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COMMENTARY

VALIDATION AND USE OF CLONED, EXPRESSED HUMAN DRUG-METABOLIZING ENZYMES IN HETEROLOGOUS CELLS FOR ANALYSIS OF DRUG METABOLISM AND DRUG–DRUG INTERACTIONS

RORY P. REMMEL*† and BRIAN BURCHELL‡

*Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN, U.S.A.; and ‡Biochemical Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee, U.K.

The ability to study individual products from complex gene families for the major endogenous compound and xenobiotic metabolizing enzymes has been achieved through the application of modern molecular biological techniques. Cloning of cDNA sequences encoding a single enzyme and subsequent transient or stable expression into bacteria, yeast, or mammalian cell lines has increased greatly our understanding of enzyme selectivity for a wide variety of substrates. Recent reviews and papers on the use of expressed enzymes from the cytochrome P450 family [1, 2], UDP-glucuronosyltransferases (UDPGTs§) [3–5], sulfotransferases [6, 7], glutathione-*S*-transferases (GSTs) [8, 9], epoxide hydrolases (EHs) [10, 11], and *N*-acetyltransferases (NATs) [12–14] have been compiled [15, 16]. This approach has been particularly beneficial in our understanding of drug metabolism in humans, as access to "normal" human liver in the large amounts needed for traditional enzyme purification is limited. Cloning and expression may be the only method to study those enzymes that are expressed in only minor amounts *in vivo*. A simplified system that allows expression and study of a single gene product is inherently attractive for mechanistic studies of xenobiotic metabolizing enzymes or to examine mechanisms of drug–drug interactions. However, it is equally important to understand some of the inherent difficulties that may arise in the use of these systems as predictors of xenobiotic metabolism. In this commentary, the authors wish to discuss the need for proper validation of these systems and address some of the inherent problems that arise in the use of these *in vitro* models of drug metabolism and drug interactions in humans.

Some of the aspects of validation, standardization, and use of expressed enzyme that should be considered are as follows:

(1) Initial screening of a given substrate against all available forms of the enzyme family and assessment of the relative affinity for this substrate.
(2) Development of specific analytical procedures for the metabolites.
(3) Correct use of controls to determine background activity.
(4) Estimation of the amount of expressed enzyme for kinetic experiments.
(5) Relating the concentrations of expressed enzyme in cells to that in human tissues.
(6) Factors affecting catalytic activity of the enzymes.
(7) Consideration of the *in vivo* relevance of the *in vitro* experiments.

INITIAL SCREENING OF A GIVEN SUBSTRATE

Initial screening procedures to determine the capability of an individual enzyme to metabolize an endogenous compound or a xenobiotic (drug) substrate should be performed (in an ideal situation) with a complete bank of expressed gene products from the various families of a given enzyme system. When the metabolic profile for a drug or xenobiotic is well defined in animals or humans, general screening of a substrate for the production of a specific metabolite or group of metabolites with a single expressed enzyme will provide highly useful information, especially with regard to drug–drug interactions. Qualitative estimates of oxidative metabolism with cytochromes P450 and conjugative metabolism catalysed by GSTs, UDPGTs, sulfotransferases, and acetyltransferases may be obtained when all available forms are used in the initial screening. The ability to obtain a complete metabolic profile will undoubtedly require cooperation between groups working in this area to ensure that all enzymes are screened.

Locating a specific enzyme for a selected substrate may not be possible due to the multiplicity of the drug-metabolizing enzymes. For example, conjugative enzymes such as UDPGTs and sulfotransferases often display broad substrate tolerance, such that simple phenols like 1-naphthol and 4-methylumbelliferone are conjugated by at least three UDPGTs, in the *UGT1* family [17]. On the other

† Corresponding author: Dr Rory P. Remmel, Department of Medicinal Chemistry, University of Minnesota, 8-101 Health Sciences Unit F, 308 Harvard Street S.E., Minneapolis, MN 55455. Tel. (612) 624-0472; FAX (612) 624-2974.

§ Abbreviations: acetyl-CoA, acetyl-coenzyme A; EH, epoxide hydrolase; GSH, glutathione; GST, glutathione-*S*-transferase; NAT, *N*-acetyltransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; UDPGA, uridinediphosphate glucuronic acid; and UDPGT, UDP-glucuronosyltransferase.

hand, a cell expression system for all of the major enzymes responsible for a selected metabolic pathway may not be available. This is exemplified with the UDPGTs where the major enzymes responsible for the glucuronidation of such important substrates as morphine and the tertiary amine drugs such as imipramine have not yet been cloned and expressed.

During the initial screening, it may be desirable to determine the relative affinity of the substrate for an individual enzyme through kinetic experiments in order to evaluate the importance of an individual catalytic step. Pre-existing knowledge of therapeutic concentrations *in vivo* will be important in the interpretation of the relative contribution of an individual enzyme, especially when more than one form of the enzyme is involved in a specific metabolic step. For investigational drugs whose metabolic profile is undetermined, initial investigations will require development of analytical procedures to identify and quantitate the generated metabolites.

DEVELOPMENT OF SPECIFIC ANALYTICAL PROCEDURES FOR THE METABOLITES

The identified products should be compared on both a quantitative and qualitative basis with the profile obtained in humans or in liver preparations that contain all potential gene products. For investigational drugs, it may be desirable to use these systems to predict a complete metabolic profile in humans. In this case, it is critical that as many enzymes as possible be screened and that phase II pathways such as glucuronidation and sulfation also be considered. Prediction of a complete metabolic profile, while possible, may be difficult in some cases. Competition between oxidative and conjugative metabolism may occur with some substrates. Sequential metabolism of oxidative metabolites should also be investigated. In cases where multiple enzymes are involved in the production of a single metabolite, several factors must be weighed in the interpretation of results (see discussion below). Consequently, testing of a single or a limited number of enzymes may yield information that is misleading, especially if the gene product that is studied is present in only small amounts *in vivo* or is only responsible for production of a minor metabolite.

For investigational drugs, these initial studies may take a considerable amount of time as the products must be isolated and identified prior to development of quantitative analytical systems with synthetic or biosynthetic standards. Technological advances such as GLC–Fourier transform infrared spectroscopy (GLC–FTIR), HPLC–mass spectrometry, and tandem mass spectrometry will greatly enhance our ability to identify products in small amounts. For general screening, a universal assay that employs radiolabeled cofactors such as [^{35}S]3′-phosphoadenosine 5′-phosphosulfate ([^{35}S]PAPS), [^{14}C]-uridine-diphosphate glucuronic acid ([^{14}C]UDPGA), and [^{14}C]acetyl-coenzyme A ([^{14}C]acetyl-CoA) can be extremely useful in the initial screening of a large variety of potential substrates towards a given enzyme. These studies may then be used to generate data on structure–activity relationships for an individual enzyme. Radiolabeled cofactors can also be used to locate metabolites in complex extracts with radioactive TLC scanners or radioactive flow detectors for HPLC or GC.

The ability to scale up enzyme expression systems into large scale fermentations in yeast or in transformed cells grown in liquid culture without a solid support will be important if large quantities of metabolites are needed for identification techniques such as NMR, for use as analytical standards, or for pharmacologic or toxicologic testing. Alternatively, long-term incubations with intact cells, i.e. *ex vivo* incubations, may generate large amounts of stable metabolites. Large scale production may be very valuable, especially when chemical synthesis of the metabolite is difficult. This is especially true of conjugated metabolites such as glucuronides, sulfates, or glutathione conjugates. With the recent initiatives of the regulatory agencies to require development of a single stereoisomer of investigational drugs, stereospecific syntheses of several metabolites may be a costly and involved process. Thus, biosynthesis in cell expression systems should prove to be a cost-effective alternative to chemical synthesis of metabolites.

CORRECT USE OF CONTROLS TO DETERMINE BACKGROUND ACTIVITY

Control experiments for screening of enzymatic activity in expressed cell systems towards a given substrate must be carefully considered. The background activity of the cells used for expression, i.e. bacteria, yeast, or mammalian cells, must be correctly subtracted. For example, V79 cells have been used for the stable expression of cytochrome P450 enzymes [18] and UDPGTs [19]. V79 cells have low or non-existent P450 and UDPGT activity when transfected with an expression vector that does not contain a viable coding sequence [19, 20]. For enzymes such as epoxide hydrolase and GST, basal activity may be more substantial as these activities are ubiquitous in most cells [21]. In addition, typical analytical controls for cell homogenates or microsomes that lack enzyme cofactors (-NADPH for cyt. P450; -UDPGA for UDPGT; -PAPS for sulfotransferase, -acetyl-CoA for acetyltransferase, -glutathione (GSH) for GST, etc.) or that consist of incubations with boiled enzyme preparations should also be run.

Choice of a cell vehicle for expression may be influenced by other factors related to metabolism other than the enzyme itself. For example, HepG2 cells, a hepatocellular carcinoma cell line, have been used as an expression vehicle for cytochromes P450, because it is assumed that this liver-derived cell line has all the necessary cofactors, coenzymes, and transport systems required for xenobiotic metabolism [22]. HepG2 cells selectively retain some, but not all P450 enzyme activities compared with freshly isolated hepatocytes [22, 23] and also contain other metabolizing enzymes such as epoxide hydrolase, GSTs, UDPGTs, and sulfotransferases that may compete for substrates or catalyse sequential metabolism of oxidative metabolites generated by an expressed P450 enzyme. This property may be useful to study sequential metabolism in an *ex vivo* incubation, especially if the generated oxidative metabolites are unstable, e.g. arene oxides.

However, the presence of these additional enzymes may be confounding factors for interpretation of the role of a single enzyme in the metabolism of a specific substrate. Thus, an investigator may wish to use more than one cell environment depending upon the questions that are to be answered.

ESTIMATION OF THE AMOUNT OF EXPRESSED ENZYME AND CATALYTIC ACTIVITY

An accurate estimate of the catalytically-active enzyme content is critical for comparison between cell lines containing different forms of the enzyme or for comparisons with human liver microsomes. Levels of expression may vary widely depending on the mode of transfection and the cell type used for expression. For example, expression of rat P450IIA1 was 0.02% of total cellular protein in yeast, 0.2% in HepG2 cells with vaccinia virus-mediated transfection, and 2.0% in *Spodoptera frugiperda* cells with a baculovirus-mediated expression [24]. The amount of active expressed enzyme per milligram of protein will depend upon such factors as rates of translation and transcription, mRNA stability, subunit formation, regulation of enzyme activity by protein phosphorylation, and enzyme degradation rates. For cytochrome P450 activity, additional factors such as heme synthesis and degradation [24] and amounts of cytochrome P450 reductase [25] will affect enzymatic activity. Therefore the molar amount of active enzyme per milligram of cellular or "microsomal" protein will be variable, although consistent stable expression is achievable within a single recombinant cell line. Some methods that have been employed to estimate catalytically-active enzyme concentrations include direct spectral measurement, estimation of concentration based on the units of enzyme activity present for a selected substrate, quantitation of mRNA, and use of antibodies as tools for quantitation. Each of these techniques will be discussed in detail below.

Direct measurement of active enzyme

For cytochrome P450, the problem of measuring active enzyme is somewhat alleviated as the nanomoles of enzyme present can be estimated by the CO binding to reduced cytochrome at 445–455 nm. For experiments with P450, the expression of kinetic rates could be based on the nanomoles of P450 rather than on a milligram protein basis. However, this may not always be possible. For example, spectral measurement of P450-mediated 17-α-hydroxylase activity transiently expressed into COS-1 cells was difficult because of the low level of expression [26]. Measurement of the expression of mitochondrial P450s was reported to be impossible due to high background absorption observed in COS-1 cells [27]. Thus, in cases of low expression, the amount of P450 may be approximated by immunoblot analysis after titration of the antibody with a specific P450 standard. For most of the other drug-metabolizing enzymes, no direct spectral measurement is possible, and when a specific antibody or purified protein is not available, results are often expressed on the basis of the milligrams of total cellular or microsomal protein.

Estimation of enzyme amounts by comparison with a specific substrate activity

For enzymes such as UDPGT, one potential option for comparison with the activity in liver would be to compare kinetic results on the basis of a defined unit of activity for a selected substrate. For this method to be effective, the chosen substrate should have a high turnover rate and should be specifically catalysed by only a single enzyme. The limitation of slow turnover substrates may be overcome by an "*ex vivo*" experiment, wherein the substrate is incubated with the expressed cells in tissue culture for long periods of time (24–48 hr), thus allowing the metabolite(s) to accumulate. As discussed previously, identification of a specific substrate may not be possible because of enzyme multiplicity. To use a selected substrate activity as a quantitative tool, it is important that all potential forms of the enzyme family be screened. The combined use of selected inhibitors and activators, correlation of activities, development of specific antibodies, and availability of each of the expressed isozymes can ensure the specificity of this approach.

Measurement of mRNA

If direct measurement of the protein is not possible, another potential method to measure expression levels is to estimate the mRNA levels by northern blotting with a specific cDNA sequence. Unfortunately, this method is imprecise and the mRNA concentrations may not necessarily reflect the concentrations of catalytically-active enzyme. An alternative method to quantitate mRNA with better sensitivity and precision is by an RNAse protection assay [28]. There are several examples where mRNA concentration does not correlate with enzymatic activity or with western blots. For example, liver obtained from persons with Crigler–Najjar Type I disease contains very low to nonexistent activity towards bilirubin, yet mRNA for HP2 and HP3, two UDPGT isoforms responsible for bilirubin conjugation, can be detected by northern blotting (Sutherland L, Ebner T and Burchell B, unpublished results). Individuals with the slow acetylator phenotypes have normal liver levels of NAT mRNA; however, the mRNA of the M1 phenotype containing two point mutations was poorly translated [12]. In contrast, protein produced from the M2 slow acetylator phenotype containing a point mutation resulting in a single amino acid change was found to be 4-fold less stable than the rapid acetylator protein. The proteins for both the M1 and M2 phenotypes had identical binding properties for sulfamethazine and acetyl-CoA. Given the variability in transcription and translation rates and enzyme stability, use of mRNA levels as an indicator of expressed protein cannot be recommended without considerable validation.

Quantitation of enzyme amounts with antibodies

In cases when spectral measurement of active enzyme is impossible, the preferred, albeit indirect, method for quantitation of the expression of enzyme in cell lines is through the use of antibodies. Quantitation with antibodies is indirect, because the antibody may not necessarily distinguish between active and inactive proteins. Densitometric evaluation of western blots or development of an ELISA assay may be used to compare the level of expression

between cell lines or between cell lines and human liver. For accurate quantitation, it is necessary to have a specific antibody and a source of pure protein, so that the relationship between antibody binding and the molar amount of enzyme may be estimated. The first problem in this approach is in the development of a specific antibody. Application of modern molecular biological techniques coupled with the increased knowledge of protein structure and folding may solve many of the problems associated with lack of specificity in antibodies prepared against purified proteins by either polyclonal or even monoclonal techniques. An excellent example of problems associated with the lack of specificity of monoclonal antibodies was described recently by Wrighton *et al.* [29] in an attempt to quantify human cytochrome P450IIB in liver. Monoclonal antibodies raised to two separate epitopes on rat P450IIB1 unexpectedly recognized human IIE1, despite a lack of binding to rat IIE1, thus obviating the use of the rat antibody as a quantitative tool for human enzymes. To overcome lack of specificity, antipeptide antibodies prepared against hydrophilic regions of membrane bound proteins such as P450 and UDPGT have shown high or absolute specificity for single isozymes [30, *]. This may be accomplished either by coupling a synthetic peptide to a carrier protein or by preparing fusion proteins of an unrelated protein to a specific peptide to generate an immunogen [31]. For example, the latter method was used to prepare a specific antibody to P450IIB2 that did not cross-react with P450IIB1, despite a sequence homology of 98% for these two isoforms [31]. Again, it is important to have access to all of the individual enzymes so that one can ensure the specificity of the antibody. In addition, development of a quantitative assay such as an ELISA will require the availability of pure protein, so that the relationship between antibody binding and milligrams of enzyme is achieved. Pure protein in large amounts should also be much easier to obtain from the expressed enzyme systems than from liver. Overexpression and purification from bacterial or yeast expression vehicles may be useful in this regard, as these cells are easy to grow in large quantities. However in bacteria, protein that is produced may not be correctly folded, and therefore immunoreactivity may be different than for microsomal proteins that are expressed into cells containing endoplasmic reticulum. It should also be possible to purify protein from expressed mammalian cell systems, especially if affinity chromatography is used. Specific affinity columns such as hexyl glutathionyl-Sepharose for GST [32, 33], ATP-agarose for sulfotransferase [34] or UDP-hexanolamine-Sepharose [35] for UDPGT may be used, or an alternate attractive approach would be to prepare an affinity column with the raised antibody. Once a specific antibody is obtained, a simple titration of antibody response with purified protein would allow one to use the antibody for quantitative purposes and a correlation with the enzyme in human tissues.

* Clarke DJ, Coughtrie MWH and Burchell B, Anti-peptide antibodies to a human phenol UDP-glucuronosyltransferase (HP1-UDPGT)–Analysis of biological variations in UDPGT expression. *Workshop on Glucuronidation: Its Role in Health and Disease*, Noordwijkerhout, The Netherlands, 1991 (abstr.).

RELATING THE CONCENTRATIONS OF EXPRESSED ENZYME IN CELLS TO THAT IN HUMAN TISSUES

After titration of the antibody with protein is established, then it should be possible to evaluate the level of expression for an enzyme activity in a cell line and in human liver. One must be aware, however, that antibody techniques do not necessarily measure catalytically-active enzyme as they may bind to an apoenzyme, e.g. P450 without heme, or to a gene product that has been processed incorrectly after translation or is localized in an "unnatural" subcellular compartment. In human tissues, mutant gene products that are catalytically inactive may be measured. If an ELISA assay is used, partially degraded protein with an intact immunoreactive site may give a false positive signal.

A non-specific antibody may also be useful in the comparison of the levels of expression of individual gene products from a single family expressed separately in different cell lines. If the antibody is equally cross-reactive, the level of expression and thus catalytic activity towards a given substrate can be adjusted based on western blotting intensity. For example, in the *UGT1* gene family, all forms share a highly conserved constant region [36–38]. This region probably encodes the UDPGA binding site [3]. Similarly, sequence analysis of the P450IID family revealed a highly conserved exon in all four genes that encodes a region corresponding to the heme-binding site, an area that is conserved in all P450 gene families [39]. An antibody raised to these constant regions should cross-react equally with all isoforms, and such an antibody could be used to compare the abilities of cell homogenates containing the individual enzymes to catalyse multiple substrates in screening experiments. However, the use of such an antibody may be problematic for comparisons with human liver as the individual enzymes may not be separable on a sodium dodecyl sulfate–polyacrylamide gel.

If the antibody is also inhibitory to catalytic activity, the antibody itself may be a useful tool to estimate the contribution of a single enzyme to the overall catalytic activity towards a specific substrate in human liver samples. This approach has been employed successfully to estimate the amount of pharmacogenetically controlled enzymes such as *S*-mephenytoin-4-hydroxylase [40] and the debrisoquine/sparteine-metabolizing enzyme, P450IID6 [41, 42], towards drug substrates in human liver microsomes. Immunoinhibition with a selective antibody has also been used to characterize the sex-specific expression of hydroxysteroid and estrogen sulfotransferases [43]. Once a battery of specific antibodies becomes available, it will be important to estimate the relative abundance of the individual enzymes in human tissues in order to predict the relevance of a specific enzyme-catalysed pathway to overall drug elimination.

FACTORS AFFECTING CATALYTIC ACTIVITY OF EXPRESSED DRUG-METABOLIZING ENZYMES

Homo-oligomeric or hetero-oligomeric enzymes

GST is an enzyme that forms both homodimers

and heterodimers. Relating catalytic activity of cellular-based expression systems to activity in tissues for GSTs may be difficult, since only homodimers will be formed when a single enzyme is expressed [32]. If multiple gene products are expressed, the factors controlling formation of relative amounts of homodimers and heterodimers may be different in the expression cells compared with tissues such as liver [44]. One approach to study catalysis by heterodimers may be to form a chimeric protein that contains both subunits in a single protein product [45].

Similarly, UDPGTs have been proposed to be dimers and tetramers in hepatic microsomes based on radiation inactivation analysis [46, 47]. UDPGT tetramers may be responsible for the formation of bilirubin diglucuronide from bilirubin monoglucuronides, whereas a monomeric form of the enzyme apparently catalysed the initial conjugation of bilirubin [46]. These catalytically-active membrane bound complexes of UDPGTs may prove to be homo-oligomers, since cloned expressed bilirubin UDPGT catalysed the formation of bilirubin diglucuronides [17].

How does cytochrome P450 complex formation affect functional activities? Obviously interaction with P450 reductase is essential [1] and association with cytochrome b_5 may be required for certain xenobiotic oxidations [48]. The existence of oligomeric forms of cytochrome P450 has been suggested based on sedimentation equilibrium experiments [49]. Cross-linking experiments have indicated that P450 LM2 has a hexameric structure [50]. Further study will be necessary to ascertain if hetero-oligomeric interactions between P450s affect their function.

Membrane incorporation, protein orientation, and latent activity

For microsomal drug-metabolizing enzymes, an additional and perhaps uncontrollable factor is the nature of the membrane environment. Certain drug-metabolizing enzymes, notably UDPGT, exhibit high levels of latency in hepatic microsomes, which is affected by the membrane environment [3, 5]. Therefore, an additional problem in the expression of drug-metabolizing enzymes may be the variable nature of the endoplasmic reticulum membrane, especially when the enzymes are expressed in bacteria, yeast, insect cells, or in cells derived from tissues in which the enzymes do not normally exist. However, latency of UDPGT may be retained if expressed in alternate cells, as rat and human phenol UDPGTs stably expressed in Chinese hamster fibroblast cells were activated by the detergent Brij 58 and stimulated by UDP-*N*-acetylglucosamine.*

Unfortunate cloning of mutant enzymes

The heterogeneity of the human population must be considered as an additional factor when dealing with genetic material from a single source. Commercial human cDNA liver libraries have commonly been used as the source of the genetic code for cloning of individual enzymes. The library may be generated from a single individual, and it is possible that this individual may not be representative of the "normal" human population. Point mutations are common and a mutation in the active site may lead to an enzyme that is catalytically different or has an altered substrate selectivity compared with the "wild type" enzyme. Furthermore, cloning by polymerase chain reaction (PCR) techniques may also introduce unfortunate mutations into the DNA. If a mutant enzyme is cloned and expressed, one could be misled in initial kinetic experiments. However, through the growing use of PCR analysis for pharmacogenetic studies [2, 15], it should be possible to determine if the expressed enzyme is genetically identical to the most prevalent enzyme present in the normal population. This widely used procedure can serve as an additional check to ensure that the enzyme is representative. Kinetic analysis of mutant enzymes may be valuable for probing the structure of the active site of enzymes and determining their mechanism of catalysis.

Value of apparent kinetic parameters for cloned, expressed human drug-metabolizing enzymes

One of the most difficult problems in the use of expressed enzyme systems is in the interpretation of kinetic experiments. Parameters such as V_{max} and the V_{max}/K_m ratio (catalytic rate or potential, an estimate of intrinsic clearance) are dependent upon the amount of active enzyme present and should therefore be based on an estimate of the molar amount of active enzyme as determined by the methods described above. Kinetic analysis of individual substrates may provide useful predictive data, especially when different enzymes are expressed *in vivo* at similar levels. Specific high affinity of a single enzyme for a certain substrate may indicate that the substrate would be suitable as a probe indicator of the enzyme activity in human tissues. Kinetic experiments may be performed with purified, reconstituted enzymes, microsomal preparations, cell homogenates, or whole cells. If whole cells are used, the concentrations of enzyme cofactors, e.g. NADPH for P450, PAPS for sulfotransferase, UDPGA for UDPGTs and GSH for GSTs, may be significantly different than the cofactor concentration in the liver *in vivo* or in isolated hepatocytes. In some cases catalytic activity may be improved in cells by incorporating biosynthetic precursors into the medium. For example, the rate of glucuronidation can be improved in expressed cells by incorporating glucose into the media (Burchell B, unpublished observations). By adjusting media conditions, kinetic data similar to metabolic clearance values *in vivo* could be obtained, thereby providing some prediction of metabolic handling for extrapolation to humans.

Analysis of potential drug–drug interactions with recombinant cells in culture

One of the most useful applications of a cell-based expression will be to evaluate the extent and investigate the mechanism of drug–drug interactions with human enzymes. One of the major cytochromes P450 expressed in liver and intestine is P450IIIA. Screening experiments with cloned expressed P450IIIA3 and P450IIIA4 have revealed that this

* Pritchard M, Fournel-Gigleux S, Bock KW, Siest G and Burchell B, Manuscript in preparation.

enzyme is responsible for the metabolism of over twenty drugs [51]. Important drug interactions between macrolide antiobiotics such as erythromycin and substrates such as cyclosporine A have been studied in this system [51]. Recently, Rettie *et al.* [52] have implicated P450IIC9, a high affinity *S*-warfarin hydroxylase, in the numerous drug interactions involving the oral anticoagulant, warfarin. Human microsomal epoxide hydrolase expressed in yeast has been used to study the interaction between valproic acid and the major active metabolite of carbamazepine, carbamazepine epoxide [11]. This methodology should prove to be extremely useful in the prediction of potential drug interactions for investigational drugs prior to Phase II clinical trials in patients.

CONSIDERATION OF THE *IN VIVO* RELEVANCE OF THE *IN VITRO* EXPERIMENTS

Undoubtedly, there will be significant variability in expression of individual forms of an enzyme in the population, due to gender, age, and genetic and environmental factors. However, general estimates of the abundance and variability of a selected enzyme may become apparent if one examines selected populations, e.g. males vs females, children vs adults, smokers, persons taking inducing anticonvulsant drugs, persons with liver failure, genetically poor metabolizers, and persons with inborn errors of metabolism such as Crigler–Najjar syndrome. This information is critical, because the importance of drug interactions or the catalytic activity of a selected enzyme towards an investigational drug may be greatly overestimated if the enzyme in question is expressed in low amounts. While some of this information is already available for the cytochrome P450 enzymes, relatively little is known about the abundance of individual forms of UDPGTs, sulfotransferases, NATs, GSTs, and EHs.

An approach that has been used frequently to associate activities to a specific enzyme in human liver is to correlate the activity of a selected substrate towards that of a known substrate in a variety of human liver samples. For example, ethinylestradiol glururonidation has been highly correlated to bilirubin conjugation in human liver microsomes ($r = 0.92$), and this steroid substrate is conjugated by HP3, an isozyme of the *UGT1* superfamily [53]. High correlation values (>0.9) with nifedipine oxidase activities have been used to implicate the metabolism of selected substrates for cytochrome P450IIIA activity [54, 55]. However, at least four forms of human P450IIIA have been sequenced (P450IIIA3, A4, A5, and A7) and activities associated with P450IIIA such as nifedipine oxidation and erythromycin N-demethylation may be catalysed by several isoforms, thus complicating the interpretation of this method [56]. For example, P450IIIA4 catalyses both nifedipine oxidation and erythromycin N-demethylation, whereas P450IIIA5 catalyses only nifedipine oxidation [23]. Other P450 enzymes may be involved in erythromycin N-demethylation in some species as significant correlations between rates of $^{14}CO_2$ production in the erythromycin breath test and either immunoreactive cytochrome P450p (P450IIIA) or holocytochrome P450 have been observed in rats induced with phenobarbital and dexamethasone, but not in untreated rats [57].

An excellent example of the difficulties inherent in the multiplicity of enzymes is found in the metabolism of aflatoxins. One of the uses that has been proposed for P450 expression systems is for mutagenicity testing [58]. Stably expressed rat cytochrome P450IIB1 in V79 cells was shown to activate aflatoxin B_1 to a mutagenic metabolite by Doehmer *et al.* [18]. It was demonstrated that P450NF (P450IIIA) appeared to be the principal P450 enzyme involved in the bioactivation of aflatoxin B_1 in human liver by a variety of techniques including immunoinhibition and correlation with nifedipine oxidase activity [59], although other P450s were also implicated in some persons. Shortly after this, it was demonstrated that five out of twelve expressed forms of P450 (P450 IA2, IIA3, IIB7, IIIA3, and IIIA4) were capable of activating aflatoxin B_1 to mutagenic metabolites [22]. Immunoinhibition experiments with specific polyclonal anti-rat liver P450 antibodies revealed that inhibition of aflatoxin B_4 activation in human liver microsomes ranged from 10% for anti-P450IIA3 antibody to 65% with anti-P450IIIA3/A4 antibody. Thus, depending on the relative concentrations of the various enzymes in humans and the relationship to environmental factors such as smoking, interpretation of the role of a single enzyme is problematic. These studies also demonstrate that experiments based on correlation of activities must be interpreted with caution. As long as multiplicity of isoenzymes is recognized for a gene family, data gathered with antibodies that broadly recognize specific gene families may still be highly useful for pharmacogenetic studies and have been important in our understanding of polymorphism of drug metabolism.

CONCLUDING REMARKS

The availability of expressed, catalytically-active gene products of the major drug-metabolizing enzyme systems will provide useful tools for the study of drug metabolism in humans. Examination of the role of expressed enzymes in the metabolism of endogenous compounds will also be important. For example, drugs that cause jaundice can be screened for their ability to directly inhibit bilirubin UDPGTs. However, correlations between *in vitro* systems and the *in vivo* metabolism and clearance of drugs, while attractive, will be problematic without more extensive development as described above. Additional considerations should include evaluation of drug metabolism and particularly drug interactions at relevant therapeutic concentrations and the possibility of metabolic switching between oxidative, reductive, and conjugative pathways. Consideration of absorption and bioavailability, extrahepatic metabolism, the contribution of protein and tissue binding, drug transport, and other pathways of elimination (renal and biliary) to the overall disposition of drugs is also necessary. In this commentary, the authors have attempted to discuss some of the problems that may be associated with the use of protein expression systems. Given the

current constraints on the availability of human tissue and animal experimentation, it is likely that these systems will be widely used. An understanding of some of the difficulties associated with their use, coupled with additional input from the scientific community, should help to optimize the development and utilization of cell-based expression systems for individual drug-metabolizing enzymes.

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