

Drugs Behave as Substrates, Inhibitors and Inducers of Human Cytochrome P450 3A4

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Abstract: Human cytochrome P450 (CYP) 3A4 is the most abundant hepatic and intestinal phase I enzyme that metabolizes approximately 50% marketed drugs. The crystal structure of bound and unbound CYP3A4 has been recently constructed, and a small active site and a peripheral binding site are identified. A recent study indicates that CYP3A4 undergoes dramatic conformational changes upon binding to ketoconazole or erythromycin with a differential but substantial (>80%) increase in the active site volume, providing a structural basis for ligand promiscuity of CYP3A4. A number of important drugs have been identified as substrates, inducers and/or inhibitors of CYP3A4. The ability of drugs to act as inducers, inhibitors, or substrates for CYP3A4 is predictive of whether concurrent administration of these compounds with a known CYP3A4 substrate might lead to altered drug disposition, efficacy or toxicity. The substrates of CYP3A4 considerably overlap with those of P-glycoprotein (P-gp). To date, the identified clinically important CYP3A4 inhibitors mainly include macrolide antibiotics (e.g., clarithromycin, and erythromycin), anti-HIV agents (e.g., ritonavir and delavirdine), antidepressants (e.g. fluoxetine and fluvoxamine), calcium channel blockers (e.g. verapamil and diltiazem), steroids and their modulators (e.g., gestodene and mifepristone) and several herbal and dietary components. Many of these drugs are also mechanism-based inhibitors of CYP3A4, which involves formation of reactive metabolites, binding to CYP3A4 and irreversible enzyme inactivation. A small number of drugs such as rifampin, phenytoin and ritonavir are identified as inducers of CYP3A4. The orphan nuclear receptor, pregnane X receptor (PXR), have been found to play a critical role in the induction of CYP3A4. The inhibition or induction of CYP3A4 by drugs often causes unfavorable and long-lasting drug-drug interactions and probably fatal toxicity, depending on many factors associated with the enzyme, drugs and the patients. The study of interactions of newly synthesized compounds with CYP3A4 has been incorporated into drug development and detection of possible CYP3A4 inhibitors and inducers during the early stages of drug development is critical in preventing potential drug-drug interactions and side effects. Clinicians are encouraged to have a sound knowledge on drugs that behave as substrates, inhibitors or inducers of CYP3A4, and take proper cautions and close monitoring for potential drug interactions when using drugs that are CYP3A4 inhibitors or inducers.

Keywords: CYP3A4, inducer, inhibitor, substrate.

INTRODUCTION

The human cytochrome P450s (CYPs, EC 1.14.14.1), comprising 57 genes, are a family of monooxygenases that catalyze the metabolism of a wide variety of endogenous and exogenous compounds including xenobiotics, drugs, environmental toxins, steroids, and fatty acids. The catalytic mechanism appears to be common to all CYPs and involves a two-electron reduction of molecular oxygen to form a reactive oxygen species and water. Of the human CYP enzymes, CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4 have been described to contribute to the metabolism of the vast majority of drugs compared with other Phase I oxidative enzymes. The human CYP3A subfamily, include CYP3A4, 3A5, 3A7 and 3A43, with CYP3A4 having the highest abundance in the liver (~40%) and intestine and metabolizing more than 50% of the clinically used drugs [1]. The substrates of CYP3A4 are structurally diverse and exhibit a wide range of sizes and affinities. Typical metabolic pathways for these substrates include *N*-oxidation (e.g. voriconazole), *C*-oxidation (e.g. nicotine), *N*-dealkylation (e.g. fentanyl and sufentanil), *O*-dealkylation (e.g. fluoxetine), nitro-reduction (e.g. clonazepam), dehydration (e.g. bromperidol and haloperidol), and *C*-hydroxylation (e.g. terfenadine) [2]. Significant interindividual variability in the expression and activity of CYP3A4 has also been observed, which is considered a result of interplay of environmental, physiological and genetic factors. The crystal structures of unbound and bound human CYP3A4 have been revealed [3]. Surprisingly, a small active site is identified, with little conformational change when the protein is bound by either a substrate or an inhibitor [3].

Like other CYPs, CYP3A4 is subject to induction and inhibition by a number of compounds. Induction of hepatic CYP3A4 by drugs can occur by increasing the intrinsic clearance thereby increasing the extraction ratio or by increasing the hepatic blood flow. The inhibition of CYP3A4 activities can occur by two main mechanisms: reversible inhibition and mechanism-based inactivation (including quasi-irreversible and irreversible inhibition) [4]. Reversible inhibition refers to competition of two drugs for a CYP3A4. Mechanism-based inhibition of CYP3A4 involves the inactivation of the enzyme via the formation of metabolic intermediates (MIs) that bind tightly and irreversibly to the enzyme [4, 5]. The MI can then exert its inhibitory effect by forming a direct covalent interaction with an amino acid in the active site, a non-covalent tight binding complex, or an inactive enzyme product that is released from the inhibitor [5]. The aim of this paper is to update the knowledge on drugs that behave as substrates, inhibitors or inducers of CYP3A4.

THE CYP3A4 GENE AND CYP3A4 PROTEIN

The CYP3A family of enzymes consists of 4 isoforms, CYP3A4, CYP3A5, CYP3A7 and CYP3A43. All of the genes that encode for these enzymes, as well as three pseudogenes, are found in a cluster spanning roughly 200kb on chromosome 7. The *CYP3A4* gene and its 5' flanking region has been found to be 27,592 base pairs long with a coding region that includes 13 exons [6]. It is located on chromosome 7 q21.3-22.1. The promoter region includes a basal transcription element, AP-3 binding site, a p53 binding motif, a hepatocyte nuclear factor-4 (HNF-4) element, two hepatocyte nuclear factor-5 (HNF-5) elements, a glucocorticoid response element (GRE), a pregnane X receptor element (PXRE) and an oestrogen response element [6]. There have been demonstrated genetic polymorphisms in both the promoter and coding regions. The protein translated from the gene transcription is 502 amino acids in length, weighing 57.29 kDa making it the largest

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human CYP enzyme and consists of a complex array of helices and sheet structures.

CYP3A4 is categorized by the Enzyme Commission number EC1.14.13.97, identifying the chemical reaction that it catalyses. It is also the enzyme that is present in the largest quantities in the human body, making up roughly 28% of our total P450s [7]. CYP3A4 is expressed predominantly in the liver where it makes up 33% of hepatic CYP enzymes and is partly responsible for the characteristic color of liver tissue. Its expression in the small intestines means it plays a role in the bioavailability of many substances via first-pass metabolism. It is also present in the brain and kidneys however its role in these organs has yet to be fully elucidated. Its presence in the prostate and breast have been the source of research in to the possible role of polymorphisms and the incidence of cancer [8]. CYP3A4 is constitutively expressed but is also subject to transcriptional induction by a wide array of substances. However there is a large inter-individual variation in the response to known inducers. Inter-individual variation influencing hepatic expression has been shown to be as high as 40- to 100-fold [2]. These variations cannot be explained purely by genetic polymorphisms and clearly have a significant effect on drug metabolism. CYP3A4 is subject to both transcriptional induction and a complex series of constitutive regulation. Finally there are differences in the basal expression and xenobiotic induced transcription between the sexes. It appears that sex hormones are responsible for influencing basal transcription via their effects on growth hormones. CYP3A4 is surprisingly not expressed in the human fetus where CYP3A7 occurs instead [9]. In the weeks after birth however CYP3A7 ceases to be expressed and is gradually replaced by CYP3A4.

The presence of multiple binding sites on the CYP3A4 enzyme adds another level of complication to the regulation of the enzyme. It has been shown that there are 2 substrate binding domains as well as an allosteric effector binding site on the enzyme [2]. It is these three binding sites that allow homotropic and heterotropic cooperativity to influence the metabolism of CYP3A4 substrates. Capacity in the active site of the enzyme for two substrates can act to increase metabolic activity if the presence of one substrate ensures that the second is held in a more favorable position for catalysis. It can also act to decrease metabolism via a type of competitive inhibition by preventing a second substrate from attaining a favourable catalytic position. Supporting an allosteric model, resulting in conformational change to CYP3A4, is the discovery of different enzyme conformations in the presence of different substrates [2]. These conformations have been shown to alter the catalytic properties of the enzyme. This can cause an increase in activity as seen with diclofenac metabolism in the presence of quinidine, or a decrease in activity as seen in the form of non-competitive inhibition of diclofenac metabolism in the presence of 7,8-benzoflavone [10, 11]. These types of CYP cooperativity are controlled by the concentration of the various substrates and allosteric effector molecules. These interactions make drug-drug interactions harder to predict because of the complexity of multiple dose-related responses. Finally the activity of CYP3A4 has shown to be further influenced by ionic strengths, divalent cations and buffer systems making precise predictions of drug-drug interaction understanding that much more complex.

IDENTIFICATION OF DRUGS AS SUBSTRATES OF CYP3A4

Drugs as Substrates of CYP3A4

A substrate is a drug which requires the metabolism to a more hydrophilic compound for ultimate elimination. CYP3A4 was originally called nifedipine oxidase due to its ability to metabolize the calcium channel blocker nifedipine [12]. Apparent polymorphisms in the metabolism of nifedipine lead to the isolation of CYP3A4 in 1986 [13]. It was shown that nifedipine oxidation was well correlated with levels of CYP3A4 mRNA and protein levels.

CYP3A4 was then shown to be involved in the hydroxylation and termination in activity of steroid hormones, especially testosterone, oestrogen and cortisol, the detoxification of bile acids and the activation of several potent carcinogens [14]. Other xenobiotic substrates of CYP3A4 include codeine, diazepam, simvastatin and R-warfarin [14]. To date, more than 50% therapeutic drugs are found to be metabolized by human CYP3A4 [15].

The substrate specificity of the CYP3A4 enzymes is very broad, with an extremely large number of structurally divergent chemicals being metabolized often in a regio- and stereo-selective fashion [16]. CYP3A4 is known to metabolize a large variety of compounds varying in molecular weight from metyrapone (Mr = 226 Dal) to cyclosporine (Mr = 1203 Dal). CYP3A4 exhibits a relatively large substrate-binding cavity that is consistent with its capacity to oxidize bulky substrates such as cyclosporine, statins, taxanes, and macrolide antibiotics. Although the active site volume is similar to that of CYP2C8, the shape of the active site cavity differs considerably due to differences in the folding and packing of portions of the protein that form the cavity. Compared with CYP2C8, the active site cavity of CYP3A4 is much larger near the heme iron [17].

CYP3A4 contains an unexpected peripheral binding site located above a phenylalanine cluster, which may be involved in the initial recognition of substrates or allosteric effectors [3]. CYP3A4 has been shown to display cooperative behavior with the binding of substrates in previous studies [18-20], and this is often explained by a flexible and large CYP3A4 active site, which can accommodate multiple and bulky substrates such as cyclosporine, statins, taxanes, and macrolide antibiotics. However, two published CYP3A4 crystal structures, in contrast to CYP2B6, have little conformational change in the ligand-free and ligand-bound forms [3, 17]. One possible reason might be that the ligand in the crystal is relatively small and does not cause marked conformational change of CYP3A4. Large ligands, when used in crystallization, either stay outside of the active site pocket (e.g. for progesterone) or have yet to be cocrystallized with the protein (e.g. erythromycin). In contrary to above reports, a recent study indicates that CYP3A4 undergoes dramatic conformational changes upon binding to ketoconazole or erythromycin with an differential but substantial (>80%) increase in the active site volume [21], providing a structural basis for ligand promiscuity of CYP3A4. The binding of two molecules of ketoconazole to the active site of CYP3A4 and multiple binding modes for erythromycin may provide an explanation for the atypical kinetics of CYP3A4.

In vitro studies suggest that the metabolism of ~50% of drugs used in humans involves CYP3A4-mediated oxidation. It is clear that CYP3A4 is of major importance in the metabolism of drugs by humans. In one study involving 165 CYP3A4 substrates, most of the compounds have only one metabolite catalyzed by CYP3A4, whereas ~27% of its substrates have two or more metabolites [22]. For the most reactions catalyzed by CYP3A4, 43% are aliphatic or aromatic hydroxylation, 27% are *N*-dealkylation, and 7% are *O*-dealkylation [22].

The anticancer drugs metabolized by CYP3A4 include docetaxel, paclitaxel, cyclophosphamide, ifosfamide, etoposide, tamoxifen, irinotecan, vinblastine, and vinorelbine. Although there is a marked interindividual variation of pharmacokinetic parameters between patients, and such variation in patient response is often attributed to polymorphism in *CYP* genes, CYP3A4 is an exception because only a small percentage of the variation in activity can be attributed to genotype. Interactions with coadministered drugs are therefore likely to be particularly important in explaining variations in anticancer drug pharmacokinetics and side effects. If a patient experiences significant toxicity during chemotherapy, the clinician will usually reduce the dose of the cytotoxic drug, reducing the anticancer effect. A more appropriate action might be to substitute a different comedication that will not interact with the therapy and so maintain dose intensity of the cytotoxic drug.

Probes of CYP3A4

A small number of drugs are used as model substrates of CYP3A4 when assessing *in vitro* and *in vivo* phenotyping activity and drug interactions Table 1. A recent Food and Drug Administration report [23] has shown that testosterone is the most commonly used *in vitro* CYP3A4 probe (50% of reported studies), followed by midazolam (15–20% of *in vitro* estimates of CYP3A4 activity). Nifedipine, felodipine, and erythromycin were used in <10% of studies. At substrate concentrations of ≤ 250 μM , testosterone-6 β -hydroxylation rate primarily reflects the CYP3A4 activity, and thus can be used to probe drug interaction potential of a new drug toward this enzyme *in vitro* [24]. CYP3A4 catalyzes the oxidation at C-6 β of cortisol to form 6 β -hydroxycortisol, and the urinary excretion ratio 6 β -hydroxycortisol/cortisol has extensively been used as a suitable noninvasive index for evaluating *in vivo* CYP3A activity and potential drug-drug interactions [25]. Although numerous studies have described successful utilization of the urinary ratio 6 β -hydroxycortisol/cortisol, the ratio does not always correlate with the disposition of CYP3A substrate drugs, including typical phenotyping probe drugs such as [^{14}C]-erythromycin and midazolam [26, 27]. This may be due to the fact that both compounds are also substrates for P-glycoprotein (P-gp) [28, 29].

All above probe drugs for CYP3A4 have also been used to investigate the mechanism-based inhibition of CYP3A4 *in vitro* by a variety of drugs [4, 5]. There is evidence indicating the existence of distinct and preferential binding domains for each substrate subgroup, namely, midazolam, testosterone, and nifedipine [30]. However, only some of these probes be used in assays with intact cells (e.g. hepatocytes), as these substrates should be non-cytotoxic and readily cross cell membranes. Because of safety concerns, terfenadine and testosterone are not for *in vivo* studies. Because the *in vitro* findings obtained with one probe substrate for CYP3A4 are often extrapolated to the potential of test compounds to influence all CYP3A substrates and the inhibition of CYP3A4 by drugs is often substrate-dependent [31], it is important to use the right probe substrate and to conduct the experiment under optimal conditions [23].

Midazolam is considered as one of the best *in vivo* probe drugs for the study of CYP3A4 activity [32–34], because of several reasons: a) it can be administered both orally and intravenously, which can provide a measure of CYP3A4 activity relative to intestinal and hepatic metabolism, respectively; and b) midazolam metabolism at lower concentrations exhibits a regio-selective difference which can be used to discriminate among individual subjects with or without CYP3A5, as CYP3A5 has a much higher 1'-OH/4-OH ratio of midazolam metabolism than CYP3A4. The probes for CYP3A4 differ in their *in vitro* complexities, from standard hyperbolic kinetics (midazolam and quinidine) to positive (testosterone) and negative cooperativity (nifedipine) [35]. In addition to *in vitro* differences, the substrates selected vary in their *in vivo* characteristics that may impact the prediction of CYP3A4-mediated drug-drug interactions, e.g., significance of intestinal metabolism, involvement of P-gp (minimal role in case of midazolam, in contrast to other substrates [34]), and contribution of CYP3A5 to the overall clearance. Substrate auto- and heteroactivation, partial inhibition, substrate inhibition, and pathway differential kinetics observed for CYP3A4 are attributed to the different binding domains for the substrate and modifier within the enzyme active site [36, 37]. Involvement of multiple binding sites may result in an inhibition effect at only one site or a differential effect at each site, confounding a straightforward prediction of a potential *in vivo* interaction. For example, quinidine possesses the ability to stimulate meloxicam metabolism by CYP3A4 and increases the contribution of CYP3A4 over CYP2C9 to the overall metabolism through a heteroactivation mechanism [38].

Dual Substrates of CYP3A4 and P-glycoprotein

Like CYP3A, P-gp seems to have broad substrate specificities. A striking feature of P-gp function relates to its ability to transport a large number of structurally divergent compounds. Moreover, there is an overlap in substrate and inhibitor specificity for P-gp and that for CYP3A4, although the overlap is not absolute [39, 40]. A considerable number, but not all (e.g. benzodiazepines), of CYP3A substrates interact with P-gp either as substrates and/or inhibitors (e.g. calcium-channel blockers, azole antifungal agents, immunosuppressants, natural product anticancer agents and macrolide antibiotics). Accordingly, the coadministration of two CYP3A4 substrates can result in interactions that reflect inhibition of metabolism alone, reduced P-gp efflux only, or a combination of both effects. A dual substrate of CYP3A4 and P-gp, e.g. erythromycin [28], also makes the interpretation of *in vivo* data difficult. Recently, there is evidence that erythromycin breath test actually reflects both CYP3A4 and P-gp activity [28]. However, from the point of drug disposition, this phenomenon of substrate overlap is of importance because P-gp and CYP3A4 are frequently expressed in the same cells, such as the enterocytes and hepatocytes. Thus, for drugs that are dual substrates of P-gp and CYP3A, the complementary functions of these proteins likely result in greater than expected reduction in systemic exposure to such drugs. Moreover, having such redundancy may allow for the maintenance of drug elimination function when either CYP3A or P-gp function is inhibited. In terms of P-gp drug substrates, a number of drugs used in cancer chemotherapy, immunosuppression, hypertension, allergy, infection, and inflammation are substrates of this transporter. However, not all members of a therapeutic class interact in the same manner with P-gp. For example, among calcium channel antagonists, diltiazem and verapamil have been shown to be transported by the P-gp [41]. However, nifedipine, a well-recognized CYP3A4 substrate, is not a P-gp substrate [40].

There is substantial overlapping in substrate specificity between CYP3A4 and P-gp, and several modulators/substrates of P-gp and CYP3A4 have been shown to coordinately up-regulate the expression of these proteins *in vitro* [42]. P-gp and CYP3A4 appear functionally linked components of a drug detoxification cascade that limits the bioavailability of several drugs [42]. P-gp is present on many barrier sites throughout the body, such as the blood-brain and blood-testis interfaces, and could decrease the concentration of their substrates such as protease inhibitors in these organs [43]. P-gp can also alter the intracellular concentration of CYP3A4 inhibitors and inducers and hence the magnitude of the inhibitory and inductive responses. Furthermore, P-gp, like CYP3A4, can also be induced by many drugs including certain anti-HIV agents [44]. P-gp mediated efflux of many structurally divergent substrate drugs can often limit their intestinal absorption and blood-brain barrier penetration, as well as facilitate their active excretion into bile and urine. In conjunction with drug-metabolizing enzymes, P-gp provides a protective physiological barrier capable of altering the rate and extent of xenobiotic entry into the systemic circulation. Complex modulatory interactions with P-gp can occur; i.e., certain compounds can function as combinations of substrate, inhibitor, and activator. Quinidine is a P-gp substrate and inhibitor. Ketoconazole and loperamide activate P-gp-mediated secretory transport of vinblastine and digoxin *in vitro* and inhibit their secretory transport at higher concentrations, whereas the other P-gp modulators including cyclosporine, verapamil, and nifedipine inhibit secretory transport of these probe P-gp substrates in a concentration-dependent manner but did not activate the secretory transport. Inhibition and/or activation of P-gp can potentially alter the pharmacokinetic profile of a drug, possibly resulting in concurrent changes in pharmacodynamic response. However, altering the tissue distribution of a drug that is a P-gp substrate, via coadministration of a P-gp inhibitor, can be therapeutically advantageous. In *mdr1a* wild-type mice, coadministration of

Table 1. Commonly Used Model Substrates of CYP3A4

Model Substrate	Brand Name	Metabolic Reaction	Inhibitory Drugs Tested <i>In Vitro/In Vivo</i>
Alfentanil	Alfenta	<i>N</i> -Dealkylation	Troleandomycin, erythromycin, verapamil, diltiazem
Alprazolam	Xanax	1'-Hydroxylation	Troleandomycin, fluoxetine, erythromycin
Atorvastatin	Lipitor	1'-Hydroxylation	Ketoconazole, itraconazole, fluconazole
Cerivastatin	Baycol	Demethylation & hydroxylation	Ketoconazole, itraconazole, fluconazole, erythromycin
Clarithromycin	Biaxin	<i>N</i> -Demethylation	Troleandomycin, cyclosporine, carbamazepine
Cortisol	Contone	6 β -Hydroxylation	Gestodene, cyclosporine, desogestrel
Cyclosporine	Neoral, Sandimmune	<i>N</i> -Demethylation	Nicardipine, nifedipine, verapamil, diltiazem, clarithromycin, erythromycin
Dapsone	Avlosulfon	<i>N</i> -Hydroxylation	Gestodene, troleandomycin, ethinyl estradiol
Diazepam	Valium	<i>N</i> -Demethylation & 3-hydroxylation	Diclofenac, ketoconazole, troleandomycin
Diclofenac	Voltaren	5-Hydroxylation	Quinidine
Erythromycin	Ery	<i>N</i> -Demethylation	Troleandomycin, cyclosporine, delavirdine
Ethinyl estradiol	Estinyl	2- & 4-Hydroxylation	Gestodene, ritonavir, desogestrel
Felodipine	Plendil	Dehydrogenation	Halofantrine, quinidine, haloperidol
Flunitrazepam	Rohypnol	<i>N</i> -Demethylation & 3-hydroxylation	Ketoconazole, ritonavir
Lidocaine	Xylocaine	<i>N</i> -Deethylation & 3-hydroxylation	Troleandomycin, erythromycin, ketoconazole, troleandomycin
Lovastatin	Altoprev, Mevacor	6' β -Hydroxylation	Ketoconazole, itraconazole, fluconazole, cyclosporine, erythromycin, troleandomycin
Mexazolam	Melex	1'-Hydroxylation	Pravastatin, simvastatin, fluvastatin, atorvastatin, cerivastatin
Midazolam	Versed	1'-Hydroxylation	Isoniazid, clarithromycin, diltiazem, fluoxetine, quinidine, mibefradil, nicardipine, verapamil
Nifedipine	Procardia	Dehydrogenation	Gestodene, ritonavir, quinidine, haloperidol
Nisoldipine	Sular	Dehydrogenation	Ketoconazole, itraconazole, fluconazole, troleandomycin
Paclitaxel	Taxol	3'-Hydroxylation	Troleandomycin, cyclosporine, felodipine, ketoconazole
Quinidine	Quinidex	3-Hydroxylation & <i>N</i> -oxygenation	Ketoconazole, itraconazole, fluconazole
Rifabutin	Mycobutin	Demethylation & deacetylation	Ketoconazole, itraconazole, fluconazole, clarithromycin, troleandomycin
Simvastatin	Zocor	3'-Hydroxylation & 3',5'-dihydroxylation	Quinidine, haloperidol, ketoconazole, itraconazole
Tacrolimus	Prograf	Demethylation	Ketoconazole, itraconazole, fluconazole, gestodene, troleandomycin
Terfenadine	Seldane	Hydroxylation	Clarithromycin, gestodene, ritonavir, fluoxetine
Testosterone	Striant	6 β -Hydroxylation	Irinotecan, tamoxifen, dihydralazine, diltiazem, mifepristone, verapamil, nicardipine, raloxifene, bergamottin, erythromycin, mibefradil, ketoconazole, quinidine, itraconazole, diclofenac
Triazolam	Halcion	1'- & 4-Hydroxylation	Delavirdine, nelfinavir, ritonavir, erythromycin, fluoxetine, clarithromycin, diltiazem

Data are compiled from Zhou *et al.* [5, 102] and Galetin *et al.* [30].

the potent P-gp inhibitor LY335979 elicited a marked increase in the penetration of nelfinavir into the testes and brain, tissues that express P-gp and normally function as viral sanctuary sites for HIV [45].

INHIBITION OF CYP3A4 BY DRUGS

An inhibitor is a drug which impairs the ability of drug metabolizing enzymes such as CYP3A4 to metabolize other co-administered substrates. Reaction phenotyping studies using cDNA-expressed CYPs, chemical inhibitors and inhibitory antibodies are designed to determine the contribution of individual CYP isoforms to the metabolism of a drug. Chemical inhibitors provide a simpler and more cost-effective alternative to immunoinhibitory antibodies and can be used in cells. However, proper use of chemicals may require foreknowledge of the reaction kinetics under investigation.

Chemicals used as selective inhibitors of CYP3A4 include a small number of compounds inhibiting CYP3A4 in an irreversible (e.g. triacetyloleandomycin, gestodene) and/or reversible (e.g. ketoconazole) manner [5]. Ketoconazole is most widely used, probably because of advantages in potency, selectivity, commercial availability, and ease of use. However, selectivity of ketoconazole for CYP3A4 is often less than ideal. For example, CYP1B1, CYP2B6, and CYP2C8/9/19 enzymes are significantly inhibited (20-60%) at concentrations required to inhibit CYP3A4 by 95% [46].

Reversible inhibitors of CYP3A4

There are two main classes of enzyme inhibitors; these are reversible and irreversible inhibition, which is differentiated according to their affinity for the enzyme. Sometimes the loss of activity can be reversible by simply removing the inhibitors, however, it

may also be irreversible, where the loss of activity is time-dependent and cannot be recovered during the timescale of interest. When an inhibited enzyme is completely inactive (e.g. by a potent mechanism-based inhibitor), irreversible inhibition acts as a time-dependent loss of enzyme concentration, therefore, a lower V_{max} results. In other cases where there is incomplete inactivation, there may be time-dependent changes in both K_m (defined as the concentration of substrate that gives half life) and V_{max} . However, in most cases for the majority of enzyme catalyzed processes, the effects of reversible inhibitors are more important.

Reversible enzyme inhibitors can be classified as competitive, non-competitive or mixed inhibitors. The classification of drugs into these three groups depends on the site in which the inhibitor binds to on the enzyme, as well as the order with which it binds relative to the substrates. Competitive inhibitors compete for the enzyme's active site with the substrate; non-competitive inhibitors, in which the drug binds with the enzyme at another site rather than the active site, does not compete for the active site; in the case of mixed inhibition, the inhibitor and the substrate can bind to the enzyme's active site at the same time.

Mechanism-based inhibitors of CYP3A4

A number of drugs with widely differing structures and therapeutic targets have been reported to be mechanism-based inhibitors of CYP3A4 Table 2. The identification of drugs causing irreversible CYP3A4 inhibition, and the mechanisms involved, are important in term of rational use of therapeutic drugs [47]. Mechanism-based inhibition of CYP3A4 can decrease a drug's first-pass clearance in the liver and gut, thus greatly altering the kinetic behavior. Moreover, the decrease in functional catalytic activity of the damaged CYP can lead to enhanced exposure of other drugs that are normally cleared by CYP3A4, thus setting the stage for potential drug-drug interactions. It is therefore not surprising that during drug development new compounds that are potent CYP3A4 inhibitors are regarded with high skepticism and often withdrawn from further development.

To date, the identified clinically important mechanism-based CYP3A4 inhibitors mainly include macrolide antibiotics (e.g., clarithromycin, and erythromycin), anti-HIV agents (e.g., ritonavir and delavirdine), antidepressants (e.g. fluoxetine and fluvoxamine), calcium channel blockers (e.g., verapamil and diltiazem), steroids and their modulators (e.g., gestodene and mifepristone), and several herbal and dietary components Table 2. Large numbers of acetylenes, particularly those synthetic steroids such as gestodene, norethisterone, ethinyl estradiol, and norgestrel, have been demonstrated to cause mechanism-based inactivation of CYPs [48]. However, most of the alkynes that inactivate CYPs are terminal acetylenes. Studies have shown that internal acetylenes such as several different methyl-substituted aryl acetylenes (propynylaryl acetylenes) and 10-dodecynoic acid also cause mechanism-based inactivation of CYPs [49, 50]. Mifepristone, an internal acetylene that has a methyl group substituting for the hydrogen on the external carbon of the triple bond, is a potent and selective mechanism-based inactivator of CYP3A4 via irreversible modification of the apoprotein [51]. Most of these CYP3A4 inactivators are also substrates and reversible inhibitors of CYPs (in particular CYP3A4), and some of which are also inducers of CYP3A and other CYPs. Three glitazones, troglitazone, rosiglitazone and pioglitazone, are mechanism-based CYP3A4 inhibitors, and their order of potency for inactivation is troglitazone > rosiglitazone > pioglitazone [52]. Structurally, the three glitazones share a 2,4-thiazolidinedione functionality. Reactive metabolites from bioactivation of 2,4-thiazolidinedione moiety can inactivate CYP3A4. However, troglitazone is the only one containing a chromane moiety; instead, rosiglitazone has a dialkylamino-pyridine and pioglitazone has a dialkylpyridine group. Formation of quinone methide from chromane might contribute to the greater potency of troglitazone for inactivating CYP3A4. The less

effective formation of covalent adducts in CYP3A4 by rosiglitazone and pioglitazone, combined with the much lower doses generally prescribed (less than 10 mg/day) may explain the lacking of idiosyncratic hepatotoxicity and pharmacokinetic drug-drug interactions of those drugs, compared with troglitazone, in clinical settings [52].

A pharmacophore built with SKF-525A, erythromycin, am-prenavir, and norverapamil indicate that four hydrophobic features and a hydrogen bond acceptor are important for these MI complex forming compounds [53]. *In silico* modeling studies indicate that hydrophobic and hydrogen bond acceptor features are required for MI complex formation [53]. The drugs that inactivate CYP3A4 often possess a number of structural similarities such as a tertiary amine function, a furan ring, methylenedioxy, or an acetylene functional group [4]. These functional groups are easily converted to radical or carbene species that can react with the apoprotein or the heme of the CYPs. The formation of the MI complex has been reported to play a critical role in the inhibition mechanism of CYPs by methylenedioxyphenyl compounds. It is also known that such MI complexes exhibit a characteristic absorbance peak at 455 nm, with a secondary peak at 430 nm, and are referred to as type 3 binding spectra. However, drugs such as indinavir and nelfinavir lacking these functional groups can form MI complex via their metabolites [54], indicating other chemical moieties are also involved. The presence of an amine function does not always imply that a molecule will form a MI complex [55]. For example, 3-hydroxy tamoxifen and 4-hydroxy tamoxifen do not form MI complex while tamoxifen and *N*-desmethyltamoxifen form MI complex *in vitro* [56]. Some macrolide antibiotics such as clarithromycin form MI complex with the CYP [57] while others like miocamycin do not [58], suggesting that the amine may be sterically hindered in the later case such that it cannot orientate correctly in the CYP3A4 binding site.

In some cases, the ability of a series of compounds to behave as mechanism-based inhibitors of CYP3A varies depending on the structures of the analogs, even though the compounds contain the same reactive functional group that theoretically could lead to the formation of a reactive intermediate. For example, the antidiabetic drugs troglitazone, pioglitazone, and rosiglitazone are all thiazolidinedione derivatives causing mechanism-based inactivation of CYP3A4. However, the potencies for inactivation by the three compounds varied 6-fold [52]. Troglitazone is the most potent CYP3A4 inactivator, containing other functional groups that could be responsible for production of reactive intermediates [59]. Similarly, tienilic acid is a potent inactivator of CYP2C9 [60, 61] and the inactivation is due to the metabolism of tienilic acid at its thiophenyl structure [62]. However, ticlopidine and clopidogrel, which also contain the thiophenyl substructure, show much weaker inhibition of CYP2C9, but inhibit CYP2C19, 3A4 and 2B6 in a mechanism-based manner [63]. This suggests that a single potential reactive functional group may not be the only contributing factor for inactivation and that other functional substructures within the compounds may also contribute to mechanism-based inactivation. It appears that the chemical properties of a drug critical for CYP3A4 inactivation include i) being a CYP substrate, ii) formation of reactive metabolites; iii) preponderance of CYP inducers and reversible inhibitor, and iv) being a P-gp substrate. Many mechanism-based CYP3A4 inhibitors are able to induce CYP3A4 and inhibit this enzyme in a reversible manner [64]. It is not a surprise that many mechanism-based CYP3A4 inhibitors are also P-gp substrates given that these inhibitors are often metabolized by CYP3A reactive metabolites [64].

The first step of inactivating CYP3A4 by a drug is the bioactivation of drugs to reactive MIs which is often via various CYPs. The diversity of CYPs means that a wide range of drugs can be bioactivated by either a single CYP or multiple CYPs. Investigations of mechanism-based inactivation can lead to detailed informa-

Table 2. Drugs and Some Herbal Components Reported to be Mechanism-Based CYP3A4 Inhibitors

Drugs	Reversible Inhibitor	k_{inact} (min^{-1})	K_I (μM)	Mechanism of Inhibition (Protein, Heme or Both)	Plasma Conc. in Humans (ng/ml)	Ref.
(-)-Hydrastine (Goldenseal, <i>Hydrastis Canadensis</i>)	Yes	0.23	110	Heme	ND	[103]
14-OH-clarithromycin	No	0.05	0.5	Heme	350-800	[53]
4-Ipomeanol (Naturally occurring alkylating furan)	Yes	0.15	20	Protein	ND	[104]
6', 7'-Dihydroxybergamottin (Grapefruit juice)	Yes	0.16	59	Protein & heme	ND	[105, 106]
Amiodarone (Cordarone)	Yes	ND	ND	Protein	500-3,500	[107]
Amitriptyline (Elavil)	Yes	ND	ND	Protein	50-350	[107]
Amlodipine (Norvasc)	Yes	0.35	2.6	Heme	100-300	[53]
Amprenavir (Agenerase)	Yes	0.073	1.4	Heme	3,500-118,000	[54, 108]
Atazanavir (Reyataz, BMS-232632)	Yes	ND	IC_{50} : 0.31	Heme		[109]
Azamulin (Pleuromutilin class antibiotics)	Yes	ND	ND	Heme & protein	ND	[110]
Azithromycin (Zithromax)	No	0.0158	623	Heme	240-440	[111]
Bergamottin (Grapefruit juice)	Yes	0.30	4.2	Protein & heme	ND	[112, 113]
Bromocriptine (Parlodel)	Yes	ND	ND	Protein	4-6	[47]
Buprenorphine (Buprenex)	Yes	ND	ND	Protein	1-50	[47]
Clarithromycin (Biaxin)	No	0.072	5.49	Heme	1,000-4,000	[53, 57, 72]
Clozapine (Clozaril)	Yes	ND	ND	Protein	120-320	[47]
Cyclosporine A (Neoral, Sandimmune)	Yes	ND	ND	Protein	655-1,802	[114]
D-617 (Active metabolite of verapamil)	Yes	0.07	7.9	Heme	50-200	[53]
Delavirdine (Rescriptor)	Yes	0.44	9.5	Heme & protein	27,000-99,500	[115]
Desipramine (Norpramine)	Yes	ND	ND	Heme	150-850	[107]
Diclofenac (Voltaren)	Yes	0.246	1640	Protein	417-1,417	[116]
Dihydralazine (Apresoline)	Yes	0.05	35	Protein	36-58	[117]
Diltiazem (Cardizem LA)	Yes	0.11	2.0	Heme	50-200	[57, 118-120]
DPC 681 (Phase I/II)	Yes	0.22	0.24	Heme	779-6,916	[121]
Erythromycin (Ery)	No	0.08	46.6	Heme	1,100-1,700	[53, 122-124]
Ethinyl estradiol (Estinyl, contraceptive)	Yes	0.04	18	Heme & protein	5-20	[78, 85]
Felodipine (Plendil)	Yes	ND	ND	Protein	30-120	[107]
Fluoxetine (Prozac)	Yes	0.017	5.26	Heme	15-55	[53, 57]
Fluvoxamine (Luvox)	Yes	0.05	3.7	Heme	90-550	[53]
Gestodene (Femoden ED, Trioden ED)	Yes	0.40	46	Heme & protein	7-13	[48]
Glabridin (Licorice)	Yes	0.14	7.0	Heme	ND	[125]
Gomisin C (Schisandra fruit as herbal medicine)	Yes	0.092	0.4	Protein or heme	ND	[126]
Indinavir (Crixivan)	Yes	ND	ND	Heme	12,050-23,400	[54]
Irinotecan (Camptosar)	No	0.06	24	Protein	1,700-3,400	[127]
Isoniazid (Nydrazid)	Yes	0.08	228	Protein & heme	70,000-350,000	[128]
K11002 (Irreversible peptidomimetic cysteine protease inhibitor)	Yes	0.026	0.5	ND	ND	[129]
K11777 (Irreversible peptidomimetic cysteine protease inhibitor)	Yes	0.054	0.06	ND	ND	[129]
L-754,394 (Phase I)	Yes	1.62	7.5	Heme & protein	Rat: 156-4,172 Dog: 203-2,328 Man: ND	[106, 130, 131]
Levonorgestrel (Jadelle)	Yes	ND	ND	Heme & protein	5-10	[48]
Lidocaine (Xylocaine)	Yes	ND	ND	Heme	1,500-5,000	[66]
Limonin (Evodia fruit)	Yes	0.27	23.2	ND	ND	[132]
Lopinavir (Kaletra, with ritonavir)	Yes	0.11	1.0	Protein	6,100-13,500	[54]
Mibefradil (Posicor, withdrawn from the market)	Yes	0.40	2.3	Protein	300-600	[133]
Midazolam (Versed)	Yes	0.15	5.8	Protein	90	[80]

(Table 2) contd....

Drugs	Reversible Inhibitor	k_{inact} (min ⁻¹)	K_{I} (μM)	Mechanism of Inhibition (Protein, Heme or Both)	Plasma Conc. in Humans (ng/ml)	Ref.
Mifepristone (Mifeprex, RU486)	Yes	0.089	4.7	Protein	1,980	[51, 53]
<i>N</i> -desmethyldiltiazem (Metabolite of diltiazem)	Yes	0.027	0.77	Heme	10-40	[53, 57]
<i>N</i> -desmethylerythromycin	No	0.34	5.7	Heme	350-1,200	[53]
<i>N</i> -desmethyltamoxifen (Active metabolite of tamoxifen)	Yes	0.08	2.6	Heme	152-706	[56]
Nefadozone (Serzone, withdrawn from the market in 2003)	Yes	0.27	12.5	Protein	100-300	[53, 134, 135]
Nelfinavir (Viracept)	Yes	0.18	5.6	Heme	3,200-4,800	[136]
Nicardipine (Cardene)	Yes	2.0	0.6	Heme	36-133	[53, 120]
Norclomipramine	Yes	ND	ND	Heme	175-450	[107]
Nortriptyline (Aventyl)	Yes	ND	ND	Heme	30-200	[53, 107]
Norverapamil (Metabolite of verapamil)	Yes	0.30	10.3	Heme	100-350	[137]
Oleandomycin (Amimycin)	No	ND	ND	Heme	300-1,500	[138]
Oleuropein (Olive products)	ND	0.09	22.2	ND	ND	[139]
Phenelzine (Nardil)	Yes	ND	ND	Heme	5-30	[114]
Pioglitazone (Actos)	Yes	0.0112	10.4	ND	500-1,500	[52]
Prazosin (Minipress)	Yes	ND	ND	Protein	5-60	[47]
Propoxyphene (Darvon)	Yes	0.41	1.4	Heme	50-100	[53]
Raloxifene (Evista)	Yes	0.16	9.9	Protein	0.5-1.36	[140-143]
Resveratrol (Red wine)	Yes	0.20	20	Protein	ND	[122]
Ritonavir (Norvir)	Yes	0.078	0.07	Heme	7,600-14,800	[54, 108, 144]
Rosiglitazone (Avanda)	Yes	0.0195	11.9	ND	76-598	[52]
Roxithromycin (Roxar)	No	ND	ND	Heme	800-2,500	[107]
Rutaecarpine (Evodia fruit)	Yes	0.39	107.7	Heme	ND	[132]
R-Verapamil	Yes	0.39	6.5	Heme	100-350	[53]
Saquinavir (Invirase, fortovase)	Yes	0.26	0.65	Protein	2,300-3,500	[54]
Selegiline (Zelapar)	Yes	ND	ND	Heme	3.3-4.5	[114]
Sertraline (Zoloft)	Yes	ND	ND	Protein	20-150	[47]
Silybin (Milk thistle, <i>Silybum marianum</i>)	Yes	0.06	32	ND	ND	[145]
SKF-525A	Yes	0.25	0.4	ND	Heme	[53]
SN-38 (Active metabolite of irinotecan)	No	0.10	26	Protein	30-60	[127]
S-Verapamil	Yes	0.64	3.0	Heme	100-350	[53]
Tabimorelin (NN703)	ND	0.08	4.7	ND	ND	[146]
Tadalafil (Cialis)	Yes	0.21	12	ND	250-390	[147]
Tamoxifen (Soltamox, Nolvadex)	Yes	0.051	0.2	Heme	71-183	[56]
ThioTEPA (Thioplex)	Yes	0.35	300	ND	1,300-1,850	[148]
Tiamulin (Denagard)	Yes	ND	ND	Heme	ND	[149]
Tranylcypromine (Parnate)	Yes	ND	ND	Heme	10-200	[114]
Troglitazone (Rezulin, withdrawn from the market in 2000)	Yes	0.0335	5.0	ND	920-2,820	[52]
Troleandomycin (TAO)	No	0.15	0.18	Heme	1,000-5,000	[71-73, 122, 141]
Verapamil (Calan, Isoptin)	Yes	0.09	1.7	Heme	125-400	[118, 120]

K_{I} = the half-maximal rate of inactivation; k_{inact} = the maximal rate constant of inactivation at saturation; ND = Not determined.

tion on the interaction of the compound and the enzyme, i.e., the nature of the reactive intermediate formed, the efficiency of the inactivation process, and amino acid residues located within the enzyme active site [65]. For some CYP3A4 inactivators, the reactive MIs have been largely identified. Diltiazem, nicardipine and verapamil (all calcium channel blockers) contain an amine functional group and undergo *N*-dealkylation, resulting in MIs [66, 67]. Macrolides such as erythromycin, clarithromycin, troleandomycin,

and oleandomycin with 14-membered rings can be metabolized by CYP3A4 to form reactive nitrosoalkanes via *N*-demethylation which interact with CYP to result in MI complex [68-77]. Ethynyl estradiol, the major constituent of many oral contraceptives, was metabolized by CYP3A4 to one major metabolite, 2-hydroxyethynylestradiol, and at least three additional catechol metabolites [78, 79], which are believed to inactivate CYP3A4 [78]. However, for most known CYP3A4 inactivators, their reactive MIs,

and bioactivation and inactivation pathways are largely unknown. The CYP3A4-mediated metabolism of several protease inhibitors including amprenavir, nelfinavir, and ritonavir results in unknown reactive metabolites which then inactivate CYP3A4 [5]. Midazolam is a potent CYP3A4 inactivator and such enzyme inactivation is suggested to be related to the 1'-hydroxylation metabolic pathway [80]. Additionally, several antiprogesterins (e.g. mifepristone, lilepristone and onapristone) are suggested to be oxidized by CYP3A4 to reactive nitroso species that complex the heme of the enzyme [81], thereby inactivating it.

Mechanisms of CYP inhibition by a drug can be divided into three categories: reversible, quasi-irreversible, and irreversible [82]. Quasi-irreversible and irreversible inhibitors require at least one cycle of the CYP catalytic process, and are thus signified by both NADPH- and time-dependent inhibition. These catalytic processes result in reactive metabolites that leads to chemical modification of the heme, the protein, or both as a result of covalent binding of modified heme to the protein [83, 84]. Covalent labeling of the apoCYPs has been shown to be the mechanism for inactivation of CYPs by terminal acetylenes such as 1-ethynylpyrene, 2-ethynyl-naphthalene, and some other polycyclic arylacetylenes, furan-containing compounds such as 8-methoxypsoralen, coriandrin, and bergamottin, and sulfur-containing and halogenated compounds such as parathion and chloramphenicol [5]. CYP3A4 inactivators such as delavirdine, ethynyl estradiol, and midazolam possibly bind covalently to the CYP apoprotein and inactivate it [5]. Midazolam is found to be a mechanism-based CYP3A4 inhibitor [80]. The reactive intermediates of acetylenic compounds formed by several CYPs have been known to alkylate the prosthetic heme group as well as to bind covalently to the protein [84, 85]. Whether the inactivation was due to alkylation of prosthetic heme or to modification of the apoprotein was determined by the site of addition of the oxygen to either the internal or terminal carbon of the carbon-carbon triple bond, respectively. However, the factors determining to which carbon of the triple bond the oxygen is transferred, and whether heme or protein modification or both occur, are not clear. An inactivation mechanism involving both heme destruction and covalent binding of inactivator to apoprotein has been observed with bergamottin for CYP3A4 and 2B6, *t*-butyl acetylene for CYP2E1, and ethynyl estradiol for CYP3A4 and 3A5 [4, 85].

Mechanism-based inhibition of CYP3A4 is irreversible based on its definition, and there are three different mechanisms by which the reactive intermediate is able to inactivate the CYP: covalent adduction to an amino acid residue within the enzyme active site, arylation or alkylation of the prosthetic heme moiety, and destruction of the heme group, leading to heme-derived products that form cross-links with the CYP apoprotein. However, such inactivation can be reversible. Dexter and Hager [86] reported for the first time the transient heme *N*-alkylation of the enzyme chloroperoxidase by terminal alkenes and alkynes. Chloroperoxidase was inactivated in a CYP-type reaction involving the mechanism-based formation of *N*-alkylporphyrins during the oxidation of allylbenzene and 1-hexyne. These structurally distinct compounds inactivated chloroperoxidase in a time- and concentration-dependent manner with losses in the enzymatic activity corresponding to losses in the native heme and the formation of *N*-alkyl heme adducts. The inactivated chloroperoxidase generated in these reactions then underwent a spontaneous loss of the heme adducts (as observed by electrospray mass spectrometry analysis) with a restoration of enzymatic activity and native heme [86]. While characterizing small *tert*-butyl acetylenes as mechanism-based inactivators of CYP2E1 and 2B4, Hollenberg and coworkers [87] observed the reversible inactivation of an acetylene-inactivated T303A mutant of CYP2E1. The reversibility was associated with the structure of the inactivator and the positioning of conserved amino acid residues, threonine 303 (alanine in the mutant) and glutamate 302, in the enzyme active site [88]. Reversibility was also observed with both wild-type CYP2B4 and the T302A

mutant of 2B4, although this inactivation and reversibility did not seem to depend on the T302 residue [87-89]. Enzyme active site architecture and the location and distance of critical amino acids residues from the inactivator itself seem to influence the mechanism of reversibility. In addition, the size and chemical nature of the compound will determine whether it is an irreversible or a reversible mechanism-based inactivator. The observation that mechanism-based inactivators can fulfill all the criteria set forth by Silverman [90] and yet can inactivate in a reversible manner is interesting while its clinical significance is unknown.

INDUCTION OF CYP3A4 BY DRUGS

Drugs as Inducers of CYP3A4

Inducers are drugs that increase metabolic activity by increasing the synthesis of the CYP enzymes involved. Due to the transcriptional processes involved this takes longer than inhibition.

Compared to substrates, a much smaller group of drugs behave as CYP3A4 inducers. The most potent inducers of CYP3A4 are rifampin and rifabutin, whose coadministration can reduce a drug's plasma concentration 20- to 40-fold, effectively negating drug efficacy [16]. Commonly used anticonvulsants such as carbamazepine and, to a lesser extent, phenytoin, primidone, and phenobarbital, also can significantly increase CYP3A4 activity. The antimycotic clotrimazole and HMG-CoA reductase inhibitors (statins) are also inducers of CYP3A4. Paclitaxel, cyclophosphamide and ifosfamide are also CYP3A4 inducers.

Drugs that induce CYP3A4 and other CYPs are structurally diverse, however all have one major similarity, lipophilicity. Inducers of CYP3A4 and other CYPs can be separated into five classes: a) archetypical - phenobarbital-like inducers (e.g. phenobarbital, and phenytoin); b) polycyclic aromatic hydrocarbon-like inducers (e.g. cigarette smoke, and omeprazole); c) pregnenolone 16 α carbonitrile & glucocorticoid type inducers (e.g. dexamethasone, rifampin, and erythromycin); d) ethanol-like (e.g. ethanol, and isoniazid); and e) *peroxisome proliferators-type* (e.g. clofibrate, and phthalates used in plasticizers).

In principle, there are three main mechanisms for CYP3A4 induction: a) increased anabolism of the enzyme(s) by upregulation of gene expression; b) increased anabolism through stabilization of mRNA molecules; and c) decreased rate of catabolism (degradation of the protein) (e.g. post-translational stabilization). Unlike hepatic enzyme inhibition, induction takes time (hours or days) and is a function of chronic exposure.

Many known substrates of CYP3A4 also play a role in the induction of the CYP3A4 enzyme. This regulation is mainly transcriptional (e.g. dexamethasone and rifampicin), however, this increased enzyme expression may also be achieved via mRNA stabilization (e.g. erythromycin) [91]. Modulators belonging from various chemical groups such as drugs, steroids and herbal sources have somewhat of an impact on the induction of the highly inducible CYP3A4 enzyme. An increase in the levels of enzyme is due to the fact that CYP3A4 induction is both time-dependent and concentration-dependent [92]. The induction of the CYP3A4 enzyme has been found to cause significant decrease in pharmacological potency of various drugs. For example, studies looking at CYP3A4 inducers such as rifampicin, carbamazepine and phenytoin, showed that these drugs had the ability to reduce the plasma concentration of many drugs which may lead to the diminishment of the efficacy.

Many known *in vitro* inhibitors of CYP proteins actually serve to induce those proteins when administered *in vivo*. For example, clotrimazole and other imidazoles, SKF 525-A, chlorpromazine and metyrapone, all of which are inhibitors of CYP2B and CYP3A *in vitro*, induce these proteins *in vivo* [93]. Similarly, troleandomycin, a specific inhibitor of CYP3A, highly induces this protein *in vivo* [94].

Some drugs can induce their own metabolism mediated by CYP3A4, including rifampin, cyclophosphamide, ifosfamide, spiro lactone, and phenytoin Table 3. This is called auto-induction, which results in an increase in clearance and shortened $t_{1/2\beta}$ values following repeated drug administration. Auto-induction of cyclophosphamide and ifosfamide metabolism has been observed within 12 to 24 hr after the start of treatment. Auto-induction is typically observed between day 1 to day 5, but metabolic rates are always reset to initial base level at the start of a consecutive course 3 weeks later [95].

Table 3. Examples of Drugs that Induce their Own Metabolism

Drug	Example
Analgesics	Phenylbutazone
Antibiotics	Rifampicin
Anticancer drugs	Cyclophosphamide, ifosfamide
Anticonvulsants	Phenytoin, carbamazepine
Antimalarials	Quinine
Diuretics	Spiro lactone
Sedatives	Barbitone

Role of Nuclear Receptors in the Induction of CYP3A4

Induction of CYP3A4 appears to occur through an expansion of the number of hepatocytes expressing the enzyme as opposed to an increase in concentration of the enzyme within cells [96]. The cell specific expression of CYP3A4 has shown that in the liver, only those cells closest to the central veins within hepatic lobules demonstrate basal expression. However with the administration of an inducing substance the 1-2 cell wide rings expand outwards towards the portal triad to become as much as 10 cells deep around the central vein [96].

Studies have indicated that several nuclear receptors play an important role in the induction of CYP3A4. Nuclear receptors are ligand-inducible proteins found within a cell and are responsible for sensing presence of hormones or other molecules [97]. The main role of these receptors is to mediate the transcriptional response in target cells to hormones such as sex steroids (progestins, estrogens, and androgens), adrenal steroids (glucocorticoids and mineralocorticoids), vitamin D₃, and thyroid and retinoid (9-*cis* and all-*trans*) hormones, as well as various other metabolic ligands [97]. When the ligands bind to the nuclear receptors, they cause a conformational change and activate the receptor. The nuclear receptors then have the ability to bind directly to DNA segments and regulate expression of adjacent genes such as *CYP3A4*, *CYP2B6* and *MDR1*.

The CYP3A4 inducers bind to the pregnane X receptor (PXR), the constitutively androstane receptor (CAR), HNF4 α , glucocorticoid receptor (GR), or vitamin D receptor. Like CYP3A4, PXR is also highly expressed in both the intestine and liver. PXR is activated by a large number of endogenous and exogenous chemicals including steroids, antibiotics, antimycotics, bile acids, and hyperforin, a constituent of the herbal antidepressant St. John's Wort. The activated PXR complex forms a heterodimer with the retinoid X receptor (RXR) which binds to the PXR response element region, called xenobiotic responsive enhancer module, of the *CYP3A4* gene. The xenobiotic responsive enhancer module is a regulatory region of the *CYP3A4* gene, and binding causes a cooperative interaction with proximal promoter regions of the gene, resulting in increased transcription and expression of CYP3A4. Upon ligand binding, the PXR transactivates everted repeat with a 6 bp spacer

element (ER6) upstream of the *CYP* genes [92]. The binding of PXR/RXR to ER6 causes coactivator proteins such as steroid receptor coactivator-1 and transcriptional activation of respective gene to follow. In addition to *CYP3A4*, PXR up-regulates the expression of phase II conjugating enzymes such as glutathione *S*-transferases and transporting proteins such as P-gp.

The induction of CYP3A4 occurs against the background of constitutive expression. This basal expression is regulated by CAR. This nuclear receptor is associated with a co-activator called the steroid receptor co-activator 1. The CAR/SRC-1 complex dimerises with the RXR before binding with the promoter region of *CYP3A4*. It is in the 5'-UTR of the gene where the CAR/RXR complex binds, to a sequence containing two copies of the nuclear receptor organized as ER6, and controls the expression of pre-mRNA. Induction of CYP3A4 can occur via up-regulation of this process following an increase in the affinity of complex for the ER6 element.

It appears that HNF4 α is critically involved in the PXR- and CAR-mediated transcriptional activation of CYP3A4. A specific cis-acting element in the *CYP3A4* gene enhancer confers HNF4 α binding and thereby activates *PXR* and *CAR* genes [98]. Although unusual, *CYP3A4* is highly induced by glucocorticoids such as hydrocortisone and dexamethasone, despite the enzyme lack of the consensus GR response element. Recently, glucocorticoids have been found to act as ligands for PXR, hence, further suggesting that the induction of *CYP3A4* by glucocorticoids occurs primarily through PXR activation [99]. Both the PXR and RXR α are induced by GR. Thus, activation of GR by glucocorticoids, such as dexamethasone will lead to the induction of PXR/RXR and an immediate increase in CYP3A4 induction by endogenous and exogenous compounds.

CONCLUSIONS AND FUTURE DIRECTIONS

Drug-drug interactions remain an important issue in clinical practice and drug development [100]. Adverse pharmacokinetic drug interactions may occur when drugs that are substrates, inducers and/or inhibitors of the same metabolizing enzymes are co-administered, potentially altering the expected rate of metabolism of one or both compounds. The clinical consequences can range from a lack of therapeutic efficacy to severe toxicity and, in extreme cases, fatality. With our recently advanced knowledge of the human CYP3A4 and its critical role in drug metabolism, more systematic approaches to the study of drug interactions have evolved. Previous to this knowledge, studies of drug-drug interactions for new drugs were carried out empirically; combinations of drugs chosen for investigation of drug-drug interactions were selected based on the potential for alteration in the pharmacokinetics or dynamics of a narrow therapeutic index drug (e.g., digoxin, warfarin, theophylline, and phenytoin) or whether there was a high likelihood that the new compound would be frequently coprescribed with another agent for a given condition [100].

However, with an increased understanding of drug-metabolizing enzymes and their roles in the metabolism of specific drugs, a more mechanistic approach to assessing drug-drug interactions can be taken. The results of clinical drug-drug interaction studies with one drug can be extrapolated to other drugs that are cleared by the same enzyme [101]. *In vitro* drug-drug interaction data are necessary for devising mechanistically based clinical drug-drug interaction study strategies. The effects of new drugs on well characterized drug metabolism reactions known to be specific for various human drug-metabolizing enzymes are routinely examined using *in vitro* approaches.

The relative selectivity of some currently available drugs to modulate CYP3A4 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 [16]. Drugs for which CYP3A4-mediated metabolism importantly contributes to the overall clearance process would be more likely to be affected

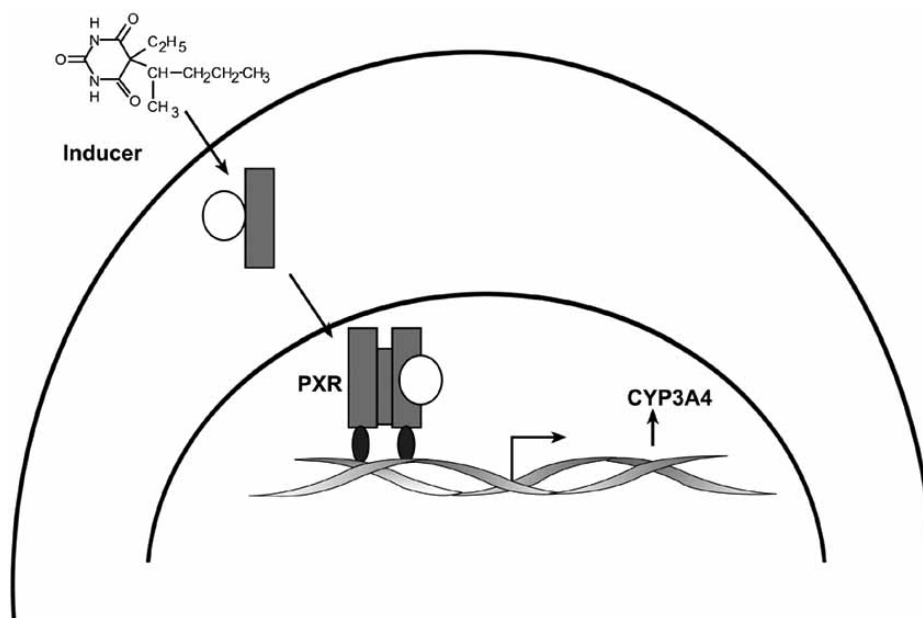


Fig. (1). Induction of CYP3A4 by inducers via activation of pregnane X receptor (PXR). The ligand enters the cells and binds PXR in cytosol to form a complex which is translocated into the nucleus. Activation of the PXR by structurally diverse ligands results in the binding of the PXR-RXR (retinoid X receptor) heterodimer to responsive elements in target genes such as *CYP3A4*.

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 CYP3A4 substrate. That is, drugs that modulate CYP3A4 activity in the nanomolar to low micromolar range are more likely to result in a drug interaction than those requiring high micromolar levels. 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.

The broad substrate selectivity makes CYP3A4 susceptible to relatively frequent reversible or irreversible (mechanism-based) inhibition by a wide variety of drugs. Irreversible inhibition of CYP3A4 due to enzyme inactivation or complexation occurs when some therapeutic drugs are converted by CYP enzymes to reactive metabolites capable of covalently binding to CYP3A4 protein or heme moiety. Mechanism-based inhibition of CYP3A4 is characterized by NADPH-, time- and concentration-dependent enzyme inactivation. A number of therapeutic drugs have been identified as mechanism-based CYP3A4 inhibitors.

Understanding the role of nuclear receptors in regulating expression of CYP3A4 of the P450 family offers an exciting promise to the future, as it may be possible to further define the physiologic function and interindividual differences of CYP3A4 in patient health and different disease states. The activity of CYP3A4 enzyme is highly variable from person to person, therefore, this explains the variability in sensitivity to drug interactions involving CYP3A4 inhibition or induction. A small number of receptors have been investigated and found to play a role in CYP3A4 induction by transcriptional activation of the enzyme. CYP3A4 induction is a complex regulatory network and requires more mechanistic investigations to predict or prevent drug-drug interactions. It is important to elucidate all the mechanisms involved in the translocation of recep-

tors such as PXR and CAR. Furthermore, extra assessment of PXR as a drug target may lead to drugs that improve the pharmacokinetics of other drugs by mechanisms such as the inhibition of PXR action, chemically modifying the drugs so that they retain their pharmacological properties, but lack the ability to activate PXR mediated gene expression. This may ultimately provide drug regimens with fewer and less severe side effects and possibly enhanced beneficial activity.

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ABBREVIATIONS

CAR	=	Constitutively androstane receptor
CYP	=	Cytochrome P450
E_{inact}	=	Inactivation efficiency
GR	=	Glucocorticoid receptor
HMG-CoA	=	3-Hydroxy-3-methylglutaryl-coenzyme A
HNF4 α	=	Hepatocyte nuclear factor-4 α
K_i	=	Apparent inhibition constant
K_I	=	The concentration required for half-maximum inactivation
K_{inact}	=	The maximum rate of inactivation at saturation
MI	=	Metabolic intermediate
P-gp	=	P-glycoprotein
PXR	=	Pregnane X receptor
RXR	=	Retinoid X receptor
SSRI	=	Selective serotonin reuptake inhibitor
$t_{1/2\text{inact}}$	=	Inactivation half-life.

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