reversible) complex.

‡ Relatively high-affinity selective CYP2A6 substrate, $K_m \sim 0.5\ \mu$M [55].

in each case, since most of these compounds at high enough concentrations will inhibit one or more additional CYP forms. This becomes very critical when the metabolism of a given drug is mediated by two or more CYP proteins, e.g. ketoconazole is relatively selective for CYP3A activities in human liver microsomes at concentrations equal to or below 2.0 μM, but inhibition of CYP2C forms can occur at higher concentrations [70]. Diethyldithiocarbamate was originally proposed as a CYP2E1 selective inhibitor, but is now known to also inhibit CYP2A6 activities [64, 65]. It is also important to carry out inhibition experiments with samples of human microsomes that have equivalent levels of as many CYP forms as possible. The use of microsomes from induced subjects should be undertaken cautiously, since the elevated levels of a particular CYP form (e.g. CYP3A4) may bias the results.

Identifying which forms of CYP are involved in the metabolism of a given drug is only part of an investigation. In turn, the drug candidate should also be studied as an inhibitor of selected monooxygenase activities. There are a number of examples where a given drug is metabolized by one CYP form but inhibits a second form of the enzyme. A well-documented example is the CYP3A substrate quinidine, which has been shown to be a potent inhibitor of CYP2D6 [66]. Inhibition experiments can be readily performed using human liver microsomes and CYP marker activities (Table 1). However, it is important to bear in mind that many of these activities are characterized by distinctly different K_m values; high-affinity substrates are less likely to be (competitively) inhibited than low-affinity substrates. A well-documented example involves 7-ethoxyresorufin (O-deethylation) and theophylline (N_3-demethylation). Both reactions are catalyzed almost exclusively by CYP1A2 in human liver microsomes, but with very different K_m values: ~0.2 μM and 0.46 mM, respectively [71, 72]. A similar situation exists for CYP2C9-mediated *S*-warfarin 7-hydroxylation ($K_m \leq 4.0\ \mu$M) and tolbutamide methyl hydroxylation ($K_m = 60$–$176\ \mu$M) [42, 67, 68]. Therefore, lack of inhibition of 7-ethoxyresorufin or *S*-warfarin oxidation does not preclude the inhibition of the metabolism of lower affinity substrates. The consideration of the relative K_m values for two or more substrates will also apply *in vivo*, although the relative (*unbound*) liver concentrations and the contribution of a given metabolic pathway to the overall metabolism of each drug (fraction of a dose metabolized by a particular pathway *in vivo*, fm) should also be taken into account.

Predicting induction of drug metabolism in humans

It has been known for some time that many drugs can induce their own metabolism (autoinduction) or the metabolism of concurrently administered drugs. Induction is often clinically significant and represents the net increase in the levels of one or more drug-metabolizing enzymes, as a result of increased *de novo* protein synthesis or protein stabilization [73–75]. The induction potential of a given compound is usually first recognized in rodent models, as part of early toxicity testing (e.g. 2–4 week dose period). More often than not this manifests itself as hepatomegaly and proliferation of the smooth endoplasmic reticulum and/or peroxisomes [75]. The next logical step is to determine if induction can also occur in humans, with the use of primary cultured human hepatocytes, various cell lines (e.g. HepG2 cells), or possibly even precision-cut liver slices [12, 28, 76–79]. First, however, it must be understood that induction in rodents does not necessarily mean that induction will occur in humans. Second, the use of these models as tools to *quantitatively* predict *in vivo* induction in humans is still in its infancy. However, some information can be obtained, such as an *in vitro* rank order of inductive potency for a series of compounds [77]. Furthermore, the inductive potency can be related to the metabolic profile of a given drug, especially when searching for inducing metabolites. These models are also useful for determining the comparative induction profile of a given drug, i.e. which drug-metabolizing enzymes are induced and which are not [76, 78, 79]. The

usefulness of these models will largely be dependent on many of the points that have already been discussed:

(1) Ideally, induction studies with human cells should be conducted with positive controls (e.g. phenytoin, phenobarbital or rifampicin) and alongside more established rodent models such as primary cultured rat hepatocytes [80]; the emphasis here is also on the *in vivo–in vitro* correlation of data in order to strengthen the predictions made with the human models.

(2) Before studying induction of drug-metabolizing enzymes, it is important to determine baseline activities. Induction cannot be clearly defined without detailed knowledge of the changes in basal activity throughout the typical culture period of 1–5 days [76–82]. This means that the various activities should be characterized thoroughly; the difficulty of doing this will depend largely on the sensitivity of the assays employed.

(3) The issue concerning the use of clinically relevant drug concentrations will be paramount. It may be wise to test induction at a number of concentrations, so that the linearity of the inductive response can be determined (i.e. % increase/μM). However, the detection of induction at lower concentrations may be hampered by assay sensitivity, and at higher drug concentrations there is always the possibility of cellular toxicity. Therefore, measurement of leakage of enzymes such as lactate dehydrogenase should be considered.

(4) The possibility of differential drug-uptake may have to be addressed, especially when comparing the relative inductive potency of a series of compounds in one species, or a single compound in multiple species.

CONCLUSIONS

The use of *in vitro* models for studying human drug metabolism, as part of the drug development process, is likely to expand. The major areas of application are likely to be the prediction of metabolic profiles, drug–drug interactions, and screening for metabolically stable compounds. However, their usefulness will largely depend on the quality of human tissue, the extent to which the model(s) is characterized, the *in vivo* relevance of the drug concentrations used, the continuous correlation of *in vitro* with *in vivo* data, and the application of the right model to the right problem. In this way the predictive power of these models can be enhanced, especially if they are used in an integrated manner.

One of the major challenges faced by drug companies is that regulatory agencies are requiring more information at a molecular level, especially concerning the enzymes (e.g. CYP forms) involved in the metabolism of a given drug candidate [14, 83]. While many of these companies are on the "front line", they cannot invest time or resources in the development of tools such as CYP selective antibodies and heterologously expressed enzymes to answer these questions. Therefore, academic institutions must play an important role in this process. The ultimate challenge for all concerned is to apply the growing amount of *in vitro* information to a clinically relevant *in vivo* setting. Once this is achieved, it may be possible to minimize the use of animal models, and to implement better guided, safer, and more cost-effective clinical trials. Given the current economic and ethical trends, this goal appears to be justified.

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