DRUGS AS CYP3A PROBES, INDUCERS, AND INHIBITORS

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Human cytochrome P450 (CYP) 3A subfamily members (mainly CYP3A4 and CYP3A5) mediate the metabolism of approximately half all marketed drugs and thus play a critical role in the drug metabolism. A huge number of studies on CYP3A-mediated drug metabolism in humans have demonstrated that CYP3A activity exhibits marked ethnic and individual variability, in part because of altered levels of CYP3A4 expression by various environmental factors and functionally important polymorphisms present in CYP3A5 gene. Accumulating evidence has revealed that CYP3A4 and CYP3A5 have a significant overlapping in their substrate specificity, inducers and inhibitors. Therefore, it is difficult to define their respective contribution to drug metabolism and drug-drug interactions. Furthermore, P-glycoprotein and CYP3A are frequently co-expressed in the same cells and share a large number of substrates and modulators. The disposition of such drugs is thus affected by both metabolism and transport. In this review, we systematically summarized the frequently used CYP3A probe drugs, inducers and inhibitors, and evaluated their current status in drug development and research.

Key Words: CYP3A4; CYP3A5; P-glycoprotein; Drug metabolism; Drug transport; Probe; Substrate; Induction; Inhibition; Drug-drug interactions.
INTRODUCTION

The human CYP3A gene subfamily consists of four known members—CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (Lamba et al., 2002; Xie et al., 2004). Of their gene products, CYP3A4 and CYP3A5 are abundantly expressed in liver (Lamba et al., 2002; Shimada et al., 1994). In addition, they are also expressed in some extra-hepatic tissues and organs that are important in drug disposition and metabolism, such as gut lumen (Paine et al., 2006) and kidney (Haehner et al., 1996; Schuetz et al., 1992). In fact, CYP3A (mainly CYP3A4 and CYP3A5) is the greatest piece of the human liver and intestine P450 “pie,” accounting for approximately 40% and 80% of total P450 content, respectively (Paine et al., 2006; Shimada et al., 1994). Obviously, CYP3A4 and CYP3A5 are the most abundant and important drug-metabolizing enzymes in humans. On the other hand, accumulating evidence has shown that CYP3A mediates the biotransformation of approximately half of all marketed drugs (Rendic, 2002). Accordingly, altered CYP3A activity is predicted to lead to changes in intestinal metabolism, hepatic biotransformation and renal clearance of affected drugs.

There are marked ethnic and individual variations in CYP3A activity (Xie et al., 2001; Lin and Lu, 2001), which are the result of altered genetic and non-genetic factors (Lamba et al., 2002; Xie et al., 2004), including drug-herb interaction (Venkataramanan et al., 2006; Xie and Kim, 2005) and drug-drug interaction (Lin and Lu, 2001; Thummel and Wilkinson, 1998) that result in either induction or inhibition. Of importance for drug disposition is that CYP3A and drug transporters (such as P-glycoprotein, or P-gp) are frequently co-expressed in the same cells (or tissues) and share a large number of substrates and modulators (Wacher et al., 1995). Furthermore, the expression of CYP3A and P-gp is also regulated by nuclear receptors (Tirona and Kim, 2005). That makes defining the CYP3A-mediated drug metabolism difficult.

Most importantly, CYP3A5 shares approximately 90% sequence identity of its cDNA with CYP3A4, and similar substrate specificity makes it difficult to dissect their respective contribution to overall CYP3A-mediated drug metabolism (Xie et al., 2004). To address these issues, a large number of efforts have been made to identify and validate phenotyping tests that could be used to assess CYP3A activity (in vitro and in vivo) and its alterations that are caused by drug-drug interactions. Initially, much work was conducted to screen and identify in vitro probes for CYP3A. However, the validation of in vivo CYP3A probes is more complicated. Although some probes are only validated in vitro, they are useful tools for early-phase research and development of new drug candidates. In this review, we attempted to systematically summarize and evaluate the probe drugs, inducers and inhibitors of CYP3A that are frequently used as tool drugs for the drug development and research.

COMMON CYP3A PROBES

The CYP3A probe is defined as any drug whose metabolism is principally or uniquely catalyzed by CYP3A. Thus, the amount of drug’s metabolite(s) generated through CYP3A-mediated metabolic pathway(s) can be used to reflect the level of CYP3A activity. The following drugs have been identified and/or validated as CYP3A probes, and their relative specificity of CYP3A4 and CYP3A5 is shown in Table 1.
DRUGS AS CYP3A PROBES, INDUCERS, AND INHIBITORS

Midazolam

Midazolam (MDZ), a short-acting benzodiazepine, is one of the most commonly used probes for the determination of intestinal and/or hepatic CYP3A activity, in vitro and in vivo, because it is generally recognized that MDZ is a “pure” CYP3A probe (Kim et al., 1999; Takano et al., 1998). MDZ undergoes significant intestinal and hepatic first-pass metabolism and is eliminated almost entirely by CYP3A (Paine et al., 2000). Thus, apparent oral clearance or systematic clearance of MDZ is currently considered as the criterion standard for in vivo assessment of CYP3A activity, whereas the amount of 1’-hydroxy- and/or 4-hydroxymidazolam generated in the hepatocytes and microsomes can also reflect the activity of CYP3A in the cells. However, MDZ is a drug with an intermediate hepatic extraction ratio, and its systemic clearance may vary by individual differences in hepatic blood flow.

The major metabolic pathways of MDZ are CYP3A-catalyzed 1’-hydroxylation and to a lesser extent, 4-hydroxylation (Yuan et al., 2002). For human subjects, oral administration of MDZ is used to measure total (intestinal and hepatic) CYP3A activity, whereas systematic clearance of MDZ (after intravenous dosing) only reflects hepatic CYP3A activity (Streetman et al., 2000; Thummel et al., 1994). In human liver microsomes (HLMs), some studies have demonstrated that the rate of 1’-hydroxymidazolam generation correlated well with CYP3A4 protein content (r=0.92) and, to a lesser extent, with CYP3A5 protein content (r=0.60) (He et al., 2006). However, other studies had inconsistent results. For example, correlation of the rate of MDZ 1’-hydroxylation formation with CYP3A5 content was higher than that with CYP3A4 content, whereas correlation of the rate of MDZ 4-hydroxylation formation with CYP3A5 content was similar to that with CYP3A4 in the HLMs from Japanese subjects (Yamaori et al., 2004).

Similarly, Huang et al. found that CYP3A5 was more potent than CYP3A4 in catalyzing total MDZ hydroxylation (3-fold) using heterogeneously expressed CYP3A4 and CYP3A5 and phenotyped HLMs (Huang et al., 2004a). In addition, intrinsic clearances (CL_{int}) for CYP3A4 were found to be similar to those for CYP3A5 (Galetin et al., 2004; Soars et al., 2006; Williams et al., 2002). Patki et al. used recombinant CYP3A4 and CYP3A5 and found that rCYP3A4 and rCYP3A5 both produced 1’-hydroxy- and 4-hydroxy- metabolites from MDZ and that the metabolic activity of CYP3A4 was greater than that of CYP3A5 (Patki et al., 2003). Although oral or intravenous MDZ is validated to measure intestinal/hepatic CYP3A activity, whether the formation of different hydroxyl

Table 1 Validated probe drugs for CYP3A4 and/or CYP3A5.

<table>
<thead>
<tr>
<th>Probe</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Midazolam</td>
<td>+</td>
<td>+</td>
<td>Patki et al., 2003</td>
</tr>
<tr>
<td>Triazolam</td>
<td>+</td>
<td>+</td>
<td>Patki et al., 2003</td>
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<tr>
<td>Alfentanil</td>
<td>+</td>
<td>+</td>
<td>Klees et al., 2005b</td>
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<td>Testosterone</td>
<td>+</td>
<td>+</td>
<td>Kamdem et al., 2004</td>
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<tr>
<td>Cortisol</td>
<td>+</td>
<td>+</td>
<td>Huang et al., 2004b</td>
</tr>
<tr>
<td>Alprazolam (α-hydroxylation)</td>
<td>−</td>
<td>+</td>
<td>Hirota et al., 2001; Williams et al., 2002</td>
</tr>
<tr>
<td>Tacrolimus (demethylation)</td>
<td>−</td>
<td>+</td>
<td>Kamdem et al., 2005; Dai et al., 2006</td>
</tr>
<tr>
<td>Vincristine</td>
<td>−</td>
<td>+</td>
<td>Dennison et al., 2006</td>
</tr>
<tr>
<td>Quinidine (3-hydroxylation)</td>
<td>+</td>
<td>−</td>
<td>Mirghani et al., 2003; Mirghani et al., 2002</td>
</tr>
<tr>
<td>Erythromycin (EBT)</td>
<td>+</td>
<td>−</td>
<td>Baker et al., 2004</td>
</tr>
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metabolites of MDZ can be used to differentiate relative contribution of CYP3A4 and CYP3A5 to CYP3A-mediated drug metabolism remains uncertain.

Erythromycin

Erythromycin is not a “pure” CYP3A probe because it also undergoes P-gp transport (Kim et al., 1999). The erythromycin breath test (EBT), a widely used in vivo phenotyping method to measure hepatic CYP3A activity, is based on the fact that $N$-demethylation of intravenous $^{14}$C-erythromycin is catalyzed by hepatic CYP3A, which is expressed as the amount of the derived $^{14}$CO$_2$ that is expired over a fixed time period (Watkins, 1994). In general, greater than 4% $^{14}$C expired per hour is often considered indicative of an “induced” state. It is assumed that the EBT responds predictably to CYP3A4 induction or inhibition and drug-drug interaction in vivo (DeVane et al., 2004; Nicandro et al., 2007). However, erythromycin is also a P-gp substrate (Kim, 2002).

As expected, hepatic P-gp is shown to be a potential confounding factor that affects CYP3A activity in liver (Baker et al., 2004). In addition, the CL$_{int}$ of CYP3A5-catalyzed erythromycin $N$-demethylation was 41% of that by CYP3A4 (Huang et al., 2004a), suggesting that CYP3A4 plays an important role in the metabolism of erythromycin. Furthermore, administration of $^{14}$C-erythromycin produced plasma concentrations frequently below CYP3A4 saturation (Baker et al., 2004). Therefore, the EBT can be used as a measuring of hepatic CYP3A4 activity. But this “gold standard” has two important limitations: (1) the intestinal CYP3A4 is bypassed; (2) erythromycin is a probe for both CYP3A4 and P-gp (Lemahieu et al., 2003). Obviously, no information is available from the above EBT to show the involvement of P-gp present in the intestinal tract (Lemahieu et al., 2003). But, when orally and intravenously administered [N-methyl-$^{14}$C] erythromycin is used to evaluate $^{14}$C-labeled excretion dynamics in breath and urine, both intestinal and hepatic CYP3A4 and P-gp activity can be measured, respectively (Lemahieu et al., 2003).

Triazolam

Triazolam, a drug with low hepatic extraction ratio, is metabolized by intestinal and hepatic CYP3A (Greenblatt et al., 2004; Kronbach et al., 1989; von Moltke et al., 1996). Triazolam is not a P-gp substrate (von Moltke et al., 2004). Both rCYP3A4 and rCYP3A5 produce 1-hydroxy and 4-hydroxy metabolites from triazolam, but the catalytic activity of CYP3A5 was less than that of CYP3A4 (Patki et al., 2003). For example, the CL$_{int}$ of 1-hydroxy triazolam mediated by rCYP3A4 is 2.5-fold greater than that by rCYP3A5, and 1.6-fold higher for 4-hydroxy triazolam (Patki et al., 2003). Similar to MDZ, triazolam can be used to measure intestinal and hepatic CYP3A activity.

Alprazolam

Alprazolam is metabolized by CYP3A, producing $\alpha$-hydroxyalprazolam and 4-hydroxyalprazolam (Greenblatt and Wright, 1993). Subsequently, experimental evidence has demonstrated that CYP3A5 exhibits a higher $\alpha$-hydroxyalprazolam clearance by 5.5-fold but a lower 4-hydroxyalprazolam clearance by 2-fold than CYP3A4 (Galetin et al., 2004). Similarly, there are good correlations between the CL$_{int}$ for $\alpha$-hydroxylation and CYP3A5 content, and between the CL$_{int}$ for 4-hydroxylation and CYP3A4 content.
expressed in 10 HLM samples (Hirota et al., 2001). Furthermore, using recombinant CYP isoforms expressed in insect cells, the CL_{int} of alprazolam 4-hydroxylation by CYP3A4 is about 2-fold greater than that by CYP3A5, whereas the CL_{int} of α-hydroxyalprazolam by CYP3A5 is about 3-fold greater than that by CYP3A4 (Hirota et al., 2001), suggesting that CYP3A5 plays an important role in the metabolism of alprazolam (Park et al., 2006), and that the formation of α-hydroxyalprazolam is mainly catalyzed by CYP3A5 rather than by CYP3A4 (Hirota et al., 2001; Williams et al., 2002). Accordingly, the formation of α-hydroxyalprazolam may be used as a selective marker for CYP3A5 activity (Galetin et al., 2004; Soars et al., 2006), although relative contribution of CYP3A5 to this metabolic pathway varied between 3 and 47% (Galetin et al., 2004).

In addition, the average CL_{int} value (sum of 4- and α-hydroxylation) obtained using three HLM samples is 4-fold greater than that obtained using 3 small intestinal microsomal samples from the same donors, suggesting a minor contribution of intestinal metabolism to alprazolam disposition (Hirota et al., 2001). Furthermore, the oral bioavailability of alprazolam is >90% after s.c. administration in rats, indicating the little role of intestinal first-pass metabolism (Lau et al., 1997).

**Alfentanil**

Alfentanil is a particularly useful probe both *in vitro* and *in vivo* for intestinal and hepatic CYP3A activity, because a change in subject’s pupil diameter is a surrogate for its plasma concentrations as a non-invasive assessment of CYP3A activity (Kharasch et al., 2004; Klees et al., 2005a). Alfentanil undergoes extensive CYP3A-mediated metabolism via two major pathways, forming noralfentanil and N-phenylpropionamide (Klees et al., 2005a). In the HLM samples, alfentanil is metabolized by CYP3A5 in addition to by CYP3A4 (Klees et al., 2005b).

Furthermore, there is a relatively poor correlation between alfentanil metabolism and CYP3A4 content ($R^2 = 0.30$), but an excellent correlation when CYP3A5 is taken into account (i.e., total CYP3A content, $R^2 = 0.81$) (Klees et al., 2005b). In human body, alfentanil has a similar intestinal extraction ratio as does MDZ but a somewhat lower hepatic extraction ratio than MDZ, and is primarily metabolized by CYP3A (Kharasch et al., 2004). Similar to MDZ, oral clearance of alfentanil can reflect total (intestinal and hepatic) CYP3A activity of subjects, whereas hepatic CYP3A activity is measured by its intravenous clearance (Chaobal and Kharasch, 2005; Kharasch et al., 2004).

**Quinidine**

Quinidine is a frequently prescribed anti-arrhythmic drug. The 3-hydroxylation of quinidine is a specific probe for CYP3A4 activity in the HLM preparations, whereas its N-oxidation is a somewhat less specific marker reaction for CYP3A4 activity as CYP2C9 and CYP2E1 also catalyze the formation of minor proportions of the N-oxidation metabolite (Nielsen et al., 1999). Similar results confirmed that quinidine 3-hydroxylation can be used as specific CYP3A4 probe in subjects (Andreasen et al., 2007), HLM samples and human rCYP3A4 (Mirghani et al., 2002, 2003). The relative ratio of quinidine 3-hydroxylation generated by CYP3A4 to that by CYP3A5 increases more than 4-fold between 5 and 500 μM quinidine concentrations (Galetin et al., 2004). Based on the formation of metabolite mediated by rCYP3A4 and rCYP3A5, quinidine 3-hydroxylation is a selective CYP3A4 marker (Galetin et al., 2004). In addition, some evidence has suggested that
CYP3A5 appears incapable of mediating (3-S)-3-hydroxylation of quinidine (Nielsen et al., 1999; Wrighton et al., 1990). Quinidine was also used to predict a range of reported CYP3A4 drug interactions with azole inhibitors and provide a good link between the in vitro data and in vivo inhibition (Galetin et al., 2005).

**Cortisol**

CYP3A mediates the formation of 6β-hydroxycortisol from the endogenous cortisol, and 6β-hydroxycortisol (a minor metabolite of cortisol) is thus excreted in urine as an unconjugated form (Furuta et al., 2003). In addition to CYP3A4, CYP3A5 catalyzes cortisol 6β-hydroxylation in liver and kidney tissues, and cortisol 6β-hydroxylation rate correlates well with 6β-hydroxyltestosterone (Huang et al., 2004b). After systematically reviewing 277 papers that used 6β-hydroxycortisol urinary excretion, Galteau and Shamsa suggested that urinary 6β-hydroxycortisol is a validated test for evaluating CYP3A-mediated drug induction or drug inhibition although it is not reliable enough to measure CYP3A activity during the period of actual reaction (Galteau and Shamsa, 2003).

**Testosterone**

Testosterone 6β-hydroxylation is CYP3A-mediated (Yuan et al., 2002). Kamdem et al. (2004) studied the relative contribution of CYP3A5 to total CYP3A activity as measured by testosterone 6β-hydroxylation, and found that the specific activities of CYP3A4 and CYP3A5 towards testosterone are comparable in baculovirus-expressed CYP3A4 and CYP3A5, but the contribution of CYP3A5 to 6β-hydroxylation of testosterone in 47 Caucasian liver microsomes was limited, possibly due to the much lower constitutive expression levels of CYP3A5. In contrast, another study using the HLM preparations of Japanese subjects indicated that CYP3A5 contents correlated well with the amount of 6β-hydroxyl-testosterone among subjects carrying at least one CYP3A5*1 allele, and that the contents of CYP3A5 (and to a lesser extent, CYP3A4) correlated well with the rate of testosterone 6β-hydroxylation (Yamaori et al., 2004). Other studies also documented inconsistent roles in the 6β-hydroxylation of testosterone played by CYP3A4 and CYP3A5, respectively (Gillam et al., 1995; Patki et al., 2003; Williams et al., 2002). Thus, testosterone 6β-hydroxylation is the probe for both CYP3A4 and CYP3A5, but in the subjects carrying no CYP3A5*1 allele, the role of CYP3A5 seems little.

**Vincristine**

Vincristine is an important chemotherapeutic agent whose pharmacokinetic properties display significant inter-individual variations, possibly due to CYP3A-mediated metabolism (Yao et al., 2000). Recently, Dennison et al. incubated vincristine with a library of cDNA-expressed P450s and found CYP3A4 and CYP3A5 were the only P450s involved in its metabolism. Furthermore, CYP3A5 was more efficient in catalyzing the formation of its major metabolite M1 than CYP3A4 (9- to 14-fold higher CL_int for CYP3A5), regardless of the absence and presence of cytochrome b5 (Dennison et al., 2006). Accordingly, the metabolism of vincristine to M1 can be used as a specific marker for CYP3A5 activity in vitro. In addition, because of the polymorphic expression of CYP3A5, the use of this drug may be related to marked inter-racial and inter-individual variability in its metabolism (Bohme et al., 1995; Fedeli et al., 1989).
Tacrolimus

Tacrolimus, an immunosuppressant used for organ transplantation, has a narrow therapeutic range and its pharmacokinetic variability complicates its daily dose assessment (Op den Buijsch et al., 2007). Tacrolimus is predominantly metabolized to 13-O-demethyltacrolimus in liver and intestine by CYP3A (Kamdem et al., 2005). Recombinant CYP3A5 metabolized tacrolimus with an CL_{int} (V_{max}/K_m) that was 64% higher than that of CYP3A4, but the contribution of CYP3A5 to 13-O-demethylation of tacrolimus in the HLMs varied from 1.5% to 40% (median, 18.8%) (Kamdem et al., 2005). Dai et al. observed similar results that the CL_{int} of CYP3A5 was twice that of CYP3A4 (Dai et al., 2006). Thus, the demethylation of tacrolimus to 13-O-demethyltacrolimus can be used as a specific probe of CYP3A5 in vitro, but the contribution of CYP3A5 in vivo should be dependent on the genotype of CYP3A5 and concomitant CYP3A4 activity.

Fluorescent Probes

In the early phase of the drug development, high-throughput methods were needed to determine metabolic inhibition of a large number of chemicals being tested (Crespi et al., 1997; Stresser et al., 2000). Assays of the P450 activity by fluorometric probes are highly sensitive and high-throughput (Stresser et al., 2002). The currently available fluorescent probes include 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-benzyloxyquinoline (BQ) (Stresser et al., 2002), 3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one (DFB) (Chauret et al., 1999; Nicoll-Griffith et al., 2004), 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBC) (Renwick et al., 2001), benzyloxyresorufin (BzRes), dibenzylfluorescein (DBF) (Stresser et al., 2000), 2-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (2TFBC), and 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBC) (Baririan et al., 2006). These substrates produce metabolites that emit fluorescence as measured by either fluorescent plate reader (Stresser et al., 2000) or HPLC combined with spectrofluorometric analysis (Baririan et al., 2006). Although the fluorescent probes are not enzyme-specific, they are sensitive, high-throughput for the analysis of enzyme inhibition.

Consistency of Prediction by Various Probes

For the various CYP3A probes, sometimes it is difficult to predict each other as a result of their different molecular and pharmacokinetic properties, such as structural diversity, atypical kinetic profiling due to multiple binding sites associated with CYP3A (Galetin et al., 2005; Ueng et al., 1997), extra-hepatic metabolism (Lown et al., 1994), their interactions with inhibitors with different inhibition types (Thummel and Wilkinson, 1998; Wang et al., 1997), and CYP3A genetic polymorphisms that may selectively affect the metabolism of probes. Therefore, the interactions observed with one CYP3A probe may not be representative of those with others. Kenworthy et al. determined the relationship between 10 commonly used CYP3A4 probes using modifiers with a range of inhibitory potency and recommended that multiple CYP3A4 probes are used for the in vitro assessment of CYP3A4-mediated drug interactions (Kenworthy et al., 1999).

However, a recent report by Galetin et al. focused on four most commonly used CYP3A4 in vitro probes for the prediction of 26 reported in vivo interactions with azole inhibition, demonstrating simple in vitro inhibition profiles of MDZ and quinidine by
azoles with a good accuracy and precision for prediction (Galetin et al., 2005). In addition, two research groups debated on the accurate prediction of CYP3A probes. Dr. Benet believed that useful CYP3A probes will never be developed as all prior attempts to develop probes for the most clinically important CYP3A failed (Benet, 2005). In contrast, Kharasch et al. (2005) challenged some of Dr. Benet’s interpretations and concluded that a probe that predicts 50% of interpatient variation in elimination kinetics may be useful.

Rational Use of Probes

According to these studies, there are various results available for the same probe. The following causes may contribute to such inconsistency.

1. Different sources of the drug-metabolizing enzymes: the HLM preparations, recombinant enzymes (which are produced from transfected bacteria, E. coli, yeast, insect cells, and human lymphoblastoid cells), and primary cultured human hepatocytes.
2. Ethnic and individual variability in CYP3A4 and CYP3A5 expression: CYP3A5*3 is the most common polymorphism present in all ethnic groups and causes the absence of functional CYP3A5 protein (Xie et al., 2004). The ranking order of estimated population frequency of the CYP3A5*3 allele is: Caucasians (90–93%) > East Asians (73%) > Hispanic (65%) > South Asians (60%) > African-Americans (32%) (Xie et al., 2004).
3. The matrix used may vary by lipid mixtures, cholate, detergent, buffer, salt compositions, and components of the NADPH-regenerating system used (Huang et al., 2004a).
4. The contents of NADPH-dependent cytochrome P450 reductase and cytochrome b₅ (which contribute to different CYP3A4 and CYP3A5 metabolic activities) may vary (Huang et al., 2004a).

CYP3A INDUCERS

Some drugs can increase CYP3A activity (Burk and Wojnowski, 2004) through transcriptional activation (induction) that is mediated by the nuclear receptors PXR and/or CAR. PXR and CAR have the potential to cross-regulate CYP3A gene expression (Quattrochi and Guzelian, 2001). CYP3A induction is generally considered clinically less important than its inhibition because it is expected to reduce the plasma concentrations of co-administered CYP3A substrate drugs rather than to impair their safety (Burk et al., 2004). However, the loss of efficacy also has a devastating effect on the treatment of life-threatening diseases or conditions, such as cancer, organ transplantation, epilepsy, oral contraceptives, and so on. The following are common CYP3A inducers and their data derived from in vitro studies are presented in Table 2.

Rifampin

Antibacterial agent rifampicin (Rif) is the most frequently used CYP3A inducer. Studies with cDNA and oligonucleotide expression arrays have shown that Rif can induce the expression of a large number of phase I and phase II drug-metabolizing enzymes, but its induction may vary, with CYP3A4 being induced by 55-fold relative to CYP3A5 and CYP3A7 by 5- and 28-fold, respectively (Rae et al., 2001). Burk et al. observed similar results that 10μM Rif produced a negligible effect on CYP3A5 expression in human
hepatocytes, which is between 1.6 and 7.9% of that measured for CYP3A4 (Burk et al., 2004). It is now assumed that induction of CYP3A by Rif is genotype specific for CYP3A.

It has been reported that Rif activated PXR and CAR and regulated the expression of CYP3A genes (Vyhlidal et al., 2006).

In addition, P-gp and CYP3A have considerable overlapping in inducers in vitro and share common regulatory mechanisms (PXR and CAR) (Matheny et al., 2004; Xu et al., 2005). In rodents, Rif can induce intestinal and hepatic CYP3A and intestinal P-gp but not hepatic P-gp (Matheny et al., 2004). Similarly, coinduction of CYP3A and P-gp by Rif has been confirmed in human LS180 colon carcinoma cell lines and human intestine rather than in rat liver (Cummins et al., 2002). Clearly, Rif can be used as both CYP3A and P-gp inducer in small intestine and as a CYP3A inducer in liver.

Phenobarbital

Phenobarbital (PB) and PB-like compounds have considerable inducing effects in liver (Bell and Michalopoulos, 2006). PB mainly induces CYP2B and CYP3A (Park et al., 2005). PB is a CAR mediator and to a lesser extent, PXR activator (Bell and Michalopoulos, 2006; Wang and LeCluyse, 2003). In cultured HepG2 cells, the level of CYP3A4 mRNA expression by PB is 3-fold greater than that under the constitutive condition, whereas CYP3A7 mRNA is weakly induced (2-fold) and CYP3A5 induction is negligible (Usui et al., 2003). Interestingly, in the human lung, CYP3A5 is the major CYP3A isoform and is induced 11-fold by PB, but CYP3A4 is not induced by any of the typical CYP3A4 inducers used in human alveolar type II cells (Hukkanen et al., 2000). Therefore, when studying CYP3A induction and CYP3A-associated drug interactions in lung, CYP3A5 gene polymorphisms should be taken into account.

Dexamethasone

Dexamethasone (Dex) is a frequently used CYP3A inducer although it is less potent than Rif in human hepatocytes (Lu and Li, 2001). Conversely, in rat hepatocytes, Dex is a
potent CYP3A inducer whereas Rif is not an inducer (Lu and Li, 2001). Similar to Rif, Dex can induce the expression of a large number of drug-metabolizing enzymes, including CYP3A4, CYP2B6, CYP2C8 and UGT1A1 in human hepatocytes (Greger and Blum, 2007).

Dex induces CYP3A4-driven luciferase reporter gene expression in a concentration-dependent manner and the glucocorticoid receptor is involved in such an induction in transfected HepG2 cells (Ogg et al., 1999). In primary human hepatocytes and HepG2 cells, low-Dex component results from the glucocorticoid receptor-mediated expression of PXR and/or CAR which, in turn, transactivate CYP3A4 in a xenobiotic-independent manner (Pascussi et al., 2001). However, at the concentrations greater than 10 μM, Dex binds to and activates PXR, thus producing the high-Dex component of CYP3A4 induction (Pascussi et al., 2001). Interestingly, in human fetal hepatocytes, induction of CYP3A4 and CYP3A7 is mediated directly by glucocorticoid receptor and does not involve PXR (Matsunaga et al., 2004).

In HepG2 cells, CYP3A mRNA is significantly induced by Dex, 5-fold for CYP3A4, 3-fold for CYP3A43, 2-fold for CYP3A7 and 1.5-fold for CYP3A5 relative to the controls (Krusekopf et al., 2003). In the human A549 lung adenocarcinoma cell line, Dex induces CYP3A5 by 4-fold (Hukkanen et al., 2003). Different from Rif (see above), Dex only induces hepatic and intestine CYP3A and not P-gp expression (Matheny et al., 2004). Accordingly, Dex can dissect the contribution of CYP3A and P-gp in the drug disposition.

Clotrimazole

In the reporter gene assay, clotrimazole highly activates PXR by ~10-fold and induces CYP3A4 mRNA by 6.8-fold (Luo et al., 2002). But in HepG2 cells, the induction of CYP3A4 and CYP3A5 mRNA expression is negligible by clotrimazole, whereas CYP3A7 mRNA level increases approximately 2-fold in cultured HepG2 cells (Usui et al., 2003). Interestingly, although clotrimazole is a potent activator of CYP3A4 transcription, it is also a potent inhibitor of CYP3A activity (Zhang et al., 2002) despite some conflicting results (Schuetz et al., 1996). It was reported that clotrimazole activates hPXR but deactivates hCAR. CAR and PXR may recognize and bind to the same response elements and likely co-regulate some of their target genes, suggesting that the net effect of a drug on gene transcription seems complex, depending on its effects on both CAR and/or PXR (Moore et al., 2000). That may help us better understand the inconsistent data on clotrimazole.

Carbamazepine

The antiepileptic drug carbamazepine is well known as a potent inducer and a substrate of CYP3A (Konishi et al., 2004). In the HepG2 cells, treatment with carbamazepine rapidly induces the level of CYP3A4 mRNA expression by 3- to 6-fold, and CYP3A5 mRNA approximately 2-fold (Usui et al., 2003). In cultured human hepatocytes, carbamazepine weakly activates PXR and induces CYP3A4 activity (Luo et al., 2002). Recently, carbamazepine is classified as a negligible or weak hPXR activator and induces CYP3A4 and CYP2B6 preferentially through hCAR activation (Faucette et al., 2007). In a clinical study performed with healthy volunteers taking quinidine as a probe for CYP3A, carbamazepine increased the 3-hydroxyquinidine formation by 181% and AUC ratio by 222% compared to the baseline (Andreasen et al., 2007).
Sulfinpyrazone

Sulfinpyrazone highly activates PXR (>10-fold) in PXR luciferase reporter gene expression, increasing CYP3A4 activity by 2- to 3-fold in primary culture of human hepatocytes (Luo et al., 2002). In addition, it can induce CYP3A4 promoter-driven luciferase reporter gene expression, but the induction is not enhanced by the glucocorticoid receptor (Ogg et al., 1999).

Phenytoin

The anticonvulsant phenytoin (PHT) is a CYP3A4 and CYP2B6 inducer and CYP2C9/CYP2C19 substrate (Jackson et al., 2004; Lim et al., 2004). PHT weakly activates PXR and induces CYP3A4 activity (Luo et al., 2002). But Raucy et al shows that PHT produces a marked increase in CYP3A4 mRNA (equivalent to that seen with Rif) and exhibits typical dose-response curves (Raucy, 2003).

PHT is a potent CYP3A4 inducer in human hepatocytes (707±188% of control) and in the reporter gene assay (5.2-fold over control) (Raucy, 2003). PHT may induce CYP3A4 content in hepatocytes through indirect PXR activation, which could explain the modest induction observed in the PXR-mediated reporter gene assay (Raucy, 2003). In the HepG2 cells, treatment of PHT gradually induces CYP3A4 mRNA level by 4.5-fold and CYP3A5 by 2-fold of control (Usui et al., 2003).

CYP3A INHIBITORS

There are two categories of the CYP3A inhibitors – chemical inhibitors (summarized in Table 3) and monoclonal antibodies against CYP3A. These inhibitors are very useful in clarifying the role of individual CYP3As in drug metabolism and in predicting the potential drug-drug interactions. But the mechanisms underlying the interactions of various inhibitors, substrates and CYP3As are complex. As a result of the atypical nature of CYP3A active site(s), the same inhibitor may cause different types of inhibitions towards various substrates (Gibbs et al., 1999; Kunze et al., 1996).

Low substrate specificity also makes CYP3A4 susceptible to reversible or irreversible inhibition by a variety of drugs (Zhou et al., 2005), in particular the mechanism-based inhibition of CYP3A4, which is characterized by NADPH-, time- and concentration-dependent enzyme inactivation, occurring when some drugs are converted by P450 isozymes to reactive metabolites capable of irreversibly binding covalently to CYP3A4 (Zhou et al., 2005). This suggests that a drug inhibition study should be performed using multiple P450 inhibitors to explain the contribution of P450 enzymes to the metabolism of a certain drug.

Chemical Inhibitors

Ketoconazole. Ketoconazole is a well-characterized reversible inhibitor of human CYP3A isoforms, both in vivo and in vitro (Cotreau et al., 2000). It strongly reduces CYP3A-mediated metabolism of MDZ, triazolam, testosterone and nifedipine in the HLM preparations and by rCYP3A4 (Patki et al., 2003). The inhibitory potency of ketoconazole toward rCYP3A5 is about 5- to 19-fold less than that toward rCYP3A4 for all 4 mentioned substrates (Patki et al., 2003). Similar results have shown that ketoconazole is
Table 3  CYP3A chemical inhibitors (summarized from in vitro data).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>(Ki) ((\mu)M)</th>
<th>(IC_{50}) ((\mu)M)</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>testosterone 6β-hydroxylation</td>
<td>0.024</td>
<td>–</td>
<td>rCYP3A4</td>
<td>Emoto et al., 2003</td>
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<tr>
<td></td>
<td>nifedipine oxidation</td>
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<td>0.097</td>
<td>HLMs</td>
<td>Patki et al., 2003</td>
</tr>
<tr>
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<td>midazolam 4-hydroxylation</td>
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<td>0.089</td>
<td>HLMs</td>
<td>Patki et al., 2003</td>
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<td>rCYP3A4</td>
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<td>1.9</td>
<td>–</td>
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<td>2.9</td>
<td>–</td>
<td>HLMs</td>
<td>Yeo et al., 2001</td>
</tr>
</tbody>
</table>

*Ki value: the concentration of an inhibitor that inhibits half of the maximum reaction velocity for reversible inhibitor and the concentration of an inhibitor required for half-maximal inactivation for mechanism-based inhibitor.

IC\(_{50}\) value: the concentration of an inhibitor required to produce half of reaction velocity of the control.

HIMs: human intestinal microsomes; HLMs: human liver microsomes.

*mechanism-based inhibitor.
more potent toward CYP3A4 than CYP3A5 (Klees et al., 2005b; Venkatakrishnan et al., 2001).

In addition, Ketoconazole is a relatively weak inhibitor of CYP2C9, CYP2D6, CYP1A2, CYP2A6, CYP2C19 and CYP2C8 (Venkatakrishnan et al., 2001). Ketoconazole is nonselective at high concentrations but relatively selective for CYP3A enzymes at low concentrations (Klees et al., 2005a). Low concentrations of ketoconazole (preferably 1 μM or lower) are shown to minimize nonspecific inhibition of other isoforms except for CYP3A (Venkatakrishnan et al., 2001). Ketoconazole exhibits mixed inhibition for testosterone 6β-hydroxylation (Ki = 0.024 μM, α = 8.8), and nifedipine oxidation (Ki = 0.011 μM, α = 32), but competitively inhibits MDZ 1′-hydroxylation (Ki = 0.028 μM) in baculovirus-expressed recombinant human CYP isoforms (Emoto et al., 2003).

Itraconazole (ITZ). ITZ is a potent inhibitor of CYP3A in vivo, and its metabolites are more potent CYP3A4 inhibitors than ITZ itself (Isoherranen et al., 2004). ITZ is not a mechanism-based inhibitor for CYP3A (Niwa et al., 2005). More importantly, ITZ is selectively metabolized by CYP3A4 and not by CYP3A5 (Huang et al., 2004a; Isoherranen et al., 2004).

Troleandomycin (TAO). TAO is a mechanism-based inhibitor of human CYP3A isoforms due to the formation of P450 intermediate metabolite complex (Cotreau et al., 2000; Lindstrom et al., 1993). Using the production of 1'-hydroxymidazolam as a marker for CYP3A activity, TAO is a time-dependent inhibitor of CYP3A4 (Ki = 0.26 μM), but does not inhibit CYP3A5 in a time-dependent manner in E. coli expressed CYP3A4 and CYP3A5 (Soars et al., 2006).

However, Klees et al. observed that TAO inhibits the expression of both CYP3A4 and CYP3A5 in expressed CYP3A enzymes using alfentanil as a probe. The IC50 values for TAO inhibition of alfentanil metabolism mediated by CYP3A4 and CYP3A5 were 0.28 and 0.62 μM for noralfentanil formation, and 0.24 and 0.75 μM for AMX formation, respectively (Klees et al., 2005a). Similar results were observed by Chang et al., TAO caused 90% and 75% inhibition of testosterone 6β-hydroxylation catalyzed by expressed human CYP3A4 and 3A5, respectively (Chang et al., 1994).

Erythromycin. Erythromycin is both a substrate and potent mechanism-based inhibitor of CYP3A (Polasek and Miners, 2006). Erythromycin inhibited the triazolam α-hydroxylation with IC50 (Ki) values of 33 (± 20) μM in human liver microsomes (Zhao et al., 1999). In human hepatocytes, erythromycin inhibited MDZ 1′-hydroxylation with a K1 of 11 μM and Kina of 0.07 min−1, similar to those determined with rCYP3A4 (K1 of 9 μM and Kina of 0.12 min−1) (McGinnity et al., 2006). Erythromycin is also widely used in vivo studies as a strong inhibitor of CYP3A (de Mey et al., 2001; Kantola et al., 1998).

Clarithromycin. Clarithromycin is also a mechanism-based inhibitor of CYP3A (Galetin et al., 2006). Clarithromycin increases both hepatic and intestinal availability of the selective CYP3A probe MDZ (Pinto et al., 2005). Clarithromycin inhibited the triazolam α-hydroxylation with IC50 (Ki) values of 56 (±43) μM in human liver microsomes (Zhao et al., 1999). Similarly, clarithromycin inhibited the MDZ α-hydroxylation with Kapp and Kina of 41.4 μM and 0.0423 min−1, respectively, in human liver microsomes (Ito et al., 2003). Clarithromycin is also widely used as a strong inhibitor of CYP3A in vivo studies (Galetin et al., 2006; Gurley et al., 2006).

Fluconazole. An inhibitor might exhibit competitive inhibition toward one substrate and noncompetitive inhibition toward another. That seems to be the case for interactions between fluconazole and CYP3A. Fluconazole displays predominantly competitive
inhibition toward CYP3A4-catalyzed 10-hydroxylation of \((R)\)-warfarin (Kunze et al., 1996), and noncompetitive inhibition toward MDZ 1’-hydroxylation (Gibbs et al., 1999). Despite different mechanisms of inhibition, \(K_i\) values from the two studies are similar (18 and 11 \(\mu\)M). This suggests that a single fluconazole binding site, when occupied, prevents \((R)\)-warfarin and not MDZ from binding to the substrate binding site (Gibbs et al., 1999). It should also be pointed out that the \(IC_{50}\) value of fluconazole against \(S\)-mephenytoin 4’-hydroxylation (CYP2C19) was comparable with that of nifedipine oxidation (CYP3A4), which is 12.3 \(\mu\)M and 13.1 \(\mu\)M, respectively (Niwa et al., 2005).

**SKF-525A.** SKF-525A is a non-specific and mechanism-based CYP450 inhibitor (Emoto et al., 2003, 2005), and competitively inhibits CYP3A4-dependent MDZ 1’-hydroxylation (\(K_i=1.2\) \(\mu\)M), whereas its inhibition toward testosterone 6\(\beta\)-hydroxylation (\(K_i=0.79\) \(\mu\)M, \(\alpha=2.9\)) and nifedipine oxidation (\(K_i=4.2\) \(\mu\)M, \(\alpha=4.0\)) is a mixed-type of competitive and noncompetitive inhibition in baculovirus-expressed human rCYP3A4 (Emoto et al., 2003). In the HLM preparations, SKF-525A potently inhibits CYP2D6, moderately inhibits CYP3A, CYP2C9, CYP2C19, and relatively weakly inhibits CYP1A2 and CYP2E1 (Emoto et al., 2005), but its inhibitory effect is reduced by pre-incubation with NADPH, suggesting that SKF-525A is metabolized in the HLM preparations (because a decrease in SKF-525A concentrations leads to a reduced inhibition) (Emoto et al., 2005).

**1-Aminobenzotriazole (ABT).** ABT is widely used as a non-specific and mechanism-based P450 inhibitor because it is known as a suicide substrate for P450 isoforms (Emoto et al., 2003). ABT exhibits mixed (competitive and noncompetitive) inhibition for testosterone 6\(\beta\)-hydroxylation (\(K_i=1.9\) \(\mu\)M, \(\alpha=1.6\)), MDZ 1’-hydroxylation (\(K_i=8.4\) \(\mu\)M, \(\alpha=1.4\)), nifedipine oxidation (\(K_i=1.5\) \(\mu\)M, \(\alpha=1.9\)) in baculovirus-expressed human rCYP3A4 (Emoto et al., 2003). ABT has a potent inhibitory effect on the CYP3A-mediated drug oxidation and to a lesser extent, on CYP2C9-mediated drug oxidation (Emoto et al., 2005). ABT has a wide range of \(IC_{50}\) values in the HLM preparations with and without preincubation, but its inhibitory effect is increased by preincubation (Emoto et al., 2005).

**Verapamil.** Verapamil, a calcium channel blocker, is a mechanism-based inhibitor of CYP3A (Wang et al., 2005) and has been widely used in the treatment of many cardiovascular diseases (Shen et al., 2004). It is mainly metabolized by CYP3A (CYP3A4, CYP3A5 and CYP3A7), CYP1A2 and the CYP2C subfamily (Busse et al., 1995), and has atypical enzyme kinetics for human cDNA-expressed CYP3As (Shen et al., 2004). Wang et al. (2005) have reported that verapamil differentially inhibited CYP3A4 and CYP3A5 via mechanism-based inhibition: verapamil inhibited CYP3A4 activity but had little effect on CYP3A5 activity in the absence of cytochrome b\(5\). In the HLMs, verapamil inhibited testosterone 6\(\beta\)-hydroxylation with \(K_{inact}\) and \(K_i\) of 0.15 min\(^{-1}\) and 2.9 \(\mu\)M, respectively (Yeo and Yeo, 2001). Long-term administration of verapamil inhibits CYP3A activity, as measured by its own disposition, with a maximum effect within 10 days in healthy volunteers (Lemma et al., 2006).

**Monoclonal Antibodies**

Monoclonal antibodies (mAbs) recognize a single antigenic determinant or epitope on the surface of a P450 molecule, resulting in the inhibition of catalytic activity. Therefore, mAbs are ideal tools to investigate the properties and molecular diversity of the P450 proteins. In terms of their chemical purity, stability, precision, specificity, and reproducibility for limitless generations of hybridomas, mAbs are considered to be superior reagents to
other chemical inhibitors and polyclonal antibodies (Mei et al., 1999). The mAbs function largely as noncompetitive inhibitors and are insensitive to substrate concentrations. Thus, mAbs are ideal reagents to accurately define the role of the P450s (Mei et al., 1999). Antibodies against CYP3A4 and CYP3A5 are commercially available. In addition to drug inhibition, CYP3A antibodies can also be used for CYP3A protein assay, qualitatively and quantitatively.

**CYP3A and P-gp**

CYP3As, abundant in both hepatocytes and enterocytes, limit the systemic bioavailability of drugs. P-gp is known as an efflux transporter, reducing the oral bioavailability of its substrates (Patel and Mitra, 2001). The considerable overlap in the substrate selectivity and tissue localization, and coinducibility of CYP3A4 and P-gp have led to the hypothesis that they work together to coordinate an absorption barrier against drugs (Benet et al., 2003).

Coinduction of CYP3A and P-gp has been observed by rifampin and carbamazepine (Cummins et al., 2002; Giessmann et al., 2004; Konishi et al., 2004; Matheny et al., 2004). Also, many P-gp inhibitors are known or suspected to be substrates and/or inhibitors of CYP3A (Wandel et al., 1999). Wandel et al. used a series of 14 P-gp inhibitors to assess the relationship between P-gp and CYP3A inhibition, and found that, although many P-gp inhibitors are potent inhibitors of CYP3A, no significant correlation exists between the ability of the compounds to inhibit P-gp and their ability to inhibit CYP3A (Wandel et al., 1999). Therefore, understanding of P-gp interactions with CYP3A inhibitors is essential for their drug interactions. Zhang et al. summarizes some examples of substrates and inhibitors of both CYP3A and P-gp (Zhang and Benet, 2001).

In addition, CYP3A and P-gp associated drug-herb interactions cannot be ignored. A recent review discussed the mechanism of P-gp and CYP3A4-related drug-herb interactions and found that herbs can pharmacokinetically act as either inhibitors or inducers (Pal and Mitra, 2006). *In vitro* studies suggested that short-term exposure to “pure” herbal agents (such as hypericin, kaempferol and quercetin) or extract of SJW resulted in higher uptake or influx of ritonavir and erythromycin, and these herbal constituents also caused a remarkable inhibition of cortisol metabolism during short-term exposure (Pal and Mitra, 2006).

Conversely, long-term exposure to herbal agents (hyperforin, kaempferol and quercetin) showed increased mRNA expression of *CYP3A4* and *MDR-1* in Caco-2 cells (Pal and Mitra, 2006). Therefore, clinical implications of CYP3A and P-gp associated drug-herb interactions should pay special attention. There are multiple constituents present in herbal medicines, of which some are inducers of CYP3A/P-gp and the others are inhibitors of CYP3A/P-gp. Therefore, the final net effect of herb-drug interaction would depend on the types and amounts of individual components present in herbs and the duration of exposure to herbs (Xie and Kim, 2005).

**CONCLUSIONS**

P450s are responsible for the metabolism of many drugs and also the targets for a number of drug interactions of clinical importance. A number of drug–drug interactions resulting from the induction and inhibition of CYP3A have highlighted the importance of understanding drug-metabolizing enzymes. CYP3A4 and CYP3A5 play an important role in the metabolism of endogenous substances and exogenous chemicals and share most of
their substrates, inducers and inhibitors with each other. There is no any probe that is exclusively metabolized by a single CYP3A isoform. Therefore, multiple probes should be used to increase the accuracy of prediction.

Various probes sometimes have difficulties to predict each other as a result of their different molecular properties and pharmacokinetic profiling. In terms of this complexity, we should take special cautions when making a conclusion. CYP3A inducers and inhibitors all are useful tools in defining the role of individual CYP3As involved in drug metabolism and in predicting the potential drug-drug interactions. As a result of the complicated interactions between enzymes and substrates, the use of multiple P450 inhibitors and inducers are also recommended in the drug development and research.

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