Invited Review

Noninvasive tests of CYP3A enzymes

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Received 10 January 1994 and accepted 5 March 1994

Introduction

Cytochromes P450 belonging to the CYP3A gene subfamily account for up to 25% of the total cytochrome P450 present in adult human liver, and account for the majority of cytochrome P450 present in human small bowel. The importance of the CYP3A enzymes is most established with respect to the metabolism of xenobiotics, particularly medications. The number of drugs metabolized chiefly by CYP3A enzymes is expanding rapidly and includes a wide variety of compounds in many different therapeutic classes (reviewed in Guengerich, 1992a; Watkins, 1992; Wrighton & Stevens, 1992). Several pharmaceutical companies are now screening new chemical entities in development to determine whether they are metabolized by CYP3A enzymes. The finding that a drug is a substrate for CYP3A enzymes has three major implications. First, because CYP3A enzymes are relatively abundant in the intestinal mucosa (Kolars et al., 1992; Watkins et al., 1987), CYP3A substrates may have poor oral bioavailability due to extensive 'first pass' metabolism at the level of the intestine (Kolars et al., 1991). Second, CYP3A substrates may be susceptible to certain drug interactions because some CYP3A enzymes are induced or inhibited by certain medications (reviewed in Watkins, 1992; Wrighton & Stevens, 1992). For example, the immunosuppressant cyclosporine A and the antiasthmatic terfenadine have been shown to be metabolized chiefly by CYP3A4 (Kronbach et al., 1988; Combald et al., 1989; Chul-ho et al., 1993). This enzyme has been shown to be inhibited by erythromycin and ketoconazole (Pichard et al., 1990), and this appears to be the reason that patients receiving these drugs may develop toxic responses when treated with cyclosporine A or terfenadine. Conversely, CYP3A4 is inducible by treatment with antiseizure drugs and the antibiotic rifampicin (Pichard et al., 1990), and this appears to explain why patients treated with these medications require increased dosing of cyclosporine A. In drug development, the discovery that a new chemical entity is largely metabolized by CYP3A enzymes therefore suggests the potential for similar drug interactions.

The third major implication of identifying a given drug as a CYP3A substrate relates to the observation that there are large interpatient differences in the content and catalytic activity of CYP3A enzymes in both liver and intestine (Aoyama et al., 1989; Wrighton et al., 1989; Schmucker et al., 1990; Waziers et al., 1990; Forrester et al., 1992; Kolars et al., 1992; Lown et al., 1993). These differences appear to exist in the absence of medications known to influence CYP3A enzyme regulation and may largely reflect genetic or possibly dietary factors. In at least some cases, it has been shown that this variability accounts for interpatient differences in the kinetics of certain drugs (Watkins et al., 1990; Turgeon et al., 1992). Finding that a new chemical entity is largely metabolized by CYP3A4 may therefore imply that significant interpatient differences in elimination kinetics will be observed.

CYP3A enzymes also play potentially important roles in the metabolism of ubiquitous and potentially harmful dietary contaminants (reviewed in Guengerich, 1992b). CYP3A substrates include aflatoxin B1 and other mycotoxins (Shimada & Guengerich, 1989; Aoyama et al., 1990), pyrrolizidine alkaloids (Miranda et al., 1991), and arylhydrocarbons (including benz[a]pyrene) (Shimada et al., 1989b) and heterocyclic amines (McKinnon et al., 1992). A logical but untested hypothesis is that interindividual variability in expression of CYP3A enzymes may account in part for interindividual differences in susceptibility to toxicity from those xenobiotics which are substrates for CYP3A enzymes (Guengerich et al., 1992b). If this is shown to be the case, it may be possible to reduce a given individual's risk from a
toxic CYP3A substrate by treating the individual with safe medications known to influence the regulation of CYP3A enzymes (i.e. therapeutically regulate the CYP3A enzyme involved up or down and thereby assure safe ‘handling’ of the xenobiotic by the body). CYP3A enzymes also appear to be inducible, at least in rodents, by many environmental contaminants, including polyhalogenated biphenyls (PCBs and PBBS) (Dannan et al., 1983). Expression of CYP3A enzymes may therefore serve as a biomarker for exposure to such agents in some situations.

Finally, CYP3A enzymes have been shown to play potentially important roles in the metabolism of some endogenous steroids, including cortisol, estradiol, and dihydroepiandrosteredione (DHEA) (Kitada et al., 1987; Waxman et al., 1988; Kerlan et al., 1992). Human uterus and placenta have recently been shown to contain a CYP3A enzyme which may be hormonally regulated in these tissues (Schuetz et al., 1993b). Reasonable hypotheses are that interindividual variability in CYP3A expression may have physiological consequences and that induction of CYP3A enzymes by environmental contaminants could result in reproductive toxicity.

In summary, the CYP3A enzymes have emerged in the last 5 years as a major subfamily of enzymes involved in the metabolism of medications, potentially harmful environmental contaminants, and probably steroid hormones. There is marked interindividual heterogeneity in the expression of CYP3A genes. Patients with unusually high or low CYP3A activity should be at increased risk for subtherapeutic or toxic responses to many commonly used medications, and may have altered risks for some environmental diseases. However, the true implications of having ‘high’ or ‘low’ CYP3A activity are largely unknown. The lack of progress in this area in part reflects the difficulty in quantitating activity of CYP3A enzymes in the living person. It is possible to perform liver and intestinal biopsies, but these are invasive procedures that are impractical or unethical in many situations.

The purpose of this review is to discuss the current status and future prospects for the development of tests for CYP3A activity based on the metabolism of carefully selected ‘probe’ compounds. These tests will be termed ‘noninvasive’ in this review, although most involve invasion of the body to some degree (blood sampling, intravenous injection, etc.). Noninvasive tests are already accepted to determine an individual’s phenotype for acetylation, debrisoquin hydroxylation (CYP2D6) and mephenytoin hydroxylation (a CYP2C enzyme). For several reasons that will be discussed, the development of similar probe-based tests for CYP3A activity has been problematic. In order to explain why this is so, it is first necessary to consider general issues for any probe-based test.

General considerations

It is obviously important that the selected probe be safe. In this regard, an endogenous compound such as a hormone, would be an ideal probe. A second essential criterion is that the enzyme of interest be the exclusive catalyst of the metabolic pathway that will be measured. This is most easily shown by demonstrating in human liver microsomes that the metabolic pathway is completely inhibited by antibodies or suicide substrates that specifically inactivate the enzyme of interest. It is important to note, however, that simply demonstrating that a specific enzyme exclusively catalyses the pathway of interest in liver microsomes does not mean that the probe will be useful in an in vivo test. Microsomal studies do not exclude the possibility that liver cytosolic enzymes or extrahepatic enzymes may be important in the metabolism of the probe in vivo. Even if the microsomal enzyme catalyses the major metabolic pathway for the probe in vivo, the intrinsic activity of the enzyme may not always be rate limiting in the in vivo metabolism. For example, a drug may be shown to be exclusively metabolized in liver microsomes by a specific cytochrome P450; however, the rate of systemic clearance by this metabolic pathway in vivo may be strictly a function of liver blood flow (if the probe is highly extracted by the liver). Hence, in general, compounds with high hepatic extraction are less likely to be useful as in vivo probes (if they are administered intravenously).

Other processes occurring in vivo (but that may not be evident from in vitro studies) may limit the usefulness of the probe. For example, alternate pathways of metabolism could be problematic, especially if the enzymes involved in alternate pathways show significant interindividual variability. It is therefore generally desirable that the target pathway be the major route of metabolism in the body. An additional potential problem would arise if metabolites generated in vivo are also substrates, inhibitors, or inducers of the enzyme of interest. It is therefore generally preferable for the metabolite generated by the probe to be rapidly excreted from the body.

Once a suitable probe is identified, a traditional pharmacokinetic study using the probe should estimate the catalytic activity of the enzyme of interest. In particular, the clearance of the probe (the volume of blood cleared of probe over time) should provide the best estimate of the amount of enzyme present in the liver. If the probe has multiple metabolic pathways in the body, then the ‘fractional clearance’ corresponding
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to the pathway of interest should be an appropriate measure. If the probe is highly protein bound, and if the degree of protein binding varies among subjects, clearance of unbound probe may correlate most closely with the total amount of target liver enzyme.

Clearance measurement is preferable to blood half life because the latter is dependent on volume of distribution of the drug, which will usually vary among subjects. However, accurate clearance measurements involve multiple blood level determinations over at least a few half-lives of the probe. An alternate approach would be to obtain a single blood sample at an appropriate time. However, any single blood level determination of the parent probe or of the metabolite of interest may not predict clearance if there are significant intersubject differences in volume of distribution and or/protein binding. The ratio of the blood concentration of metabolite and parent probe should in theory be independent of volume of distribution. provided the volume of distribution of parent probe and metabolite are closely correlated and the rate of elimination of the metabolite does not vary between subjects significantly. This ratio, determined from a single blood collection, could therefore correlate well with the fractional clearance of the probe through the pathway of interest, and hence provide a convenient alternative to multiple blood sampling.

In some cases it has been possible to estimate the catalytic activity of the relevant enzyme from measurements of the parent probe to metabolite ratio in normal bodily excretions, particularly urine or saliva. In theory, if it is shown that concentrations of parent and metabolite measured in urine or saliva are closely correlated with those in blood, the metabolic ratio determined in a single urine or saliva collection could be used to estimate the fractional clearance of the probe and hence the target enzyme activity. Parent and metabolite may be concentrated in urine, facilitating the measurements or reducing the dose of probe required for the test. However, urine measurements can be problematic because collections must be made over an interval of time (at least 1 h) for practical reasons. The metabolic ratio may change over time and determining the optimal timing of a urine collection may therefore be critical.

Breath may also be a useful excretion for probe based tests. This has been most commonly used when the target enzyme catalyses demethylation of the probe. Because the carbon atom in the cleaved methyl group largely appears in breath as carbon dioxide (Baker et al., 1983), the activity of the target enzyme should in theory be reflected in the rate at which breath CO₂ is produced from the probe (measurable because the methyl group in the probe is labelled with carbon-14 or carbon-13). However, CO₂ tests only measure the rate of demethylation; this should reflect both the liver enzyme activity and the liver concentration of the probe (which should correlate with total blood concentration of probe or perhaps of unbound probe). If there is significant intersubject variation in the volume of distribution of the probe or in protein binding, the results of a CO₂ breath test could be misleading (reviewed in Lane & Parashos, 1986).

In summary, there are many considerations and potential pitfalls in the development of an in vivo probe based assay for a specific cytochrome P₄₅₀. When dealing with truly polymorphic enzymes, many of the potential problems noted may not be important. For example, if the goal of the test is to distinguish only between rapid and slow metabolizers, and if the difference in probe clearance between the two phenotypes is great, the desired information may be obtained despite real effects of multiple variables. On the other hand, such variables may become very important when dealing with enzymes, such as the CYP3A enzymes, where the range of activities in the population may be relatively narrow, particularly if the goal of the test is to detect small activity differences between individuals.

Validating noninvasive tests

Potential means of validating a probe based assay are shown in Table 1. None of the criteria listed is fool proof and some are practical only in certain situations. Probably the 'gold standard' by which tests can be assessed is the concentration of the target enzyme determined directly in liver biopsies obtained from the individuals undergoing the test (Criteria 1, Table 1).

<table>
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<th>Table 1. Criteria that may be used to validate probe based tests of a liver enzyme</th>
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<tr>
<td>1. Correlate with the activity of the target enzyme determined in liver biopsies.</td>
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<td>2. Correlate with the fractional clearance of the probe mediated by the target enzyme.</td>
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<td>3. Correlate with the fractional clearance of other substrates of the target enzyme.</td>
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<td>4. Be reduced when subjects are treated with other substrates of the target enzyme.</td>
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<td>5. Be reduced dramatically when patients are treated with potent inhibitors of the target enzyme.</td>
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<td>6. Increase when subjects are treated with known inducers of the enzyme.</td>
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<td>7. Be reduced in patients with severe liver disease.</td>
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<td>8. Be markedly reduced during the anhepatic phase of a liver transplant operation.</td>
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The true 'gold standard' is probably total liver content of the target enzyme, which might not be reflected in liver biopsy analysis in all cases. For example, it has been shown that the biopsy (microsomal) content of some cytochromes P450 is normal in patients with advanced liver cirrhosis (Guengerich & Turvy, 1991: Lown et al., 1992); however, clearance of a probe may be greatly reduced in these patients due to the marked reduction in total hepatocyte mass. Even among patients with normal liver function, differences in liver volume should significantly influence probe clearance. It would obviously be desirable to perform liver biopsy studies on healthy individuals, but this is probably not justifiable. Such studies can generally be readily performed in animal models, but the data obtained are difficult to interpret given the potential for large species differences in the handling of probes.

A less direct way to validate a putative test is simply to show that the result correlates closely with the fractional clearance of the probe determined in a traditional pharmacokinetic study (Criteria 2, Table 1). This is not conclusive, however, because the test value might correlate closely with the fractional clearance of the probe while neither reflects the enzyme activity of interest (i.e. if the target enzyme is not rate limiting in the fractional clearance of the probe). It is therefore probably important to show that the results of the test correlate with the clearance of other structurally diverse compounds, whose only common characteristic is metabolism by the enzyme of interest (Criteria 3, Table 1).

The test value should also decrease appropriately when patients receive other substrates of the target enzyme which (when present in sufficient concentration) should compete with the probe for binding to the enzyme active site (Criteria 4, Table 1). These studies should employ substrates known to have high affinity for binding to the enzyme, as can be determined in studies with microsomes. In addition, if specific noncompetitive inhibitors of the target enzyme are known, and can be safely administered to people, it should be demonstrated that there is a pronounced decrease in the test value when subjects are treated with these inhibitors (Criteria 5, Table 1). For CYP3A enzymes, the antibiotic troleandomycin (TAO) appears to be a specific mechanism based inhibitor (Watkins et al., 1985, 1986, 1989).

If the target enzyme is known to be inducible, the test value should increase appropriately in subjects treated with the inducer(s) (Criteria 6, Table 1). This is especially important with probes with intermediate to high liver extraction since the enzyme activity may no longer be rate limiting when high levels of the enzyme are present (i.e. liver blood flow may become limiting). Induction studies could therefore help define the range of enzyme activities that can be estimated with the test. With CYP3A probes, the antibiotic rifampicin appears to be a potent inducer (Ged et al., 1989; Pichard et al., 1990).

Inhibition and induction studies should generally be performed by administering the proposed test to subjects both before and after altering target enzyme activity. Because each subject serves as his own control in these studies, the factors that may influence the absolute values of the test results (particularly volume of distribution and protein binding) would be expected to remain constant and not influence interpretation of studies (i.e. whether the target enzyme activity is rate limiting over a range of activities). However, these studies alone do not prove that the test provides a quantitation of enzyme activity suitable for intersubject comparison.

If a liver-specific test was sought, the possibility that extrahepatic metabolism was contributing to the metabolism measured by the test must be excluded. This can most easily be addressed by showing that the mean test result is low in patients with severe liver disease (Criteria 7, Table 1). The anhepatic phase of a liver transplant operation (the interval of time when the diseased liver has been removed from the recipient's body but the donor liver has not yet been connected to the blood circulation) also provides a unique opportunity to assess the contribution of extrahepatic enzymes to the test result (Criteria 8). Any metabolism measured during the anhepatic state is unequivocally extrahepatic.

In summary, there is, in general, no single criteria currently capable of validating a given probe based assay. Acceptance must therefore rely on findings that are consistent with the test being a quantitative and specific measure of the target enzyme in multiple clinical situations.

**Hepatic CYP3A enzymes**

One problem that potentially complicates the development of noninvasive means of measuring CYP3A catalytic activity is that multiple gene products are involved. Wang et al. (1983) partially purified a cytochrome P450 (termed 'HL5') from human liver in 1983 which was probably a CYP3A enzyme. In 1985, Watkins et al. (1985) purified to homogeneity a cytochrome P450 from human liver which was highly homologous to a major rat liver CYP3A enzyme. They termed this enzyme 'HLp' and showed that it was capable of metabolizing erythromycin. The following year, Guengerich et al. (1986a) reported the isolation and purification of the major human liver cytochrome
P450 involved in the metabolism of the calcium channel blocker nifedipine, and termed this form P450NR.

Using an antibody to HLP and an antibody to P450NR, several cDNA clones were identified and sequenced from the identical human liver cDNA expression library (Beaune et al., 1986; Molowa et al., 1986; Bork et al., 1989). The cDNA isolated with the anti HLP antibody (now termed CYP3A3) and the cDNAs isolated with the anti P450NR antibody (now termed CYP3A4) code for proteins that are 98% similar in amino acid sequence (Gonzalez, 1990; Nelson et al., 1993). It is currently unclear whether CYP3A3 and CYP3A4 represent the product of distinct genes at different genetic loci, or whether they represent alleles corresponding to the identical genetic locus. Regardless, given the minimal differences in the coding regions of both mRNAs, it is very likely that the purified proteins termed HLP and P450NR are functionally identical. Indeed, both CYP3A3 and CYP3A4 cDNAs have been expressed in heterologous systems and no significant differences in catalytic properties have yet been reported (Aoyama et al., 1990). For the purposes of this review, we will therefore assume that there is a single major CYP3A enzyme in adult liver and term it CYP3A3/4.

There is another CYP3A enzyme that is expressed in some but not all adult livers. This was first suggested when liver microsomes from multiple individuals were analysed on Western blots developed with antibodies to CYP3A3/4. Approximately one in five adult liver microsomes samples produced two distinct immunoreactive bands (a 'doublet') (Aoyama et al., 1989; Wrighton et al., 1989). One reactive protein co-migrated with purified CYP3A3/4 on the SDS polyacrylamide gels. Gonzalez et al. created a cDNA library from a human liver that appeared to contain predominantly the other reactive protein (Aoyama et al., 1989). A cDNA isolated from the library was found to be 90% homologous with CYP3A3/4. Wrighton et al. later purified the enzyme, now termed CYP3A5, from human liver (Wrighton et al., 1989). The catalytic properties of CYP3A5 are similar but not identical to those of CYP3A3/4 (Aoyama et al., 1989, 1990; Wrighton et al., 1990).

A fourth identified member of the human CYP3A subfamily is termed CYP3A7. CYP3A7 protein was first isolated from human fetal liver where it is the major cytochrome P450 present (Kitada et al., 1988). Schuetz et al. (1993) have recently reported that CYP3A7 mRNA can be detected on Northern blot analyses in approximately 50% of adult human livers but it is unclear if CYP3A7 protein is also present.

In summary, CYP3A3/4 appears to be the major CYP3A enzyme expressed in adult liver and CYP3A5 appears to be detectable in approximately 20% of adult livers. It is unlikely that additional CYP3A subfamily members remain to be identified. This is because a single CYP3A gene has been shown to be approximately 30 kb in length (John Schuetz, personal communication) and only approximately 90 kb of genomic DNA hybridized on Southern blots hybridized with the CYP3A3 cDNA under low stringency conditions (Molowa et al., 1986).

Extra hepatic CYP3A enzymes

It has recently become clear that CYP3A enzymes are present in extra hepatic tissues, particularly the small bowel (Watkins et al., 1987; Murray et al., 1988; Wątiers et al., 1989, 1990; Kolars et al., 1992; Schuetz et al., 1992). The major CYP3A enzymes present in small bowel appears to be CYP3A4, (Kolars et al., 1992) and there appears to be significant interindividual variation in the intestinal content and catalytic activity of CYP3A4 (Kolars et al., 1992; Lown et al., 1994). Several studies have suggested that there is significant 'first pass' metabolism of the orally administered CYP3A3/4 substrate cyclosporine A at the level of the small intestine (Kolars et al., 1991; Hebert et al., 1992; Tjia et al., 1993; Vickers et al., 1993; Weber et al., 1993). This was most directly demonstrated during the anhepatic phase of the liver transplant operation, when cyclosporine A was instilled into the small bowel (Kolars et al., 1991). Up to 50% of the cyclosporine A detected in the portal blood had been converted to metabolites characteristically produced by CYP3A3/4. Significant first pass metabolism of cyclosporine A was also indicated by a recent traditional pharmacokinetic study of cyclosporine A (Hebert et al., 1992).

The relative abundance of CYP3A4 in human intestine has potential implications in the development of orally administered in vivo probes of CYP3A enzymes. Any substrate of CYP3A3/4 that is orally administered may undergo significant metabolism in the small bowel, and the extent of metabolism in the small bowel might vary from one individual to another. This might not be an important consideration in certain instances. For example, if the use of the probe drug was to predict the oral pharmacokinetics of a CYP3A3/4 substrate, measurement of the aggregate metabolism occurring in the intestine and liver might give more useful information than measurement of CYP3A3/4 catalytic activity in the liver alone. On the other hand, it might not matter whether the metabolism measured chiefly reflects the liver or intestinal activity if the genetic, and or non-genetic factors that influence the variability in CYP3A3/4 catalytic
activity in the liver also determine the heterogeneity in the expression of these enzymes in the intestine. That is, if patients with high liver CYP3A3/4 activity also have high CYP3A3/4 activity in the intestine, and if patients with low liver CYP3A3/4 activity also have low intestinal activity, measurement of liver activity alone would correlate with aggregate liver and intestinal enzyme activity. Only one study to date has addressed this question (Lown et al., 1994) and no significant correlation between liver and intestinal activity of CYP3A3/4 was found. It therefore appears that liver and intestinal activity of CYP3A3/4 do not always mirror each other and hence, the results of a test of CYP3A3/4 activity may greatly depend on route of administration of the probe.

CYP3A enzymes have also been shown to be present in human kidney. Schuetz et al. (1992) performed immunoblot analyses on microsomes prepared from kidney biopsies obtained from seven adults. CYP3A5 was identified on the immunoblots in five of the seven kidney samples (and may have been present in the other two). In addition, RNA prepared from human kidney biopsies hybridized with a CYP3A5 specific cDNA. These investigators were able to detect CYP3A3/4 immunoreactive protein in only one of the seven kidney samples, and were unable to detect CYP3A3/4 mRNA on Northern analyses. These investigators concluded that CYP3A3/5 may be the major CYP3A enzyme expressed in adult kidney.

The presence of CYP3A5 in kidney creates a potential problem for urine based assays of in vivo CYP3A activity. If a chosen probe is readily metabolized by CYP3A5, it would seem important to exclude the possibility that metabolites measured in urine reflect in part kidney as well as liver activity.

Some evidence has recently been provided that CYP3A enzymes may be expressed at low levels in other extra hepatic tissues. We have found that CYP3A5 is expressed throughout the human gastrointestinal tract (Kolars et al., 1994). In general, CYP3A5 concentrations are low compared to CYP3A3/4; however, in the stomach of one individual, CYP3A5 appeared to be the major protein present (Kolars et al., 1994). In addition, it has recently been reported that CYP3A7 (but not CYP3A3/4 or CYP3A5) is present in human uterus and placenta (Schuetz et al., 1993). It is therefore possible that CYP3A7 could catalyse significant metabolism of some probes in women (and not men). No convincing data has yet been presented suggesting that CYP3A enzymes are expressed in human lung or salivary glands. One report suggested that CYP3A related protein may be present in peripheral white blood cells (Murray et al., 1988); however, this has not been subsequently confirmed (Kolars et al., 1992).

Special considerations for CYP3A noninvasive tests

In view of the above discussion, it is clear that some additional points must be considered when developing appropriate probes of CYP3A activity. First, the nature of the optimal probe will in part depend on the ultimate goal of the test. If the goal of the test is to assess CYP3A3/4 activity in liver only, oral administration of the probe may be problematic due to the potential for significant metabolism in the intestine. On the other hand, if the purpose of the test is to predict the oral clearance of CYP3A3/4 substrates, oral administration of the probe may be the ideal route. If a urine based assay is sought, it would be potentially important to have a probe that is not readily metabolized by CYP3A5, as metabolism in the kidney could confound the interpretation of the results. Finally, if a non invasive probe of liver CYP3A5 is sought (for example to identify the 20% of individuals who express significant amounts of this enzyme) a metabolic pathway unique to CYP3A5 (and not shared by CYP3A3/4) should be sought and a urine based assay might be problematic.

Because expression of CYP3A5 appears to be truly polymorphic and because this enzyme does not appear to be inducible (Wrighton et al., 1989), it seems likely that a genetic based assay could be developed in the future to identify patients expressing significant amounts of this enzyme in liver. However, because CYP3A3/4 is highly inducible by many medications in common use, and may be influenced by other non-genetic factors including diet, it is unlikely that a simple genetic based assay will be useful in determining the activity of this enzyme in all individuals. In addition, because CYP3A enzymes do not appear to be present in peripheral blood cells, blood activity assays are unlikely to be useful. Probe based assays therefore provide the most promising strategy for determining an individual's activity of CYP3A enzymes.

Evaluation of specific probes

There have been several probe based assays proposed to date as measures of CYP3A activity (Table 2). All the probes are reasonably safe, especially measurement of urinary 6β hydroxy cortisol which involves only urine collection. Each of the probes appear to be chiefly metabolized in human liver microsomes by CYP3A enzymes, and where known, the major metabolic pathway of each probe in the body appears to be
that catalysed by CYP3A enzymes. All the probes appear to be metabolized by CYP3A3/4. However, dapsone, cortisol, nifedipine and midazolam appear to also be readily metabolized by CYP3A5 (Wrighton et al., 1990; Aoyama et al., 1990; Fleming et al., 1992). Erythromycin appears to have relatively low affinity for CYP3A5 (Wrighton et al., 1990) and therefore may be relatively CYP3A3/4 specific.

Only lignocaine would be considered a high extraction drug, and therefore expected to be susceptible to alterations in liver blood flow. Dapsone largely undergoes acetylation, but data has been presented suggesting that acetylation phenotype does not influence the results of this urine based test (May et al., 1990). Finally, the metabolites generated by CYP3A enzymes from erythromycin (CO₂), cortisol (6β hydroxy cortisol), nifedipine (M1 metabolite) and dapsone (the hydroxylamine) promptly appear in a collectable body secretion. However, the lignocaine and midazolam based tests currently appear to require blood monitoring.

[^14C N-methyl] erythromycin breath test

The use of a breath test is especially attractive for CYP3A enzymes in that this P450 sub-family does not appear to be significantly expressed in the lung. To perform the test, a subject receives an intravenous injection containing a trace amount (less than 0.1 μmol) of ^14C-(N-methyl erythromycin (3 μCi)). At timed intervals thereafter, the subject blows into scintillation vials that contain 4 ml of a CO₂ binding solution (a 50% solution of hyamine in ethanol with a pH indicator). The subject stops blowing when the solution colour vanishes (corresponding to trapping of 2 mM CO₂), which takes approximately 40 s. Scintillation cocktail is then added to each vial and the radioactivity is determined in a scintillation counter. The rate of production of radiolabel in the breath can then be calculated at the time of each collection based on an estimate of the endogenous rate of carbon dioxide produced (which is a function of the subject’s body surface area at rest). It has become customary to express the erythromycin breath test results as the percentage of administered radio label exhaled during the hour after injection (Watkins et al., 1989). Repetitive breath collections are unnecessary; the percentage of administered radiolabel exhaled during the hour after the injection can be accurately estimated from a single breath collection obtained just 20 min after injection of the test dose of erythromycin (Turgeon et al., 1994). As a result, this test can be administered relatively quickly and the results can be available within hours.

As summarized in Table 2, the erythromycin breath test has been validated as a specific measure of hepatic CYP3A3/4 activity according to most of the criteria given in Table 1, with some qualifications. The breath test results have been shown to significantly correlate with the concentration of CYP3A3/4 protein in the liver of patients but not with the liver concentrations of four other P450 proteins (measured in the same liver biopsy specimens) (Lown et al., 1992). It may be important that the patients in this study had severe liver disease and many were receiving multiple medications. Hence, this study alone does not address the sensitivity of the ERMBT to detect small interindividual differences in CYP3A3/4 activity in a healthy population.

The breath test results have not yet been directly compared with measurements of the clearance of erythromycin. However, the ERMBT results have been shown to predict steady state trough blood levels of the CYP3A 3/4 substrates cyclosporine A (Watkins et al., 1990; Marchuk et al., 1991) and OGT 37-325 (Turgeon et al., 1993), and to predict the oral clearance cyclosporine A (Turgeon et al., 1992). The ERMBT values have been shown to fall in patients treated with the CYP3A3/4 substrate cyclosporine A (Watkins et al., 1990), consistent with competition between two substrates for in vivo metabolism by the identical enzyme. In addition, the ERMBT value has been shown to dramatically decrease in patients treated with TAO, a mechanism-based selective inhibitor of CYP3A3/4 (Watkins et al., 1989). The ERMBT value has also been shown to increase when subjects are treated with glucocorticoids or rifampicin, known inducers of liver CYP3A3/4 (Watkins et al., 1989).

The contribution of extra hepatic P450s to the erythromycin breath test result appears to be minimal.
The mean ERMBT result in patients with severe liver disease is far below that observed in subjects with normal liver function (Lown et al., 1992). Moreover, little CO₂ was produced from erythromycin during the anhepatic phase of the liver transplantation operation (Watkins et al., 1992). Finally, extensive testing in rats has shown the ERMBT to be a sensitive and specific measure of liver CYP3A activity in this species (Watkins et al., 1989).

In summary, the ERMBT is the most extensively validated measure of in vivo CYP3A/4 activity in liver and because of this, has been considered to be the current 'gold standard' (Kinirons et al., 1993). However, there are several disadvantages of the test. Most importantly, the ERMBT uses the radiolabel carbon-14. Although the dose of radioactivity is small, the very long half life of 14C raises theoretical health concerns, particularly in young individuals. It should be possible to convert the 14C erythromycin breath test to 13C, as has been done for other 14C breath tests (Schneider et al., 1978). This would currently require mass spectral analysis of breath. A second concern is that the test does not appear to measure intestinal CYP3A/4 activity (Lown et al., 1994), which may be important for some uses for a noninvasive test. We are currently investigating the possibility of administering erythromycin orally to assess aggregate liver and intestinal CYP3A/4 activity.

A final concern with the ERMBT is that since only the rate of demethylation during the first hour is measured, interindividual differences in protein binding or volume of distribution, could result in significant differences in the test result. That is, the rate of erythromycin demethylation will reflect not just the liver activity of CYP3A/4, but also the blood concentration of free erythromycin reaching the liver. This problem may be magnified because, for simplicity, the identical dose of erythromycin is administered to all individuals. Because the volume of distribution of erythromycin should be a function of body weight, at least in part, blood concentrations of probe will tend to inversely correlate with body weight. This may explain why an inverse correlation between body weight and the ERMBT results has been reported (Hunt et al., 1992). This may also in part account for why women (who are generally smaller than men) have been reported to have higher ERMBT results than men (Watkins et al., 1989). These considerations may not be very important if the variation introduced by interindividual differences in volume of distribution are relatively small compared with the differences in liver CYP3A/4 activity that exist. It may also be possible to correct for this 'weight effect' by either varying the test dose of erythromycin based on body weight or by simply multiplying the current test result by a correction factor based on the subject's body weight.

**Midazolam**

Midazolam is chiefly oxidized to 1' and 4' hydroxy metabolites and these metabolites have been shown to be characteristically produced by both CYP3A/4 and CYP3A5 (Kronbach et al., 1989; Gorski et al., 1993). To perform clearance measurements, patients are given an intravenous injection of midazolam (0.015 mg kg⁻¹) and blood is sampled at various time points to 6 h. The blood concentration of parent midazolam and/or its metabolites are then measured by gas chromatography and mass spectral analysis (Ken Thummel, personal communication).

There is now good evidence that the clearance of midazolam provides an estimate of liver CYP3A activity (summarized in Table 2). Most of the proposed validation criteria (Table 1) have been met. Midazolam total clearance has been shown to significantly correlate with the concentration of CYP3A immunoreactive protein and midazolam catalytic activity determined directly in liver biopsies (Thummel et al., 1993). Midazolam clearance has also been shown to correlate significantly with cyclosporine A clearance (Ken Thummel, personal communication). However, these studies have been exclusively performed in liver transplant recipients who, because of treatment with numerous drugs (including glucocorticoids), may have a wider range of CYP3A activities than other populations. The sensitivity of midazolam clearance measurements in other populations is therefore not clear.

No specific studies have yet been performed to systematically assess the effect of inducers and inhibitors on midazolam clearance; however, midazolam clearance has been reported to be increased in patients receiving the CYP3A inducer phenytoin (Thummel et al., 1992) and decreased in patients receiving the CYP3A substrate/inhibitor erythromycin (Olkkola et al., 1993). Clearance has also been shown to be significantly reduced in patients with liver disease (Pentikainen et al., 1989) but no studies performed during the anhepatic state have been reported.

In summary, the clearance of intravenously administered midazolam appears to provide a good estimate of the hepatic activity of CYP3A/4 and probably CYP3A5, in at least the liver transplant population. The advantage of this test relative to the erythromycin breath test is that no radioisotopes are required. The disadvantages of the test include the requirement of intravenous administration of a pharmacological dose of midazolam, which will produce sedative and
amnestic effects in some subjects. The test also involves multiple blood sampling over a prolonged interval and the analytical techniques involved are relatively complex. The latter problem would be partially overcome if the clearance of midazolam could be accurately estimated by a metabolic ratio determined in a single blood sample, or better still, in a saliva or urine sample (the latter may not be possible due to the CYP3A5 present in kidney). These possibilities are currently being examined (Ken Thummel, personal communication). In addition, because the ratio of 1-OH to 4-OH midazolam metabolites produced by CYP3A5 is significantly greater than that produced by CYP3A3/4 (Gorski et al., 1993), it may be possible to identify the one in five adults who express CYP3A5 in their liver by examining the ratio of these metabolites in blood. It may also be possible to administer midazolam orally and thereby measure aggregate liver and intestinal CYP3A activity (Ken Thummel and Grant Wilkinson, personal communication).

Urinary 6β hydroxy cortisol

The ratio of 6β hydroxy cortisol to free cortisol (6βF/FF) in the urine has been proposed as a measure of 'mixed function oxidase' activity in the liver for over two decades. It may be possible to determine this ratio from a single 'spot' urine collection because, although the rates of excretion of 6βF and FF show marked diurnal variability, the ratio of the two parameters appears to remain reasonably constant throughout the day (Saenger, 1983; Bienvenu et al., 1991). This urine assay may therefore be the simplest and only truly noninvasive assay proposed to date. 6βFF and FF can be measured in urine by either radioimmunoassay (Saenger, 1983) or HPLC (Gerber-Taras et al., 1981; Joellenbeck et al., 1993) techniques.

Some data obtained in vitro suggests that the test might specifically assay hepatic CYP3A enzymes in vivo (Waxman et al., 1988; Maenpaa et al., 1993); however, there is actually little objective data to support this conclusion (Table 2). Ged et al. (1989) measured urinary 6βF/FF concentration and obtained liver biopsies from patients before and after they were treated with the CYP3A3/4 inducer rifampin. They reported a significant correlation between liver content of CYP3A3/4 and the urine 6βF/FF measurements when all data points were examined. However, 6βF/FF did not correlate with the liver biopsy concentration of P450 3A3/4 when the data obtained before induction or after induction were examined separately. The authors concluded that urine 6βF/FF may only be a measure of 'induction' of CYP3A3/4 (Ged et al., 1989). This is consistent with the conclusions from earlier investigations (Gerber-Taras et al., 1981; Park, 1981).

At least two studies have failed to find a significant correlation between the ERMBT and urine 6βF/FF in subjects who have received both tests (Hunt et al., 1992; Kinirons et al., 1993). By contrast, we found a significant correlation between the two test results in a study of 47 patients; however, this correlation largely resulted because women had higher values for both tests than did men (Watkins et al., 1992). Correlations between the two test results were considerably weaker when women and men were examined separately (Watkins et al., 1992). In addition, in patients with psoriasis who received fixed doses of cyclosporine A, urine 6βF/FF ratios did not predict the trough blood levels of the drug (drug blood levels were predicted by the ERMBT in these same patients) (Watkins et al., 1992).

Studies examining the effect of treatment with inducers or inhibitors of CYP3A3/4 on urine 6βF/FF have given conflicting results. Whereas treatment with rifampin generally increases the urinary ratio (Ged et al., 1989; Horsman et al., 1992), the percentage increase in this ratio did not correlate with the percentage increase in the ERMBT results in patients who received both tests (Watkins et al., 1992). Moreover, the CYP3A3/4 specific inhibitor TAO had no consistent effect on the urine 6βF/FF ratio in five subjects (Watkins et al., 1992).

Finally, in a study involving one patient undergoing liver transplantation (Watkins et al., 1992), the 6βF/FF urinary ratio fell during the anhepatic phase, but only to approximately 50% of the preoperative levels. This suggests that extrahepatic enzymes contribute to the metabolism measured in the test. Surprisingly, this patient had higher urinary 6βF/FF before surgery when his liver was severely diseased than after surgery when he had a presumably healthy liver (Watkins et al., 1992).

The reason why urine 6βF/FF measurements may not reflect liver CYP3A3/4 activity is unclear. CYP3A5 has been shown to be capable of 6β hydroxylation of steroids (Wrighton et al., 1990) and hence the kidney may contribute significantly to urinary 6βF/FF. If this is the case, 6β cortisol excretion in the urine may largely reflect hepatic CYP3A activity only in patients who have a high liver CYP3A activity. Although this theory is attractive, it would predict that all patients with relatively high ERMBT test results should also have relatively high urine 6βF/FF, but this was not the case in the study of 47 patients (Watkins et al., 1992).
In summary, the data obtained to date suggest that urine 6PF/FF at best only partially reflects liver CYP3A3/4 activity.

Nifedipine

Guengerich et al. (1986a) showed that CYP3A3/4 is the enzyme that catalyses the major pathway of metabolism of nifedipine in liver (dehydrogenation to dehydronifedipine, also termed MI). No clinical studies involving liver biopsies have been performed to date; however, at least some data supports the idea that the clearance of nifedipine chiefly reflects CYP3A3/4 activity. Within a group of subjects, the clearance of nifedipine correlates with the clearance of other dihydropyridine calcium channel blockers (Soons et al., 1993). No studies examining correlation of nifedipine clearance with the clearances of structurally diverse CYP3A substrates have been reported. Nifedipine clearance has been reported to be increased in patients treated with the CYP3A inducer rifampin (Guengerich et al., 1986b), and is decreased in patients receiving quinidine (Schellens et al., 1991), a known substrate for CYP3A/4 (Guengerich et al., 1986b). Clearance of nifedipine is reduced in patients with severe liver disease (Kleinbloesem et al., 1986) but no studies during the anhepatic state have been reported.

Neither parent nifedipine, nor MI is appreciably excreted into urine. However, because MI appears to be converted to a metabolite (termed MII) which does appear in urine, it has been proposed that the amounts of MII in urine can be used to estimate liver activity of CYP3A3/4 activity (Schmid et al., 1988). Early work by Kleinbloesem et al. had suggested that this ratio could distinguish 'rapid' from 'poor' metabolizers of nifedipine (Kleinbloesem et al., 1984); however, this urine test has not been rigorously studied according to the criteria presented in Table 1. In particular, it has not been shown that the urine test result correlates closely with the oral or intravenous clearance of nifedipine. In addition, it has been reported that nifedipine poor metabolizes (as assessed by urine MII excretion) have similar plasma AUC (area under the plasma concentration time curve) of the MI metabolite as do 'rapid' nifedipine metabolizers (Daly et al., 1992). This is unexpected if the urine ratio reflects production of MI metabolite by the liver.

In summary, the nifedipine urine ratio has not been validated as a noninvasive test of CYP3A enzymes.

Dapsone

Both CYP3A3/4 and CYP3A5 have been shown to catalyse the N-hydroxylation of dapsone, a major metabolic route for this potential probe (Fleming et al., 1992). The oral clearance of dapsone has been reported to be significantly greater in patients treated with rifampin (Pieters et al., 1988), consistent with a primary role of CYP3A3/4 in the metabolism of the probe in vivo. It has been proposed that the ratio dapsone hydroxylamine/[dapsone + dapsone hydroxylamine] measured in an 8 h urine collection following the oral dose may be a suitable test for CYP3A activity (May et al., 1990, 1993). Although hydroxyl amines are often unstable, reliable measurements can apparently be obtained using HPLC without derivatization if an antioxidant is added to the urine (May et al., 1990). The urinary ratio was shown to correlate with dapsone oral clearance in one study of seven subjects (May et al., 1992).

An advantage of the dapsone test is that, because a metabolite ratio is measured, volume of distribution should theoretically not influence the test results. Studies comparing the urinary dapsone ratio (or direct clearance measurements) with the clearance of other CYP3A substrates have not been performed. In a study of 30 healthy subjects, there was no correlation between the dapsone urine ratio and either the ERMBT test results or urine 6PF/FF (Kinirons et al., 1993). No spontaneous reports of interactions between dapsone and CYP3A substrates or inhibitors have been reported, and studies systematically examining the effects of CYP3A induction or inhibition on the dapsone urinary ratio have not been reported. The urinary ratio is only modestly reduced in patients with liver cirrhosis (May et al., 1992), suggesting that extrahepatic enzymes may significantly contribute to the metabolism measured. It seems reasonable to assume that, because dapsone is administered orally in this test, CYP3A3/4 in small bowel contributes to the metabolism measured. In addition, because dapsone can be metabolized by CYP3A5 (Fleming et al., 1992; Gorski et al., 1993), this enzyme in kidney may contribute significantly to the metabolites measured in urine.

In summary, it seems likely that there is a significant contribution of intestine CYP3A3/4 and/or kidney CYP3A5 to the dapsone urine test result. This may be an advantage in certain applications of the test: however, further validation of the test will be necessary before its usefulness can be determined.

MEGX test

Lignocaine's primary metabolite is monoethyl-glycinexyloside (termed MEGX) and this is produced chiefly by CYP3A4 in human liver microsomes (Bargetz et al., 1989). The rate of production of MEGX
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from lignocaine, which can be estimated from blood levels (measured by a simple fluorescent immunopolarization assay kit) has been proposed as a general liver function test (Lamesch et al., 1990; Oellerich et al., 1987). To date, few of the criteria present in Table 1 have been satisfied for the MEGX test as a specific measure of CYP3A enzymes, and some of the data presented has been conflicting. For example, Azoulay et al. (1993) reported the MEGX test results predicted liver biopsy CYP3A levels and cyclosporine A blood levels in liver transplant recipients (Azoulay et al., 1993). However, Cakaloglu et al. (1992) have failed to show a correlation between the MEGX test result and the ERMBT results, or between the MEGX result and kinetic parameters of cyclosporine A or FK 506 in liver transplant recipients. As previously discussed, lignocaine is subject to high liver extraction and hence interindividual differences in liver blood flow may be a more important determinant of the MEGX test result than is heterogeneity in CYP3A activity.

Conclusions

There are many considerations that must be taken into account in developing probe based assays for CYP3A catalytic activity. It is probably safe to say that the ideal tests have not yet been developed. The optimal test will undoubtedly depend on the reason for employing it. If the goal is to determine whether a certain compound is an inducer or inhibitor of CYP3A3/4 in man, the ERMBT, midazolam clearance, urine 6PF/FF, and dapsone urine tests may each be sufficient. This is because the subjects serve as their own controls (by receiving the test before and after treatment with the compound of interest) and hence many of the potential pitfalls previously discussed are less important. However, if the aim of the test is to compare liver CYP3A3/4 activity among individuals (for example, to stratify a population for risk assessment or to identify high or low metabolizers for intentional inclusion in Phase I clinical trials of a new chemical entity), the ERMBT and midazolam clearance are the best validated techniques currently available. Finally, if the aim is to measure the aggregate CYP3A3/4 activity in liver and intestine (for example, to predict oral clearance of a CYP3A3/4 substrate), it would seem logical to administer the probe orally. The dapsone urine ratio may be useful for this purpose if the contribution of kidney CYP3A5 is determined in the appropriate studies to be either small or invariant among subjects. An oral erythromycin breath test or midazolam test may also be promising in this regard.

Acknowledgements

I would like to thank Ann Daly, Jeffrey Idle, Gail May, Ken Thummal and Grant Wilkinson, for their critical review of, and helpful comments concerning, this review.

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