IN VITRO AND IN VIVO DRUG INTERACTIONS INVOLVING HUMAN CYP3A

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ABSTRACT
Cytochrome P4503A (CYP3A) is importantly involved in the metabolism of many chemically diverse drugs administered to humans. Moreover, its localization in high amounts both in the small intestinal epithelium and liver makes it a major contributor to presystemic elimination following oral drug administration. Drug interactions involving enzyme inhibition or induction are common following the coadministration of two or more CYP3A substrates. Studies using in vitro preparations are useful in identifying such potential interactions and possibly permitting extrapolation of in vitro findings to the likely in vivo situation. Even if accurate quantitative predictions cannot be made, several classes of drugs can be expected to result in a drug interaction based on clinical experience. In many instances, the extent of such drug interactions is sufficiently pronounced to contraindicate the therapeutic use of the involved drugs.

INTRODUCTION
Over the past four to five years, the assessment of human drug metabolism has undergone a major paradigm shift, particularly with respect to biotransformations mediated by the cytochrome P450 (CYP) superfamily of enzymes. Much of this change has resulted from the rapid expansion of knowledge at
the molecular level; characterization of the various isoforms with respect to their substrate specificities and regulatory determinants; recognition that of the 30 or so isoforms in humans, only a handful are significantly involved in the metabolism of most drugs; and the ready availability of human tissue preparations and individual gene products prepared from heterologous expression systems. As a consequence, drug metabolism studies are now increasingly being used in early drug development for lead optimization and the selection of compounds for more extensive investigation; in the qualitative and quantitative prediction of drug biotransformation in vivo; and in the identification of likely determinants of metabolism following drug administration to humans, including possible drug interactions. Similarly, an increasing number of undesirable effects that occur in the clinical use of drugs are being explained and avoided on the basis of knowledge about the specific CYP enzyme(s) involved in an agent’s metabolism.

Of the several human CYP enzymes, those of the CYP3A subfamily have major importance, since collectively, they are by far the most abundant of all of the human CYP isoforms; their substrate specificity is extremely broad; accordingly, many structurally diverse compounds are substrates to some extent; they are localized in organs of particular relevance to drug disposition (gastrointestinal tract, kidney, and liver); and their catalytic activity is readily modulated by a variety of compounds. In this review, recent advances in the understanding of human CYP3A activity and its in vitro and in vivo determination are discussed, especially with respect to drug interactions.

CHARACTERISTICS OF HUMAN CYP3A

Tissue Localization
A considerable amount of information exists on the characteristics of the members in the CYP3A subfamily of enzymes in both animals and humans (1). At least three functional proteins exist in humans. CYP3A4 is universally found in the liver, where it constitutes the major isoform—on average about 30% of total CYP protein (2). Relatively high CYP3A4 levels—about 50% of hepatic levels and 70% of total CYP protein—are also present in small intestinal epithelium, particularly in the apical region of mature enterocytes at the tip of the microvillus (3, 4). The amount of isoform progressively falls along the remainder of the gastrointestinal tract. In the kidney, however, CYP3A4 is present in only about 30% of renal tissue samples, mainly in the collecting ducts (5, 6); the mechanism for such polymorphic expression is not currently understood. CYP3A3 is a very closely related isoform to CYP3A4 (>98% cDNA sequence similarity), but it is not known whether this reflects a separate gene product or an allelic variant. Therefore, the term CYP3A4 is generally taken to indicate
a collective contribution of the two isoforms. By contrast, CYP3A5 is structurally distinct from CYP3A4. It is also found in the liver but only in about 10–30% of hepatic samples and then at levels 10–30% of CYP3A4 (7, 8). A point mutation resulting in the synthesis of an unstable protein may account for such polymorphism (9). CYP3A5 is the predominant CYP3A isoform that is universally expressed in the kidney (5, 6). It is also heterogeneously expressed throughout the gastrointestinal tract but, generally, in lower amounts than CYP3A4, except in parietal cells of the stomach (3, 4) and possibly the colon (10). CYP3A5 also differs from CYP3A4 in that its expression does not appear to be up-regulated by agents that are well-established inducers of CYP3A4 (7, 11). The third functional CYP3A isoform is CYP3A7, which was originally found in fetal liver; however, it may also be selectively expressed in adult livers at lower levels than CYP3A4 and CYP3A5 (12).

CYP3A Substrate Specificity
The substrate specificity of the CYP3A enzymes is very broad; accordingly, an extremely large number of structurally divergent chemicals are metabolized by a variety of different pathways, often in a regio- and stereoselective fashion (1, 13). Estimates, based primarily on in vitro studies, suggest that the metabolism of perhaps 40–50% of drugs used in humans involves CYP3A-mediated oxidation to some extent. Whether this reflects the importance of such metabolism in drug elimination in vivo is, however, not so apparent. Nevertheless, it is clear that CYP3A is of major importance in the metabolism of drugs by humans. CYP3A4 is the most thoroughly investigated isoform, and it is generally assumed that the other isoforms have essentially similar characteristics. However, limited studies suggest the likelihood of possibly important differences in the isoforms’ substrate specificities. For example, neither quinidine nor erythromycin appeared to be metabolized by CYP3A5, although they are good CYP3A4 substrates (8, 14), and only one of three primary metabolites of cyclosporine A formed by CYP3A4 was produced by CYP3A5 (14). In other studies, the level of CYP3A5-mediated metabolism, when it was present, was less than that of CYP3A4, although in one instance, namely, the 1′-hydroxylation of midazolam, CYP3A5 exhibited greater catalytic activity (15). A difficulty with such comparative studies is that the optimal in vitro conditions may not be the same for the two isoforms (see below).

An important characteristic of CYP3A is the large interindividual variability in activity, which reflects a genetic effect combined with modulation by environmental factors. For example, hepatic microsomal activity often differs by up to 40-fold (2, 13), and large variability also has been noted with intestinal (16) and renal microsomes (6). Human in vivo studies have also indicated considerable interindividual variability, but generally, this has been smaller (fivefold),
although, of course, the range can be significantly increased by deliberate modulation, i.e. inhibition and induction. The reason for such a discrepancy is not clear, but it has been observed with other CYP isoforms and indicates that care must be taken in quantitatively extrapolating in vitro studies to the in vivo situation.

Because of the difficulty in distinguishing between the isoforms’ catalytic activities, the fact that in many instances several isoforms may be present in a single organ, and in vivo the relative contribution of a specific organ to overall metabolism cannot be readily determined, the term CYP3A is usually understood to reflect the collective activity of all of the isoforms. However, it primarily reflects CYP3A4.

**CYP3A and P-Glycoprotein**

P-glycoprotein in humans is the *MDR1* gene product, which functions as a transmembrane efflux pump. Its overexpression and role in the development of multidrug resistance by tumor cells exposed to cancer chemotherapeutic agents are well recognized (17). However, P-glycoprotein is also present in several normal tissues (17), where it is considered to function to reduce drug absorption (intestinal epithelium), prevent drug distribution (blood-brain barrier), or enhance drug elimination (hepatic canalicular membrane and proximal renal tubule). Thus, P-glycoprotein is colocalized to cells in which CYP3A is also extensively expressed, e.g. enterocytes and hepatocytes. The two proteins, therefore, function in concert to collectively reduce the intracellular concentration of xenobiotics. Significantly, considerable overlap appears to exist between compounds interacting with P-glycoprotein and also CYP3A (18). This is probably fortuitous and reflective of the broad substrate specificities of the individual proteins rather than a more fundamental interrelationship. For example, a considerable number, but not all (e.g. benzodiazepines), of CYP3A substrates interact with P-glycoprotein either as substrates and/or inhibitors (calcium-channel blockers, azole, antifungal agents, immunosuppressants and macrolide antibiotics). Accordingly, the co-administration of two CYP3A substrates can result in interactions that reflect inhibition of metabolism alone, reduced P-glycoprotein efflux only, or a combination of both effects (19). This level of complexity has only been recently recognized and is an active area of current investigation, but definitive studies in humans have yet to be reported. Several studies in animals and in vitro cultured cell lines indicate, however, that the potential for interactions previously considered to only reflect inhibition of metabolism could also involve a P-glycoprotein mechanism (20–22). Moreover, Schuetz et al (23) have shown that the extent of CYP3A induction by rifampin in vitro is related to the cellular level of expression of the efflux pump (23).
MEASUREMENT OF CYP3A ACTIVITY IN VITRO

In vitro approaches to studying CYP3A activity have the advantage that conditions can be more closely controlled and altered than in vivo. On the other hand, the selected conditions may not sufficiently reflect those present in vivo, and experimental findings cannot be readily extrapolated, especially in a quantitative fashion. This is particularly true as the level of cellular integrity and organization decreases. Nevertheless, valuable information concerning many aspects of CYP-mediated metabolism applicable to drug development and clinical use has been obtained using a variety of different preparations (24, 25).

Human CYP3A Preparations

A major problem for in vitro studies is the relative lack of availability of suitable human tissue, although significant advances have been made in recent years. Postmortem sources are of little value, since the material must be as fresh as possible and appropriately preserved. Sources of material have included both waste tissue obtained at surgery and organs from donor patients that are unsuitable for transplantation. However, causes for liver rejection—including steatosis, interrupted blood flow, and physical damage—can compromise in vitro cell viability and metabolic function. Likewise, the presence of luminal and intracellular proteases in the intestine may cause rapid cellular and enzymatic degradation, and extensive fibrosis of a rejected kidney complicates preparation of subcellular fractions without significant loss of metabolic activity. Furthermore, it is not unusual for organ donors to have previously received drugs such as dexamethasone, phenytoin, and barbiturates for therapeutic reasons, e.g. treatment of brain injury. Such drug therapy for as short a period as one to three days has been found to increase hepatic CYP3A protein levels (26–28), and induction in other organs such as the small intestine would also be expected (29, 30). Because of the rapid functional deterioration of hepatocytes and liver slices, procedures involving cold preservation (0–4°C) and cryopreservation (−70°C or below) have been investigated with increasing success (24). For example, the CYP3A-mediated 1′-hydroxylation of midazolam was maintained in hepatocytes for at least two weeks (31). By comparison, widely used subcellular fractions, such as microsomes, have been cryopreserved for many years without significant loss of metabolic activity, including that of CYP3A (32, 33).

In general, the greater the architectural and cellular integrity of the tissue preparation relative to that in vivo, the more closely the findings relate to those in the whole organ. Moreover, preparations in which cellular structure is intact permit the study of enzyme induction as well as cellular toxicity. Precision-cut tissue slices best meet this requirement, since they maintain the heterogeneous structure of the organ and also contain both Phase I and Phase II enzymes, along
with the necessary cofactors to allow sequential metabolism. Liver, kidney, and
intestinal mucosal slices have all been utilized in the study of CYP3A-mediated
metabolism (34–36). However, the rigorous requirement for freshly available
tissue has limited widespread and routine application of this approach. Whole
cell preparations such as hepatocytes also retain the full complement of enzymes
and cofactors, and accordingly, they have been utilized. For example, primary
cultures of human hepatocytes have been used to study modulation of CYP3A
activity by cytokines (37), enzyme-inducing agents (38–40), and inhibitors
(38, 39). More recently, and of particular relevance to intestinal CYP3A, there
have been reports that relatively high levels of CYP3A can be expressed in
continuous cultures of colon-derived Caco-2 cells either by stable transfection
(41) or by transcriptional activation of the native CYP3A gene using 1α,25-
dihydroxy vitamin D3 (42). Accordingly, it should now be possible to study the
role and importance of CYP3A in the vectorial transport of substrates analogous
to that occurring during intestinal absorption, as well as the interaction of this
process with P-glycoprotein–mediated efflux, since Caco-2 cells constitutively
express this transporter.

Because of their robustness and well-established methods of preparations
and characterization, subcellular fractions such as the S-9 fraction and, more
commonly, microsomes have been the most widely used systems for examining
CYP3A activity and its modulation. Tissues studied include the liver (2, 7, 8),
kidney (5, 6), and various regions of the gastrointestinal tract (3, 4, 10, 16),
and numerous compounds have been examined with regard to the involvement
and importance of CYP3A-mediated metabolism and determinants of such
biotransformation (1, 13).

The increasing availability of heterologously expressed, recombinant CYP
enzymes has provided a reasonably ready means to obtain large amounts of
pure functional proteins. All three human CYP3A isoforms have been tran-
siently expressed in, for example, HepG2 cells (14), COS cells (43), yeast cells
(44, 45), Escherichia coli (46, 47), and baculovirus-transfected SF9 cells (48).
Stable expression has also been achieved in several mammalian cell lines in-
cluding V79 Chinese hamster (49, 50), human lymphoblastoid cells (51), and
immortalized breast cancer cells (52). Each of these systems have various ad-
vantages and disadvantages that require consideration relative to the purpose
of any study. In particular, purified enzyme obtained from transient expression
systems requires reconstitution along with phospholipids and various cofactors,
including NADPH-P450 reductase and cytochrome b5. Optimization of such
conditions has been more difficult to establish for CYP3A than other isoforms
(see below). Coexpression of CYP3A4 and NADPH–cytochrome P450 reduc-
tase in baculovirus-transfected insect cells has been reported to overcome some
of these difficulties (53, 54), and a functional fusion protein has been expressed
in *E. coli* (55). Stable-expression systems do not have such a limitation, but on the other hand, the level of enzyme expression is usually far lower than that obtained with transient systems, which can have product formation rates comparable to those observed with human tissue preparations.

**In Vitro Incubation Conditions**

Duplication of the in vivo intracellular milieu is impossible in vitro, yet several studies have shown that the conditions of incubation are important determinants of CYP3A activity. In the case of reconstituted systems using recombinantly expressed or purified enzyme, the usual conditions employed with other CYP isoforms have not generally been very effective (56, 57). For example, catalytic activity was increased for some substrates by the addition of negatively charged phospholipids along with the customary dilauroylphosphatidylcholine (57, 58). Also, cytochrome b₅ has been found to be required for optimal CYP3A activity, but its effect again appears to be dependent upon the particular CYP3A substrate (55, 59–61). Whether known interindividual differences in hepatic and intestinal cytochrome b₅ content or its reductase (62–64) contribute to variable CYP3A activity in vivo is not known. The selection of the buffer agent (Tris or phosphate), its ionic strength and the presence of mono- and divalent cations (NH₄⁺, Ca²⁺, Mg²⁺) can all affect the measured rate of product formation and product ratios of CYP3A-mediated reactions (55, 57, 60). Such findings make predictions of in vivo metabolism from in vitro measurements in reconstituted systems very difficult.

**Cooperativity/Allosterism**

A unique characteristic of CYP3A is that its catalytic activity for a particular substrate may be stimulated by the addition of another xenobiotic to the in vitro incubation mixture. This positive cooperativity or activation of human CYP3A occurs with 7,8-benzoflavone and other flavonoids (65–68), and some steroids (67, 68). Similarly, increasing concentrations of certain CYP3A substrates were found to stimulate their own metabolism (66, 68), which in the case of midazolam, also resulted in a change in the product formation ratio of the two primary metabolites arising from CYP3A-mediated metabolism (15, 69). However, not all CYP3A substrates are susceptible to such activation, and in some cases the addition of 7,8-benzoflavone resulted in inhibition (70–72). Moreover, findings obtained with a reconstituted enzyme system have not always been observed with hepatic microsomes (66, 68). Importantly, activation has also been shown to occur in vivo, at least in the rat (73). An allosteric effect associated with different conformers of the CYP3A active site has been postulated to account for the activation phenomenon (66, 74–76). Regardless of the mechanism(s), the activation of CYP3A activity clearly complicates the interpretation of in
vitro studies, and if it occurs in vivo in humans, similar difficulties in prediction and extrapolation will be present.

INHIBITION OF CYP3A IN VITRO

Given the numerous compounds metabolized by CYP3A, it is not surprising that many of these act as inhibitors; the only common feature is lipophilicity and relatively large molecular size (77). Several mechanisms of inhibition are possible, with some compounds exhibiting more than one type, e.g. erythromycin.

Rapidly Reversible Inhibition

Direct, rapidly reversible binding of an inhibitor or its metabolite to CYP3A has been found to result in either competitive or noncompetitive inhibition, the extent of which is determined by the relative binding constants of substrate and inhibitor for the enzyme and by the inhibitor’s concentration. The critical factor with respect to the ratio of the inhibited intrinsic clearance to that in the absence of inhibitor, the so-called “inhibition index,” is the inhibitor’s concentration relative to its $K_i$ value (78, 79). Thus, the most potent reversible CYP3A inhibitors, which include azole antifungal agents and first-generation HIV protease inhibitors, have $K_i$ values below 1 $\mu$M (Table 1). Other drugs with known in vivo inhibitory effects exhibit $K_i$ values in the low micromolar range, and important inhibition is uncommon for compounds with values greater than about 75–100 $\mu$M (Table 1) because sufficiently high levels are not clinically achieved.

Formation of MI-Complexes

$N$-Alkyl-substituted compounds—a common feature of many CYP3A drugs—often show reversible inhibition, and an even greater effect is observed after preincubation with a metabolically competent in vitro preparation. This is due to oxidation of the inhibitor to form a nitrosoalkane species that forms a slowly reversible complex (MI-complex) with reduced heme in the CYP3A molecule (97, 98). Such compounds include macrolides like troleandomycin (99), oleandomycin (100), erythromycin (101), clarithromycin (102, 103), roxithromycin (102), and dirithromycin (104); some antidepressants such as fluoxetine (105, 106), nortriptyline (107), imipramine (106), and desipramine (106, 107); and various miscellaneous drugs, including tiamulin (108), diltiazem (106), lidocaine (106), tamoxifen (106), and amiodarone (109).

Formation of an MI-complex may, however, be difficult to demonstrate in vitro because of its dependency on the rapid and relatively efficient generation of the causative metabolite. On the other hand, inhibition under in vivo conditions results from more prolonged exposure to the inhibitor and its
### Table 1  Reversible inhibitors of human CYP3A-mediated metabolism

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Apparent $K_i$ value ($\mu$M)</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>0.00025–0.15</td>
<td></td>
<td>39, 80</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.015–8</td>
<td>Noncompetitive, mixed</td>
<td>80–83</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.27</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>Miconazole</td>
<td>0.9–1.3</td>
<td>Competitive</td>
<td>38, 39</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.3–63</td>
<td>Competitive, noncompetitive</td>
<td>39, 80, 84, 85</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.017</td>
<td>Mixed</td>
<td>86</td>
</tr>
<tr>
<td>Indinavir</td>
<td>0.2</td>
<td>Competitive</td>
<td>87</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.7</td>
<td>Competitive</td>
<td>87</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>4.8</td>
<td>Competitive</td>
<td>88</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>16–194</td>
<td>Competitive</td>
<td>38, 83, 89, 90</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>10–51</td>
<td>Competitive, mixed</td>
<td>38, 83, 89, 90</td>
</tr>
<tr>
<td>Josamycin</td>
<td>12–21</td>
<td>Competitive, mixed</td>
<td>38, 83, 89</td>
</tr>
<tr>
<td>Rokitamycin</td>
<td>41</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>Sertraline</td>
<td>24–64</td>
<td>Mixed</td>
<td>91, 92</td>
</tr>
<tr>
<td>Desmethylderivative</td>
<td>20–48</td>
<td>Mixed</td>
<td>91, 92</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>66–83</td>
<td>Mixed</td>
<td>91, 92</td>
</tr>
<tr>
<td>Norfluoxetine</td>
<td>11–19</td>
<td>Mixed</td>
<td>91, 92</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>8</td>
<td>Competitive</td>
<td>38</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>10–22</td>
<td>Competitive</td>
<td>38, 82, 83, 93</td>
</tr>
<tr>
<td>Verapamil</td>
<td>24–82</td>
<td>Competitive, mixed</td>
<td>38, 83</td>
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<tr>
<td>Diltiazem</td>
<td>50–75</td>
<td>Competitive</td>
<td>38</td>
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<td>Quinidine</td>
<td>4–10</td>
<td>Competitive</td>
<td>90, 93</td>
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<td>Progesterone</td>
<td>8–45</td>
<td>Competitive</td>
<td>38, 83</td>
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<tr>
<td>Dexamethasone</td>
<td>23</td>
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<td>83</td>
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<tr>
<td>Ethinylestradiol</td>
<td>34</td>
<td>Mixed</td>
<td>83</td>
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<td>Midazolam</td>
<td>40–63</td>
<td>Competitive</td>
<td>38, 94, 95</td>
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<td>Cyclosporine</td>
<td>1–37</td>
<td>Competitive</td>
<td>83, 96</td>
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<tr>
<td>Rapamycin</td>
<td>83</td>
<td>Competitive</td>
<td>83</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>79</td>
<td>Competitive</td>
<td>83</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.8</td>
<td>Mixed</td>
<td>90</td>
</tr>
<tr>
<td>Bromocryptine</td>
<td>7–8</td>
<td>Competitive</td>
<td>38, 83</td>
</tr>
<tr>
<td>Navelbine</td>
<td>11</td>
<td>Mixed</td>
<td>90</td>
</tr>
<tr>
<td>Ergotamine</td>
<td>12–14</td>
<td>Mixed</td>
<td>38, 83</td>
</tr>
<tr>
<td>Quercetin</td>
<td>14</td>
<td>Noncompetitive</td>
<td>95</td>
</tr>
<tr>
<td>Vincristine</td>
<td>19</td>
<td>Mixed</td>
<td>90</td>
</tr>
<tr>
<td>Dihydroergotamine</td>
<td>23</td>
<td>Competitive</td>
<td>38</td>
</tr>
</tbody>
</table>

metabolite(s), and this may also be repetitive. Accordingly, the clinical relevance of MI-complex formation observed in vitro may be underestimated. For example, erythromycin is a modestly potent competitive inhibitor (Table 1); however, CYP3A-mediated metabolism of tacrolimus (110), theophylline (111), cyclosporine (112), and terfenadine (113) was observed to be markedly inhibited in vivo following pretreatment with multiple doses of the macrolide. This was associated with maximum erythromycin plasma levels (5–6 $\mu$M) that were
considerably less than the $K_i$ value for rapid reversible inhibition (Table 1). Accordingly, it is generally accepted that erythromycin’s inhibitory effect in vivo is the result of MI-complex formation rather than rapidly reversible binding to the enzyme, despite the fact that the extent of such complexation in vitro is relatively poor compared, for example, with that of troleandomycin (101, 104). Whether this occurs with other CYP3A-alkylamine inhibitors is unclear, but such a phenomenon could explain some observed discrepancies between the in vitro and in vivo potencies of inhibition, e.g. diltiazem. Finally, MI-complex inhibition has often been observed to persist beyond the period of time that the inhibitor was present in the body because, although reversible, the lifetime of the complex is generally quite long (114).

**Irreversible, Mechanism-Based (Suicide) Inhibition**

Several 17α-ethinyl substituted steroids, e.g. ethinylestradiol, gestodene, and levonorgestrol, have been found to irreversibly inactivate human CYP3A in an NADPH- and time-dependent fashion (115, 116). The antigestational agent, mifepristone (RU-486), would also fit into this category, but whether it produces suicide inactivation of CYP3A in humans has not been reported. Recently, an experimental furanopyridine, HIV protease inhibitor was also found to be a highly potent and selective CYP3A mechanism–based inhibitor (117), and this property was a factor in not developing the agent further as a clinical candidate. This raises the question of whether other furans have similar CYP3A inhibitory properties. In this regard, it is interesting that 6′,7′-dihydroxybergamottin, a furanocoumarin, is present in grapefruit juice, the ingestion of which can markedly inhibit the first-pass metabolism of CYP3A substrates (118, 119). Moreover, this effect was recently found to be associated with autocatalytic destruction of intestinal CYP3A both in vitro (120) and in vivo (121), and 6′,7′-dihydroxybergamottin has been observed to inhibit CYP3A catalytic activity in vitro (122).

The mechanism of suicide inhibition presumably involves CYP3A-mediated formation of a reactive metabolite(s) that covalently binds to the enzyme in a fashion leading to its inactivation. Accordingly, in vivo effectiveness depends on the total amount, rather than the concentration, of inhibitor to which CYP3A is exposed; the amount of CYP3A; and partitioning of the reactive metabolite between CYP3A and other macromolecules, all relative to the resynthesis rate of new enzyme (114). It is, therefore, possible that, because of the smaller amount of CYP3A present in the enterocytes compared to the liver (64), mechanism-based inhibition following oral administration of, for example, acetylenic steroids selectively inhibits intestinal rather than hepatic metabolism.
IN VITRO–IN VIVO PREDICTIONS OF CYP3A INHIBITION

Enzyme kinetics associated with various types of inhibition, e.g. competitive and noncompetitive, are well-established and provide a basis of quantifying inhibition as a function of concentration. In principle, such relationships provide the basis for extrapolating in vitro data to the in vivo situation (78, 79). However, such modeling usually requires several assumptions, many of which are difficult to validate (79). Moreover, for CYP3A there is the additional complication that following oral administration, consideration must be given to both intestinal and hepatic enzyme and the possibility that differential inhibition may occur. Also temporal aspects, related to the changing concentrations of both substrate and inhibitor following their administration, have rarely been incorporated into the modeling process (79).

A critical aspect of any a priori prediction concerns the accuracy of the inhibition constant ($K_i$) determined in vitro. Experimental conditions may significantly affect this estimation, as indicated by the fact that substantial differences exist in report $K_i$ values for a particular inhibitor (Table 1). Several factors probably contribute to such variability. First, CYP3A-catalyzed product formation is extremely sensitive to the surrounding aqueous and lipid environment (see above), and an inhibitor-enzyme interaction may vary as a function of these parameters, as demonstrated with the rat CYP3A ortholog (123). Second, the positive cooperativity effect following the addition of a second substrate (see above) might affect the measured CYP3A activity differently depending on which allosteric form of the enzyme it interacts with. This appears to be the case for the in vitro interaction between testosterone and erythromycin (124). Third, many, if not most, CYP3A inhibitors are also substrates for the enzyme. If the inhibitor’s turnover rate is appreciable, significant loss of inhibitor may occur during the incubation period, especially at low inhibitor concentrations. This would result in an underestimation of inhibitor potency. Unfortunately, determination of the time-dependent linearity of product formation in the presence of varying inhibitor concentrations needed to identify such a problem is rare. Ideally, the inhibitor:enzyme molar ratio should have a value of three or greater to avoid inhibitor depletion; in certain instances, e.g. ketoconazole, this would only be achieved when the amount of human liver microsomal protein is less than 0.1 mg. This condition may not be achievable unless a highly sensitive assay is available; a similar analytical constraint also applies if substrate turnover is relatively slow. Accordingly, nonideal amounts of enzyme are frequently used. In such cases, the estimated $K_i$ value may vary according to the amount of microsomal protein present in the incubation mixture. For
example, using less than 250 $\mu$g/ml of microsomal protein, ketoconazole’s $K_i$ value was found to be 15 nM or less (80–82), whereas much higher values (700–8000 nM) were observed at a microsomal protein level of 1–1.5 mg/ml (83, 85).

A more subtle problem relates to the fact that more than a single isoform may be involved in formation of the measured metabolic product. In principle, this may be identified by significant deviation from linearity in reciprocal plots, but in practice this is not always the case. Importantly, in a multienzyme system, the estimated $K_i$ value might not accurately reflect that of the CYP3A4-mediated pathway. This was recently illustrated in a study comparing the inhibition of CYP3A4 and CYP3A5-mediated $1'\text{-hydroxylation}$ of midazolam by flucona-zole and ketoconazole (80). For both inhibitors, the $K_i$ value for CYP3A5 was over sixfold higher than the corresponding constant for CYP3A4. When both isoforms were present, the apparent $K_i$ value was intermediate and varied as a function of the molar ratio of the two enzymes. Given that the relative amounts of CYP3A4 and CYP3A5 frequently differ between in vitro preparations and also in vivo, this factor could account for some of the reported discordanices in prediction.

Another critical issue, and one that remains largely unresolved, is the relationship between the inhibitor’s concentration in vitro and that present at CYP3A’s active site. It is usually assumed that the inhibitor’s unbound concentration at the enzyme’s active site is not only in equilibrium with, but the same as that of the compound in the bulk solution. Moreover, this is considered to be the operative level in vivo, i.e. any plasma binding of the inhibitor must be taken into account in extrapolating from the in vitro to the in vivo situation (78, 79). While the latter assumption is widely accepted, the in vitro situation is less clear. Little consideration is generally given to determining the inhibitor’s actual unbound concentration in the incubation mixture in contrast to its value based on the total amount added to a known volume. Yet there is increasing evidence that reversible microsomal binding occurs and can significantly affect estimation of intrinsic clearance values (125, 126). An analogous effect would be expected with respect to determining $K_i$ values. Along similar lines, empirical observations with several selective serotonin reuptake inhibitors have revealed that in vitro determined $K_i$ values greatly underestimated the degree of in vivo inhibition of desipramine (127), triazolam (128), midazolam (129), and alprazolam (92). Good predictions were obtained if partitioning of the inhibitor between liver tissue and water was incorporated into the predictive model. However, it is difficult to envision how such partitioning apparently increases the concentration of inhibitor in equilibrium with the CYP3A active site. Aside from factors determining the accuracy of the $K_i$ estimates, described above, possible explanations could involve a selective effect on intestinal CYP3A because of the
higher enterocyte inhibitor concentrations, compared with that in the liver, and/or a time-dependent disequilibrium between the inhibitor’s unbound plasma concentration following oral administration and its unbound level in the intestinal epithelium and liver.

Given such poorly understood and unresolved issues, it is not surprising that certain inhibitory drug interactions are not readily explainable on the basis of the determined in vitro $K_i$ value and the systemic concentration of inhibitor. For example, steady-state plasma levels of diltiazem after dosing 60 mg every 6 h for 7 days were found to be about 350 nM (130); unbound concentrations would be lower, since about 80% of the drug is bound to plasma protein. These levels are several orders of magnitude lower than the reported $K_i$ value of 50–75 µM (Table 1). Nevertheless, the oral clearance of several CYP3A substrates, including cyclosporine (112), midazolam (131), triazolam (132), morizicine (133), and quinidine (134) were found to be markedly (>75%) reduced when diltiazem was co-administered compared to the drug substrate alone. Additionally, diltiazem inhibited the CYP3A-mediated metabolism of carbamazepine (135) and theophylline (136), which have low intrinsic clearances that preclude the likelihood of the discrepancy resulting from selective intestinal CYP3A inhibition. Also, a single diltiazem dose has been found to reduce the systemic clearance of both midazolam and alfentanil (137).

By contrast, azole antifungal agents provide good examples of observed in vivo inhibition that is consistent with in vitro findings. For example, peak plasma levels of unbound ketoconazole following standard 200–800 mg/day dosage regimens (~75–300 nM) are severalfold higher than its lowest reported in vitro $K_i$ value (Table 1). Thus, significant in vivo inhibition would be expected with CYP3A substrates, and this is consistent with observations of marked drug interactions, especially after oral administration with cyclosporine (138), midazolam (139), and tirilazad (140, 141). Moreover, the reduction in oral clearance (4– to 15-fold) was greater than the change in systemic clearance or elimination half-life, suggestive of intestinal CYP3A inhibition (see below). It is, therefore, likely that coadministration of standard doses of ketoconazole will result in a significant in vivo inhibition of CYP3A-mediated metabolism, regardless of the individual drugs. Of emerging interest is whether all of the observed interaction with ketoconazole and possibly other azole antifungals is a result of metabolic inhibition. This is because ketoconazole is an inhibitor of P-glycoprotein (142) of comparable potency to that of quinidine and verapamil (RB Kim, DM Roden, AJJ Wood & GR Wilkinson, unpublished data). Accordingly, changes in intestinal absorption, intestinal secretion, and biliary excretion may theoretically also be involved (see above).

Fluconazole and miconazole are somewhat less potent than other azole antifungal agents (Table 1), and inhibition is, therefore, more dependent on the
administered dose. For example, no inhibition of midazolam metabolism was observed after a single oral dose of 150 mg fluconazole (143), but after a 400 mg dose, the benzodiazepine’s oral clearance was reduced fourfold (144). Again, a greater effect was noted with respect to the oral rather than systemic clearance, and interestingly, the increase in midazolam’s plasma levels was similar following either intravenous or oral fluconazole. A modest reduction (45%) in the CYP-mediated 10-hydroxylation of R-warfarin associated with plasma fluconazole levels of between 50 and 70 µM following a 400 mg/daily dose for 2 weeks has also been reported (145). After correcting for plasma binding ($f_p = 0.88$), these levels were still two- to threefold greater than fluconazole’s in vitro $K_i$ value (Table 1).

Most reported in vitro–in vivo predictions of rapidly reversible inhibitory drug interactions have been retrospective in an attempt to establish feasibility of the approach. But to be optimally useful, in vitro data should not only indicate the possibility of an interaction but also prospectively predict its magnitude and likely clinical importance. These characteristics are especially relevant to the development of new candidate drugs by the pharmaceutical industry, which is currently very actively involved in this area. However, as indicated above, the current state of the art is still evolving, and a critical element to its advancement will be the reporting not only of its successes but also of its failures. Only in this way will it be possible to evaluate common experiences and determine broad conclusions.

In principle, the situation for rapidly reversible inhibition is relatively straightforward, i.e. the degree of inhibition depends only on the dose and elimination kinetics of the inhibitor and its affinity for CYP3A. However, for inhibitors that form an MI-complex or exhibit a mechanism-based activity the situation is more complex, since not only are these factors important but in addition the rate of enzyme complexation/inactivation and synthesis as well as the degradation of the holoenzyme are determinants (114). Accordingly, in vivo prediction of these types of inhibition is very difficult and has yet to be achieved.

In view of the possible discrepancy between an in vitro finding and that observed in vivo, it is clear that, at the present time, assessment of the extent of any inhibitory interaction between a new drug that has demonstrated in vitro inhibitory activity and a drug which is a CYP3A substrate still requires empirical studies reflective of the likely therapeutic scenario. On the other hand, for a new compound that is a CYP3A substrate, the in vivo consequences of maximal inhibition of its metabolism can be readily assessed by the appropriate administration of ketoconazole. Analogous studies with rifampin would indicate the likely maximal extent of induction (see below) and, therefore, establish the probable range of metabolic alterations produced by any other interacting drug.
INDUCTION OF CYP3A IN VITRO

Although not as common as drug interactions caused by inhibition, those resulting from induction of CYP3A may be just as profound and clinically important. Members of the CYP3A subfamily are somewhat unique in that their level and catalytic activity are enhanced following the in vivo administration of many diverse and structurally dissimilar compounds in several animal species. Such inducers include anticonvulsant agents, rifamycins, glucocorticoids, some macrolides, and various other agents (1). In general, human CYP3A also appears to be induced by many of these compounds, as primarily evidenced by their ability to reduce plasma levels of various substrates following a period of pretreatment. More recently, induction has been demonstrated in vitro through the use of primary cultures of human hepatocytes (37–40, 146, 147). CYP3A mRNA increased within 24 h following treatment with the inducing agent (11, 148, 149), and increased protein level and catalytic activity were observed somewhat later (40, 85, 150). The most common mechanism for CYP3A induction is transcriptional activation (11, 149–153). Several binding sites for transcriptional regulatory factors within the 5′-flanking region of the CYP3A gene have been identified, including glucocorticoid response elements (154–156). However, the overall mechanism(s) is not well defined and may involve a complicated interplay between the inducer, its receptor, and circulating hormones (157, 158). Some inducers, however, appear to increase hepatic CYP3A protein levels in animals by stabilization. Such posttranscriptional regulation was first demonstrated for troleandomycin and other macrolides that form stable complexes with the enzyme (26). The mechanism by which this interaction stabilizes CYP3A against subsequent degradation is unclear but may involve a cAMP-dependent phosphorylation process that is involved in protein denaturation (159). Other compounds with a similar mechanism of induction include clotrimazole (39, 159), some macrolide antibiotics (such as erythromycin and troleandomycin) that form MI-complexes with CYP3A (11, 159), and pesticides (such as chlordane) (150). Whether this type of induction is detectable in vivo is, however, unclear because it only occurs following chronic pretreatment, and the involved drugs also inhibit CYP3A when they are present at the enzyme’s active site. In principle, induction should be observable, provided that elimination of the agent is rapid enough following its discontinuation relative to the degradation of the stabilized CYP3A protein. However, such a biphasic effect has not been reported, and the clinical interactions described for these agents in humans with CYP3A substrates are inhibitory in nature (160–164).

An important application of the primary hepatocyte culture system and CYP3A induction has been the determination of whether a particular drug is
capable of increasing the enzyme’s catalytic activity. In this fashion, it has been possible to identify compounds that might produce induction in vivo as well as those for which such an effect would not be expected (38, 40, 144, 165–167). In contrast to similar studies for putative CYP3A inhibitors (38, 39, 165, 166), quantitative extrapolation of such data has not been extensively attempted. In the case of rifampin, the increase in CYP3A’s intrinsic clearance appears to be about five to eightfold both in vitro (11, 85) and in vivo (29, 97, 164, 168). Therefore, the approach serves as a qualitative screen and the magnitude of any in vivo effect, subsequently, must be experimentally determined.

MEASUREMENT OF CYP3A ACTIVITY IN VIVO

With CYP isoforms exhibiting wide interindividual variability and/or ready modulation of their catalytic activity by environmental factors, a useful approach to characterizing such activity in an individual subject is phenotyping with an appropriate in vivo probe. This approach has been particularly valuable with CYP2C19 and CYP2D6, which exhibit genetic polymorphisms (169). In principle, this approach should also be applicable to measuring CYP3A activity in vivo and alterations caused by drug interactions. In practice, however, there are several potentially complicating factors.

Route of Administration

The potential for significant metabolism in the gastrointestinal tract, liver, and possibly the kidney is a major complication in assessing CYP3A’s in vivo catalytic activity. This is particularly true when considering drug interactions, because the route of administration of both the primary and the putative interacting drug have to be considered, along with the relative contributions of the individual organs to the overall metabolic process. A further complicating issue is the nature of the rate-limiting determinant of the measured (phenotypic) parameter. For example, clearance and elimination half-life are more reflective of hepatic liver blood flow than metabolizing ability for a high intrinsic clearance drug administered intravenously (170). However, most of the limited number of intravenously administered CYP3A substrates have low to intermediate hepatic extraction ratios, so such elimination parameters reflect more the enzyme’s catalytic level. Thus, inhibition of CYP3A likely accounts for the reduction in alfentanil’s systemic clearance after treatment with oral erythromycin (171) and troleandomycin (164), and a similar effect on midazolam’s systemic clearance with oral itraconazole, fluconazole, and erythromycin (139, 161). On the other hand, after oral administration, plasma levels are predominantly reflective of the drug-metabolizing activity of the presystemic eliminating organs (170).
The issues in this case are to what extent such activity is present in the gastrointestinal epithelium compared with the liver and the relative effects of an interacting agent at these two sites.

The potentially important role of CYP3A-mediated metabolism by intestinal enterocytes was definitively demonstrated by intraduodenal administration to patients undergoing liver transplantation of cyclosporine (172) or midazolam (173) during the anhepatic phase. With both substrates, substantial levels of the drugs’ primary metabolites were present in the hepatic portal vein and systemic circulation. Furthermore, the estimated intestinal extraction ratios were 25 to 51% for cyclosporine and 14 to 59% for midazolam, which were similar to the drugs’ hepatic extraction values estimated in healthy subjects. Thus, for these two drugs, the overall metabolizing ability of the intestinal epithelium and liver are about comparable. Intestinal presystemic elimination has also been inferred by observations indicating a greater reduction in clearance following oral compared to intravenous administration; for example, the inhibitory interactions between erythromycin and midazolam (161), erythromycin and cyclosporine (174), and ketoconazole and cyclosporine (138) and tacrolimus (175). A similar differential effect has also been noted following CYP3A induction produced by rifampin with regard to cyclosporine (176), verapamil (177), and nifedipine (178). With the calcium-channel antagonists, oral rifampin pretreatment only modestly affected the drugs’ pharmacokinetics after intravenous administration, 26 and 65%, but their oral clearances increased 14-fold and about 40-fold, respectively. Furthermore, in the case of verapamil, the stereoselectivity of CYP3A-mediated metabolism was altered by rifampin pretreatment. Additionally, after oral dosing, CYP3A in the enterocytes should be initially exposed to higher levels of an interacting agent than enzyme in the hepatocytes. Accordingly, a greater concentration-related inhibition/induction would be expected to be present in the more proximal organ. The reverse situation would also be likely to occur following intravenous administration. Experimental data supportive of such a route-dependent degree of interaction with respect to an inhibitor/inducer have not, however, been reported.

Following oral drug administration alone, the relative contributions of CYP3A localized in the intestinal epithelium cannot be separated from those in the liver, or from any changes occurring at these sites as a result of a drug interaction. From a therapeutic standpoint, such differentiation is generally not important; however, such knowledge may be of value and interest for other purposes. Two approaches to address this issue have recently been described. The first assumes that the estimated systemic clearance of a fully absorbed and totally metabolized CYP3A substrate only measures hepatic elimination, i.e. drug in the systemic circulation is not exposed to CYP3A present in enterocytes.
The findings that the intestinal extraction of midazolam was on average less than 10% in anhepatic liver transplant patients (173) and that the formation of midazolam’s 1′-hydroxy metabolite was limited following intravenous administration in such patients (179) are supportive of this assumption, at least with this CYP3A substrate. Thus, midazolam’s hepatic extraction ratio was estimated based on knowledge of liver blood flow, and the contribution of intestinal extraction to the overall first-pass effect following oral administration was determined (180). Application of this approach to the interaction of midazolam with grapefruit juice (181) indicated that inhibition of CYP3A activity was restricted to the intestinal tract and midazolam’s hepatic clearance was unaffected (119). The same pharmacokinetic approach also suggested that induction by rifampin of CYP3A-mediated metabolism of verapamil (176) and nifedipine (177) was essentially limited to enzyme localized in the intestinal epithelium. An alternative approach has been used with drug interactions involving cyclosporine, whose absorption across the intestinal mucosa is incomplete. Again, the oral administration of interacting agents such as ketoconazole and rifampin (30, 138, 176) resulted in a two- to fourfold greater effect on the combined intestinal absorption and metabolism component of presystemic elimination than did the liver. Using this same approach, Floren et al (175) recently found a doubling of the oral bioavailability of tacrolimus after ketoconazole pretreatment without any alteration in the immunosuppressant’s hepatic extraction, i.e. inhibition was selective to intestinal CYP3A.

Dosing and Temporal Factors
The extent of inhibition or induction of drug metabolizing enzyme activity is often concentration dependent (182). Therefore, a constant concentration of the inhibitor/inducer is customarily maintained studies performed in vitro. However, a more variable and dynamic situation is present in vivo because of factors related to enzyme localization relative to the route of administration, the pharmacokinetic characteristics of the interacting drugs, and temporal aspects of drug administration. Unfortunately, few studies in humans take such complicating considerations into account.

Following oral drug administration, CYP3A in the enterocytes is exposed to all of the dose of an inhibitor that is absorbed from the intestinal lumen, and intracellular concentrations are high. Subsequent vectorial drug transport into the splanchnic circulation reduces this concentration because of possible intestinal elimination and dilution by the blood that is further increased as a result of systemic distribution. Thus, inhibition of CYP3A within hepatocytes is probably less than that in the enterocytes. Accordingly, the oral clearance of an interacting drug is likely to be affected to a greater extent than its systemic clearance, i.e. the extent of inhibition will be route dependent (see above).
addition, inhibitory concentrations do not remain constant because the absorption time profile and systemic elimination result in increasingly lower levels of the inhibitor and, in the case of reversible inhibition, a restoration of CYP3A activity. By contrast, with mechanism-based inhibitors, enzyme activity is lost as a function of the integrated exposure to the inhibitor, and its return is dependent on the differentiation rate of mature villus enterocytes and/or the synthesis rate of new protein in the liver (see above).

The use of time-averaged parameters like clearance obviates such temporal considerations to some extent, but clearly, the relative times of administration of the two interacting drugs may potentially affect the extent of CYP3A inhibition, especially when only a single dose is given. For example, the extent of interaction between felodipine and grapefruit juice, as measured by enhanced drug plasma levels, decreased as the period with increasing time between juice and drug administration was increased (0 to 10 h) (183). In the case of nifedipine, oral administration along with quinidine (184) or following a 1-h pretreatment period (185) did not result in a statistical increase in the calcium-channel antagonist’s plasma levels, whereas more prolonged quinidine pretreatment was found to produce a significant change (186). Similarly, the time interval between the administration of itraconazole and triazolam determined the extent of the interaction (187). Thus, when only using a single dose of an inhibitor, an adequate period of time must be given to allow attainment of sufficiently high inhibitor levels in order for an interaction to be measurable. Unfortunately, the optimal pretreatment period to maximize any inhibition cannot be predicted a priori.

In order to overcome the above described considerations, chronic dosing of an inhibitor has been frequently used prior to administration of the second drug. This raises issues related to the pharmacokinetic characteristics of the interacting drugs. For instance, it is not uncommon for accumulation to occur with many CYP3A inhibitors when they are administered according to a typical therapeutic regimen. As a result, the effective inhibitor concentration increases until a pseudo–steady state is attained, and the extent of inhibition changes accordingly. Such a temporal effect has been demonstrated, for example, with alfentanil, in which the extent of inhibition of metabolism following administration of erythromycin was greater after seven days pretreatment compared with one day of therapy (171). Few studies, however, actually measure the plasma concentration profile of the inhibitor to determine its magnitude or whether steady state has been achieved. This is unfortunate, since such knowledge is critical for in vitro–in vivo correlations (see above). Similarly, it is uncommon for studies to compare the extent of inhibition after both acute and chronic doses of the putatively affected drug, even though on theoretical grounds (see above) this may be potentially different when significant drug accumulation occurs that alters the relationship between drug and inhibitor concentrations.
In interactions involving the induction of CYP3A, issues related to changing drug concentration are likely to be of less concern, since the time scale of induction is longer than that for inhibition, and new protein synthesis is probably a function of the time-averaged inducer concentration. However, temporal aspects are still important, mainly because of the length of time for induction to attain its maximal effect and also to disappear following discontinuance of the inducer. In principle, the time courses of both induction and de-induction are exponential with half-lives reflective of the degradation rate of the affected enzyme (188). Limited data in humans support this scenario with respect to CYP3A; for example, trough plasma levels during chronic dosing of both enantiomers of verapamil declined and then increased with half-lives of one to two days when rifampin was continuously administered and then discontinued (177). In fact, it took almost two weeks to attain maximum induction and washout. A similar time period was also demonstrated to be necessary to maximize the enhanced metabolism of prednisolone by rifampin (189).

Interindividual Variability

A major characteristic of CYP3A is the large range of interindividual variability in the level of its activity in various populations (see above), and this is particularly the case following oral drug administration. Moreover, variability in bioavailability increases both in theory (170) and in practice (190) as the extent of first-pass metabolism increases. While these considerations apply to the enzyme’s basal level, it has also become apparent that considerable interindividual variability is present with respect to the extent of CYP3A-mediated drug interactions, especially those involving inhibition.

For example, terfenadine’s plasma levels, which are usually below the assay detection limit of 5 ng/ml, were measurable in some but not all subjects pretreated with erythromycin (113, 191), and the interaction with ketoconazole resulted in 16- to 73-fold reduction in the antihistamines’ oral clearance (192). Pronounced variability was also found in the interaction of grapefruit juice with various CYP3A substrates (119). For example, the increase in relative oral bioavailability ranged from 0 to 200% for cyclosporine (193), 5 to 470% for felodopine (194–196), 26 to 100% for midazolam (181), and 21 to 485% for terfenadine (197–199).

Several factors probably contribute to such variability. One is possibly the basal level of CYP3A, since the relative change in midazolam’s oral clearance produced by erythromycin (161) and ketoconazole (200) tended to be related to the magnitude of the pretreatment clearance value. By contrast, no such relationship was observed with itraconazole (200). The observed heterogeneity in the intestinal expression of CYP3A activity in biopsied tissue (16, 173) and the finding that certain subjects have essentially no intestinal midazolam 1′-hydroxylating ability (180) also indicate that the extent of inhibition of
metabolism is likely dependent on the amount of available enzyme. As a result, interindividual variability is reduced when an inhibitory drug interaction is present.

Another likely contributing factor is the individual variability in the inhibitor’s pharmacokinetics that results in different intestinal and hepatic tissue time profiles between subjects. Such wide interindividual variability in the extent of inhibited metabolism possibly accounts for observations indicative of the lack of an interaction when other studies have clearly demonstrated such an effect (201, 202).

In Vivo Probes of CYP3A Activity
Since numerous drugs are metabolized by CYP3A, any of these could be used to determine the enzyme’s overall catalytic activity in the body and its modulation. In fact, this is the approach used to establish whether a significant interaction occurs between a specific CYP3A substrate and a known inhibitor/inducer. On the other hand, defining the determinants of CYP3A activity—e.g. race, age, sex, and others—and possible alteration by, for example, interaction with a new drug candidate require the use of an appropriate in vivo probe. Considerable effort has been extended to identifying a compound for this purpose; however, because of several characteristics of CYP3A, an ideal and universally applicable drug has yet to be defined (203). Accordingly, the choice of an in vivo probe largely depends on the purpose of the study. Of particular importance is whether the selected phenotypic trait is to be used as a qualitative indicator of changes in CYP3A function or as a quantitative and predictive measure of the enzyme’s activity.

For many years, the 6β-hydroxylation of endogenous cortisol, provided by the ratio of 6β-hydroxycortisol to free cortisol in urine, has been used as a measure of changes in overall CYP activity (204, 205). The finding that the 6β-hydroxylation step was predominantly CYP3A mediated (168, 206) suggested that it might serve as a more selective trait measure for this particular isof orm(s). Several studies, for example, showed that the urinary cortisol ratio increased following treatment with inducers known to be selective for CYP3A, e.g. rifampin (168, 207, 208) and anticonvulsant agents (208–210). However, an analogous study with the CYP3A-selective, mechanism-based inhibitor troleandomycin noted no consistent change in cortisol 6β-hydroxylation, despite the fact that the erythromycin breath test (see below) was markedly affected (211). In addition, other studies were unable to find a correlation between the urinary excretion of 6β-hydroxycortisol and CYP3A activity measured by this breath test (212, 213), consistent with the lack of a significant relationship between the urinary cortisol ratio and the uninduced level of CYP3A activity in a liver biopsy (168). Collectively, these data seriously question whether cortisol 6β-hydroxylation, as measured by the urinary cortisol ratio, is, in fact, a valid
and useful in vivo probe for measuring CYP3A activity. The reason(s) for the discrepancies is unknown but could reflect the importance of CYP3A-mediated cortisol metabolism in the kidney.

CYP3A selectively N-demethylates erythromycin and, if $^{14}$C-$\text{-N}$-methyl drug is used, the cleaved carbon of the methyl group is eventually expired as $^{14}$CO$_2$. Measurement of this radioactivity over a 1-h period, following an intravenous dose, forms the basis of the erythromycin breath test. Considerable validation and application of this simple and relatively rapid approach as a measure of in vivo CYP3A activity have been reported (203), and the test provides a measure of CYP3A activity under certain circumstances and for some types of investigation. A limiting factor of the erythromycin breath test, however, is that it only appears to measure CYP3A4-mediated metabolism and not that mediated by CYP3A5 (8, 15). Thus, overall CYP3A activity is underestimated, especially in the 25–30% of individuals with significant hepatic levels of this isoform. More importantly, however, is the fact that the erythromycin breath test predominantly reflects CYP3A4 activity in the liver (16, 160, 211, 212), and therefore, metabolism within the intestinal epithelium is not measured (16). This is an obvious limitation with respect to an orally administered drug and may account, in part, for the relatively poor correlation between the breath test value and the oral clearance of several CYP3A substrates (213–216). Unfortunately, application of an oral erythromycin breath test to overcome this problem has not been successful (PB Watkins, personal communication). Additionally, it has not been possible to quantitatively interpret either the basal level or any change in the erythromycin breath test value with regard to CYP3A activity, i.e., it is essentially a relative measure whose relationship to erythromycin’s clearance is unknown. Nevertheless, the breath test has considerable potential for monitoring drug interactions involving hepatic CYP3A4. For example, treatment with troleandomycin markedly reduced the breath test value, whereas CYP3A-inducing agents such as dexamethasone and rifampin resulted in large increases (160). Furthermore, several published reports and ongoing research with new drugs under development indicate the utility of serial erythromycin breath testing to determine whether hepatic CYP3A induction or inhibition occurs. In this fashion, the chronic administration of delavirdine was found to result in rapid and substantial reduction in CYP3A activity that was dose related and led to the yet untested prediction that delavirdine would likely cause drug interactions when coadministered with other CYP3A substrates (217). Similarly, the time course of ketoconazole-associated inhibition of CYP3A activity, after a single oral dose, was followed for 30–36 h with repeated erythromycin breath tests (218). Also, this study showed that paclitaxel’s metabolism was not affected despite CYP3A activity being inhibited, indicating the ability of the test to identify situations where a drug interaction does not occur. Such data,
therefore, strengthen the conclusions that omeprazole did not alter CYP3A activity (219) and that interferon only causes a small degree of inhibition (220).

In humans, midazolam is almost completely and rapidly metabolized to its primary 1′-hydroxy metabolite and, to a much lesser extent, to 4-hydroxymidazolam. Importantly, both of these metabolic pathways are selectively mediated by CYP3A (15, 69). Accordingly, midazolam has been investigated as an in vivo probe for CYP3A activity and found to meet most, if not all, of the necessary criteria suggested for such an application (203). For example, microsomes from both the liver (28, 180, 221) and small intestine (16, 180) were found to have high midazolam 1′-hydroxylating activity, which in the case of the liver was significantly correlated with the drug’s systemic clearance (221). Liver dysfunction markedly impaired midazolam’s elimination (222, 223), and plasma levels following intravenous administration during the anhepatic phase of liver transplantation were elevated (173). In addition, known inhibitors and inducers of CYP3A produced consistent alterations in midazolam’s clearance (161, 164, 181, 200, 224). It was also possible to scale-up results from in vitro measures of midazolam’s 1′-hydroxylation by liver and intestinal microsomes to predict the in vivo extraction ratios of these organs (225). Importantly, close agreement was obtained between such estimates and their values measured in vivo (221, 224).

Midazolam has substantial advantages over other putative probes: in particular, the fact that the drug may be given safely by both intravenous and oral routes of administration. Thus, a measure of CYP3A activity relative to first-pass metabolism is provided following an oral dose, whereas, after intravenous administration, CYP3A-mediated hepatic metabolism is primarily obtained. Importantly, using clearance as the phenotypic value allows quantification of CYP3A function in a way that can be related to drug dosage regimens and possible changes resulting from a drug interaction. This does not, of course, apply to the use of an alternative trait measure, namely, the plasma level ratio of 1′-hydroxy midazolam to midazolam at a single time point after drug administration (28, 221). However, this latter phenotypic trait value now appears to be less valid and useful than originally suggested (180).

Importantly, it is also apparent that CYP3A-mediated metabolism of midazolam, especially as measured by the drug’s oral clearance, is very sensitive to modulation of the enzyme’s level of activity. For example, rifampin pretreatment reduced the area under the drug’s plasma concentration–time curve by 96% (224), whereas ketoconazole increased this parameter by 15-fold (200), and itraconazole also resulted in a large effect (139, 200, 226). More modest changes (two- to fourfold) were found following a single dose of grapefruit juice (181) and a period of erythromycin (161), clarithromycin (227), roxithromycin (228), fluconazole (144), or diltiazem (131) administration. By contrast, several
days pretreatment with azithromycin (229–231) and terbinafine (226) had no effect on midazolam’s metabolism. Thus, at the present time, measurement of changes in midazolam’s clearance and the fractional clearance associated with the 1′-hydroxy pathway of metabolism after oral drug administration probably provides the best means by which quantitative alterations in intestinal and/or hepatic CYP3A resulting from a drug interaction activity can be determined in vivo. Moreover, the additional determination of any change in midazolam systemic clearance after intravenous administration (ideally using a differentially labeled form of the drug) permits estimation of the separate changes in CYP3A activity at these two anatomical sites (180).

In vitro studies using human liver microsomes invariably indicate a high degree of correlation between the rates of CYP3A-mediated catalysis of a variety of substrates. However, an as yet unexplained situation apparently exists in vivo whereby only relatively weak correlations have been found between various phenotypic trait measurements of CYP3A activity (212–214), and the ability of any such test to accurately predict the clearance of another CYP3A substrate has generally been poor (214, 232–236). In certain instances, this may be because some in vivo probes are not as valid as previously considered, e.g. the urinary cortisol ratio (see above) and the urinary dapsone hydroxylation index, which probably measure CYP2E1 and CYP2C9 activities in addition to that of CYP3A (237, 238). Furthermore, others, such as the erythromycin breath test, although possibly reflective of CYP3A activity, do not necessarily measure the enzyme’s catalytic level in as quantitative a fashion as, for example, a clearance value. Differences in the route of administration of the test drugs may also be contributory, although poor relationships have been observed following the intravenous administration of both putative probes, e.g. the erythromycin breath test value relative to fentanyl’s clearance by N-dealkylation (216) and midazolam clearance (239). Additional studies are required in this area, especially those based on clearance determinations rather than other more indirect types of trait measures. Other factors possibly contribute to differences in the catalytic activities and levels of CYP3A4 and CYP3A5 and, also, their differential localization in various extrahepatic tissues. However, the interaction between CYP3A and its substrates may be more complex than previously thought. For example, if the allosteric effects observed in vitro (see above) also occur in vivo, CYP3A’s catalytic activity is likely to be relatively evanescent and dependent on active site interactions with substrates and effectors at any given time.

CLINICALLY IMPORTANT DRUG INTERACTIONS

The involvement to some extent of CYP3A in the metabolism of numerous drugs leads to a plethora of potential drug-drug interactions. Some general points have emerged from the large number of reported studies. For example,
the order of in vivo inhibitory potency ofazole antifungal agents following typical therapeutic regimens is consistent with their in vitro $K_i$ values (Table 1), i.e. ketoconazole $>$ itraconazole $>$ fluconazole, with miconazole being essentially without effect (240, 241). Certain macrolide antibiotics, such as erythromycin and troleandomycin, are also fairly potent inhibitors, although less than ketoconazole, as a result of their inactivation of CYP3A by the formation of complexes with the heme moiety (104, 241–243). Other macrolides—including clarithromycin, josamycin, roxithromycin, posinomycin, and flurithromycin—are less effective CYP3A inhibitors, and azithromycin and dirithromycin do not appear to cause significant clinical drug interaction (241–243). A further group of important CYP3A inhibitors with less potency than erythromycin are the selective serotonin reuptake inhibitors whose metabolites may also be inhibitory (244, 245). The order of CYP3A inhibitory potency for these widely used drugs appears to be nefazodone $>$ fluvoxamine $>$ sertraline $>$ paroxetine $>$ venlafaxine (244). Finally, several nondihydropyridine calcium-channel blockers, such as verapamil and diltiazem, produce a weak to moderate inhibitory interaction with other CYP3A drugs in vivo (246).

A somewhat smaller group of drugs needs to be considered with respect to the induction of CYP3A activity following therapeutic dosages. The most potent inducers of CYP3A are rifampin and rifabutin, whose coadministration can reduce a drug’s plasma concentration 20- to 40-fold, effectively negating drug efficacy (241, 247). Commonly used anticonvulsants—such as carbamazepine and, to a lesser extent, phenytoin, primidone, and phenobarbital—also can significantly increase CYP3A activity (135, 248).

Not all drug interactions are necessarily of clinical importance and several factors are contributory. In general, CYP3A-mediated metabolism of the affected drug must represent a major elimination pathway and the magnitude of inhibition/induction must produce a significant alteration in the drug’s plasma concentration–time profile relative to the concentration-response relationship. In turn, this depends on such factors as the interacting drugs’ potencies as inhibitors or inducers and the relative drug concentrations and their time courses within the enterocyte and hepatocyte. Accordingly, important clinical drug interactions generally occur only with drugs having a narrow and/or steep concentration-response relationships when coadministered with the most potent of inhibitors or inducers. More modest interactions are generally of lesser concern, since their consequences are within the window of normal population variability. The distinction is, therefore, one of probability; i.e. an interaction may occur versus one that will be present.

**Nonsedating Antihistamines**

Approved in the United States in 1985, terfenadine quickly became the drug of choice for the treatment of seasonal allergic rhinitis, worldwide. Recognition
that the drug’s elimination was predominantly determined by CYP3A (249–251) provided an explanation for several case reports in the early 1990s describing serious side effects associated with impaired metabolism, including that resulting from coadministration with ketoconazole and erythromycin (252). That is, terfenadine, a potent cardiac potassium channel blocker, which prolongs the QT-interval, underwent extensive CYP3A-mediated presystemic elimination that resulted in low circulating plasma concentrations; however, inhibition of metabolism markedly elevated these levels to produce serious cardiac arrhythmias leading in some instances to death (252, 253). Numerous prospective studies with a wide variety of CYP3A inhibitors subsequently substantiated this notion (254). Despite the serious nature of terfenadine’s adverse effects, its benefit to risk ratio was still considered acceptable until early 1997, when fexofenadine, the active metabolite of terfenadine, was approved and marketed. This equally efficacious new drug does not block cardiac potassium channels (252), and therefore, it is far safer than terfenadine. As a result, terfenadine has been removed from the market in the United States. Terfenadine, therefore, provides a classic example indicating the critical importance of drug metabolism and drug interactions in the development, regulation, and ultimate economic success of drugs.

CYP3A is also the predominant isoform responsible for the metabolism of another nonsedating antihistamine, namely, astemizole (252). Not unexpectedly, its metabolism is significantly affected when inhibitors such as ketoconazole, itraconazole, and erythromycin are coadministered, and rare cases of prolonged QT-interval and syncope have been reported (255). Consequently, concomitant use of astemizole with such CYP3A inhibitors is contraindicated. Impaired metabolism of loratidine also occurs following coadministration with potent CYP3A inhibitors; however, the up to threefold increases in the plasma levels of this drug and its active metabolite do not result in increased toxicity (256).

**Immunosuppressive Agents**

In the past two decades since its discovery and clinical application, cyclosporine has had an enormous effect on organ transplantation. However, its safe and effective long-term use requires individualization of an optimal dose that prevents rejection of the transplanted organ and minimizes drug toxicity. Many of the factors involved in such optimization arise because the metabolism of cyclosporine to its three primary metabolites involves CYP3A. As a result, interpatient variability in cyclosporine’s disposition is large, and drug interactions significantly contribute to such differences. Extensive information on substantiated and suspected interactions has recently been reviewed (112), and generally, the changes in cyclosporine blood concentration–time profiles in response to the coadministration of various inducers and inhibitors (e.g. azole antifungal
agents, macrolides, and various other drugs) are consistent with expectations based on in vitro studies and findings with other drugs metabolized by CYP3A. Of particular note has been the therapeutic use of a combination of ketoconazole (257–262) or diltiazem (260, 263) along with cyclosporine to deliberately reduce the immunosuppressant’s metabolism by CYP3A. As a result, a smaller cyclosporine maintenance dose can be used with a variety of transplanted organs, which in turn, substantially reduces the cost of long-term immunotherapy without significantly affecting either efficacy or toxicity (260, 261, 263).

Tacrolimus (FK506) is also extensively metabolized by CYP3A, and therefore, it too would be anticipated that drug interactions similar to those with cyclosporine would occur. Coadministration of azole antifungal agents, erythromycin, and calcium channel blockers have been reported to increase tacrolimus blood concentrations (175, 264). However, substantiation of other possible interactions in patients is not so extensively documented, perhaps because of their predictability and also the relatively recent introduction of the drug into widespread clinical use.

Benzodiazepines
CYP3A mediates the metabolism of various benzodiazepines, and for those drugs in which such involvement is major, then drug interactions are important. This is especially the case when inhibition of metabolism occurs, since this often results in increased sedative effects. Thus, azole antifungal agents, some but not all macrolides, selective serotonin reuptake inhibitors, and calcium-channel antagonists such as verapamil and diltiazem have been shown to markedly enhance plasma levels of midazolam (131, 139, 144, 161, 200, 226–231), triazolam (132, 265–268), and alprazolam (268–272). Moreover, central nervous effects were increased and their duration prolonged. Because of the potentially hazardous consequences of such interactions, coadministration of such drugs is usually contraindicated. A similar situation also appears to apply with respect to rifampin, since the magnitude of CYP3A induction renders the affected benzodiazepine ineffective (224, 273). Concomitant administration of carbamezepine and phenytoin also markedly reduces the plasma levels of benzodiazepines, such as midazolam and alprazolam, whose metabolism involves CYP3A (274–276).

HIV Protease Inhibitors
The development of inhibitors to HIV-1 protease has dramatically altered the clinical management of HIV-infected patients. Because of their recent introduction and use, published reports concerning their metabolism and propensity for involvement in drug interactions are relatively limited; however, available information has recently been reviewed (277, 278). It is apparent that first-generation
agents are excellent substrates for CYP3A, and this has several consequences. For example, the relatively poor oral availability of HIV protease inhibitors (saquinavir < indinavir < nelfinavir < ritonavir) is in large part related to CYP3A-mediated first-pass metabolism and possibly incomplete absorption associated with P-glycoprotein–mediated efflux transport (279). Not unexpectedly, enzyme inducers such as rifampin markedly decreased the inhibitors’ plasma levels; 80–90% reduction for saquinavir, indinavir, and nelfinavir and 35% for ritonavir. Accordingly, therapeutic concentrations are not likely to be achieved, and such coadministration is generally not recommended. Similar but probably less extensive interactions are also likely with rifabutin, the nonnucleoside reverse transcriptase inhibitor nevirapine, and also clarithromycin, i.e. 30–40% reduction in plasma levels. Presumably, administration of established inducing agents such as anticonvulsant drugs will also result in a similar modest interaction. From a therapeutic management standpoint, the autoinduction of the HIV protease inhibitors’ CYP3A-mediated metabolism during single agent chronic therapy may also be important. Induction may also affect other drug-metabolizing enzymes; for example, ethinylestradiol’s plasma levels are reduced by about 50% when nelfinavir is coadministered, because of induction of glucuronosyltransferases. Such a change would be expected to significantly affect the oral contraceptive’s efficacy.

Ketoconazole and clarithromycin have been observed to modestly increase the plasma levels of ritonavir, indinavir, and nelfinavir but, generally, not sufficiently to warrant major dosage adjustments. On the other hand, the interaction of ketoconazole and saquinavir was found to be more pronounced, resulting in a threefold increase in the protease inhibitor’s plasma levels. Additionally, because the HIV protease inhibitors have low in vitro $K_i$ values (Table 1), they can potentially inhibit the in vivo metabolism of other CYP3A substrates. However, sufficiently high plasma levels are not achieved with saquinavir, and inhibitory interactions have not been demonstrated with this agent. With the other agents’ inhibition, in vivo is more significant; for example, the plasma levels of rifabutin and its active metabolite increased two- to sixfold, and clarithromycin’s concentrations were 50 to 75% higher when either ritonavir, indinavir, or nelfinavir was coadministered. In the case of CYP3A substrates that have serious adverse effects (terfenadine, astemizole, cisapride, and midazolam), their concurrent use with HIV protease inhibitors is contraindicated because of the resulting inhibition of metabolism.

The different patterns of HIV-1 resistance that emerge following use of a single protease inhibitor have led to the investigation of combination therapy, and this is an area of active investigation. Preliminary studies, however, have already demonstrated that this will be complicated by interactions involving the inhibition of metabolism. Coadministration of usual doses of any two
protease inhibitors typically increases the plasma levels of one of the agents by 2- to 8-fold, and in the case of ritonavir, this results in a 20-fold increase in saquinavir's plasma levels. With interactions of this magnitude, modifications of recommended dosage regimens are required, and these are currently being clinically evaluated.

PERSPECTIVE

Drug interactions involving the inhibition and induction of CYP3A will undoubtedly continue to be of scientific interest and clinical importance simply because of the enzyme’s important role in the metabolism of currently available and future drugs. In the past, identification and evaluation of such interactions have been largely empirical, including serendipitous clinical observations. However, recent knowledge about CYP-mediated metabolism in general, and that by CYP3A in particular, provides an opportunity for a more rational approach. The relative selectivity of currently available drugs to modulate CYP3A activity, based on clinical studies with model substrates, provides the basis for predicting their potential to interact with a new drug metabolized by the enzyme. Drugs for which CYP3A-mediated metabolism importantly contributes to the overall clearance process would be more likely to be affected than those for which such metabolism is limited. Prediction is more challenging, however, when the goal is to identify and evaluate the potential in vivo inhibition/induction characteristics of a drug. Various in vitro approaches are being used, especially during drug development, to define inhibitor and induction potencies of a new chemical entity relative to other compounds. Minimally, such studies have the potential to identify interactions that are not likely to occur in vivo and, therefore, do not require clinical investigation. Such potency determinations should also allow an overall assessment of the probability of the new drug interacting with a CYP3A substrate. That is, drugs that modulate CYP3A activity in the nanomolar to low micromolar range are more likely to result in a drug interaction than those requiring high micromolar levels. The practical problem, of course, is the drug with an intermediate potency. However, such ranking should at least allow identification of the most critical putative interactions for subsequent clinical confirmation. There is currently a hope, though, that more quantitative in vitro to in vivo extrapolation can be achieved, particularly with rapidly reversible CYP3A inhibitors. Unfortunately, such a process of prediction is still in its infancy because the necessary experimental approaches have become available only recently. Extensive validation of this approach will be required if it is to be successfully applied at a sufficiently early time in the drug development process to meaningfully affect its outcome. In turn, this validation will probably require a collective effort to evaluate all
appropriate data whether it appears in the public domain or otherwise. Finally, of course, the appropriately performed in vivo study will remain the ultimate means by which a drug interaction and its clinical importance can be assessed.

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