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Mechanism-Based Inhibition of Cytochrome P450 3A4 by Therapeutic Drugs

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Contents

Abstract . . . 279
1. Approaches to Investigating Mechanism-Based Inhibition of Cytochrome P450 (CYP) 3A4 . . . 281
   1.1 *In Vitro* Models . . . 281
   1.2 *In Silico* Models . . . 282
   1.3 *In Vivo* Models . . . 284
2. Biochemical Mechanism for Drug-Induced Inactivation of CYP3A4 . . . 284
   2.1 Formation of Reactive Metabolic Intermediates of Drugs . . . 284
   2.2 Multiple Active Sites of CYP3A4 . . . 285
   2.3 Mechanisms for the Inactivation of CYP3A4 . . . 286
3. Estimation of Inhibitory Potencies of CYP3A4 Inactivators . . . 286
   3.1 Estimation of Apparent K_I and k_{inact} . . . 286
   3.2 Reported Apparent K_I and k_{inact} . . . 287
   3.3 Relationship between K_I and k_{inact}, K_m and K_i . . . 287
4. Clinical Considerations of Drug-Induced CYP3A4 Inactivation . . . 289
   4.1 Altered Drug Pharmacokinetics and/or Pharmacodynamics due to CYP3A4 Inactivation . . . 289
   4.2 *In Vitro-In Vivo* Extrapolation Involving CYP3A4 Inactivation . . . 292
   4.3 Factors Affecting the Clinical Consequence of Mechanism-Based Inhibition of CYP3A4 . . . 293
      4.3.1 Drug-Related Factors . . . 293
      4.3.2 Patient-Related Factors . . . 294
5. Conclusion and Future Perspectives . . . 294

Abstract

Consistent with its highest abundance in humans, cytochrome P450 (CYP) 3A is responsible for the metabolism of about 60% of currently known drugs. However, this unusual low substrate specificity also makes CYP3A4 susceptible to reversible or irreversible inhibition by a variety of drugs. Mechanism-based inhibition of CYP3A4 is characterised by nicotinamide adenine dinucleotide

phosphate hydrogen (NADPH)-, time- and concentration-dependent enzyme inactivation, occurring when some drugs are converted by CYP isoenzymes to reactive metabolites capable of irreversibly binding covalently to CYP3A4. Approaches using *in vitro*, *in silico* and *in vivo* models can be used to study CYP3A4 inactivation by drugs. Human liver microsomes are always used to estimate inactivation kinetic parameters including the concentration required for half-maximal inactivation (K_I) and the maximal rate of inactivation at saturation (k_{inact}).

Clinically important mechanism-based CYP3A4 inhibitors include antibacterials (e.g. clarithromycin, erythromycin and isoniazid), anticancer agents (e.g. tamoxifen and irinotecan), anti-HIV agents (e.g. ritonavir and delavirdine), antihypertensives (e.g. dihydralazine, verapamil and diltiazem), sex steroids and their receptor modulators (e.g. gestodene and raloxifene), and several herbal constituents (e.g. bergamottin and glabridin). Drugs inactivating CYP3A4 often possess several common moieties such as a tertiary amine function, furan ring, and acetylene function. It appears that the chemical properties of a drug critical to CYP3A4 inactivation include formation of reactive metabolites by CYP isoenzymes, preponderance of CYP inducers and P-glycoprotein (P-gp) substrate, and occurrence of clinically significant pharmacokinetic interactions with coadministered drugs.

Compared with reversible inhibition of CYP3A4, mechanism-based inhibition of CYP3A4 more frequently cause pharmacokinetic-pharmacodynamic drug-drug interactions, as the inactivated CYP3A4 has to be replaced by newly synthesised CYP3A4 protein. The resultant drug interactions may lead to adverse drug effects, including some fatal events. For example, when aforementioned CYP3A4 inhibitors are coadministered with terfenadine, cisapride or astemizole (all CYP3A4 substrates), torsades de pointes (a life-threatening ventricular arrhythmia associated with QT prolongation) may occur.

However, predicting drug-drug interactions involving CYP3A4 inactivation is difficult, since the clinical outcomes depend on a number of factors that are associated with drugs and patients. The apparent pharmacokinetic effect of a mechanism-based inhibitor of CYP3A4 would be a function of its K_I, k_{inact} and partition ratio and the zero-order synthesis rate of new or replacement enzyme. The inactivators for CYP3A4 can be inducers and P-gp substrates/inhibitors, confounding *in vitro-in vivo* extrapolation. The clinical significance of CYP3A inhibition for drug safety and efficacy warrants closer understanding of the mechanisms for each inhibitor. Furthermore, such inactivation may be exploited for therapeutic gain in certain circumstances.

The cytochrome P450 (CYP) [EC 1.14.14.1] superfamily, containing 57 genes,[1] contributes to the metabolism of a variety of xenobiotics including therapeutic drugs, carcinogens, steroids and eicosanoids.[2-6] The catalytic mechanism appears to be common to all CYP isoenzymes and involves a two-electron reduction of molecular oxygen to form a reactive oxygen species and water.[2,7] Among CYP isoenzymes, the subfamily CYP3A is responsible for the metabolism of about 60% of currently known therapeutic drugs.[2-4,8,9] The CYP3A subfamily in humans includes CYP3A4, CYP3A5, CYP3A7[4] and CYP3A43.[10] CYP3A4 is the most abundant among the isoenzymes of CYP3A subfamily in the

human liver (~40%) and metabolises more than 50% of clinically used drugs,[3,11] whereas polymorphic CYP3A5 accounts for 5–50% of total CYP3A abundance[12,13] and is present in appreciable amounts in about 25% of the adult population.[14] CYP3A7 is the primary fetal isoform and is rarely detected in adults.[15,16] *CYP3A43* gene is expressed significantly in the prostate and testis, whereas the hepatic messenger RNA (mRNA) level is only 0.2–5% of that of *CYP3A4*.[10] Thus, the two latter enzymes play a minor role in drug metabolism in adults.

The low substrate specificity makes CYP3A4 susceptible to reversible or irreversible inhibition by a variety of drugs.[17] The irreversible mechanism-based inhibition of CYP3A4 refers to the inactivation of the enzyme via the formation of metabolic intermediates (MIs) that bind tightly and irreversibly to the enzyme.[18,19] A mechanism-based inhibition of CYP3A4 should have the following features:[20,21]

- preincubation time-dependent inhibition of CYP3A4
- nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent inhibition (i.e. no inhibition without NADPH in preincubation)
- inhibitor concentration-dependent inhibition with saturation kinetics (pseudo first-order kinetics)
- substrate protection (i.e. slower inactivation rate of the enzyme in the presence of substrate)
- inhibitor protection (i.e. reduced inactivation of CYP3A4 when coincubated with CYP3A4 inhibitor or inhibitory antibody)
- uncertain prevention by exogenous nucleophiles (e.g. glutathione semicarbazide, and *N*-acetylcysteine)
- irreversible inhibition (i.e. CYP3A4 activity not recovered after gel filtration or dialysis)
- stoichiometry of inactivation (i.e. 1 : 1 stoichiometry of the inhibitor and the active site of CYP3A4).

1. Approaches to Investigating Mechanism-Based Inhibition of Cytochrome P450 (CYP) 3A4

1.1 *In Vitro* Models

Human liver microsomes, hepatocytes and complementary DNA (cDNA)-expressed enzymes are the commonly used *in vitro* systems for the investigation of mechanism-based inhibition of CYP3A4.[21] By using these *in vitro* models, the qualitative features of mechanism-based inhibition of CYP3A4, such as time-, NADPH- and concentration-dependent inhibition, can be easily determined. Experimentally, mechanisms of inhibition of inhibitors could be assessed initially by comparing their inhibitory effects obtained in the presence and absence of NADPH during a preincubation period. Important kinetic parameters for mechanism-based inhibition such as the concentration required for half-maximal inactivation (K_I), the rate constant of maximal inactivation at saturation, analogous to maximum rate of metabolism by an enzyme-mediated reaction (k_{inact}), and partition ratio (R_{max}, ratio of moles of substrate activation per mole of enzyme inactivation) can also be determined.[21]

In vitro systems (particularly microsomal systems) can be used to isolate and identify the resultant covalent metabolic intermediate-CYP3A4 complex by using gel electrophoretic and mass spectrometric techniques. The addition of glutathione and *N*-acetylcysteine facilitates the identification of reactive metabolites and characterisation of adduct formation with CYP3A4.[22] An apparent attenuation of the degree of CYP3A4 inactivation in preincubation mixtures fortified with glutathione and the identification of glutathione adducts in human liver microsomal incubations would provide further evidence implicating reactive metabolites in the inactivation of CYP3A4. Each *in vitro* model has its advantages and disadvantages, and a combination use will often provide convincing data. Liver microsomes can be studied long term and are easily manipulated and optimised, but cofactor (NADPH) is necessary for CYP3A4-catalysed reactions to replace those lost as a result of the destruction of cell

integrity. Hepatocytes provide cellular integrity with respect to enzyme architecture and are useful for determining the cytotoxicity of formed CYP3A4 adducts *in vitro*. However, the enzyme activity results obtained from hepatocytes should also be interpreted with caution, especially for quantitative comparisons, as many enzyme activities decline spontaneously during hepatocyte isolation or culture.[23] Cloned cDNA-expressed human CYP3A4 is available, and in theory supplies are unlimited.[24,25] It is worth noting that levels of enzyme expression are variable across expression systems, and (especially with regard to CYP3A) reductase and cytochrome b_5 to CYP ratios are often nonphysiological.[24,26]

A number of well characterised model substrates for CYP3A4[27-29] can be used for the study of mechanism-based inhibition of CYP3A4 *in vitro* (table I). Among these CYP3A4 substrates, benzodiazepine (midazolam and triazolam), testosterone and ciclosporin are most commonly used. These probe drugs have been used to investigate the mechanism-based inhibition of CYP3A4 *in vitro* by a variety of drugs. These compounds are readily available and suitable for experiments in microsomes. However, only some of them may be used in assays with intact cells (e.g. hepatocytes), as these substrates should be noncytotoxic and readily move across cell membranes. 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,[30] it is important to use the right probe substrate and to conduct the experiment under optimal conditions.[28]

Selective inhibitors (chemicals[124] and inhibitory antibody[125]) for CYP3A4 are also useful tools for the study of mechanism-based inhibition of CYP3A4. These chemical probes and antibody are validated and readily commercially available (see http://www.gentest.com; http://www.biotrend.com). Reversible CYP3A4 inhibitors such as ketoconazole,[22,117,119,126,127] itraconazole,[128] quinidine at high concentration (≥50 μmol/L),[22] testosterone,[129] terfenadine,[130] astemizole,[130] ciclosporin[128] and inhibitory antibody against CYP3A4[22,119,128] are useful for the demonstration of reduced covalent binding by drugs to CYP3A4 and CYP3A4 adduct formation *in vitro*. Mechanism-based inhibitors for CYP3A4 such as troleandomycin,[90,131-133] clarithromycin[90,91] and erythromycin[121,133] can be particularly useful, as they are often insensitive to the concentration of the test compound and can be used as reference CYP3A4 inactivators. Some of them such as ritonavir[119,120] have been used for the investigation of mechanism-based inhibition of CYP3A4 by drugs.

Model inducers (e.g. rifampicin [rifampin],[27,134] phenobarbital[27,135] and dexamethasone[27,134]) of CYP3A4 can be used as a valuable tool in the study of mechanism-based CYP3A4 inhibition. The nuclear pregnane X receptor, also called steroid and xenobiotic receptor or pregnane-activated receptor, was found to mediate the drug-induced expression of CYP3A4.[136-140] In *in vitro* assays for CYP3A4 induction using hepatocytes,[141-146] the catalytic activity, protein or mRNA level of CYP3A4 are enhanced and thus the formation of CYP3A4 adducts can be increased, leading to increased CYP3A4 inactivation. It should be noted that this *in vitro* approach has several limitations, such as, the remarkable interindividual donor variation in response to the CYP3A4 inducers; and cell culture conditions are also an important factor contributing to the considerable variability of CYP3A4 induction.[146] In addition, the enzyme activity of CYP3A4 obtained from hepatocytes should also be interpreted with caution, especially for quantitative comparisons, as many enzyme activities decline spontaneously during hepatocyte isolation or culture.[23]

1.2 *In Silico* Models

There is increasing use of *in silico* methods to study mechanism-based inhibition of CYP isoenzymes by therapeutic drugs. The major *in silico* methods for this purpose include simple rule-based modelling, structure-activity relationships, three-dimensional quantitative structure-activity relationships, pharmacophores and homology modelling.[147-152] All of these represent useful tools for understanding inactivation reactions (formation of

Table I. Some model substrates of cytochrome P450 (CYP) 3A4 used for the study of mechanism-based inhibition of CYP3A4

Substrate	Metabolic reaction	Quantitation method	Examples of *in vitro* application	*In vivo* application	Examples of *in vivo* application
Alfentanil	*N*-dealkylation[31-33]	LC-MS and GC-MS[31,34,35]	Troleandomycin,[31] erythromycin[31]	Yes[34,36]	Erythromycin,[37] verapamil,[36] diltiazem[36]
Alprazolam	1′-hydroxylation [38]	HPLC[39]	Troleandomycin[38]	Yes[40]	Fluoxetine,[41,42] erythromycin[40]
Clarithromycin	*N*-demethylation[43]	HPLC[44]	Troleandomycin[43]	Yes[45,46]	Ciclosporin,[46] carbamazepine[45]
Cortisol	6β-hydroxylation[47-49]	HPLC[47,50,51]	Gestodene,[47] ciclosporin[47]	Yes[51,52]	Gestodene,[53] desogestrel[53]
Ciclosporin	Oxidation[54-57]	HPLC[55,58]	Nicardipine,[58] nifedipine,[57,58] verapamil,[58] diltiazem[58]	Yes[59]	Clarithromycin,[60] erythromycin[61]
Dapsone	*N*-hydroxylation[62,63]	HPLC[64]	Gestodene,[62] troleandomycin[62]	Yes[65]	Ethinylestradiol[66]
Erythromycin	*N*-demethylation[67]	Breath test,[68] spectrophotometry[69]	Troleandomycin[69]	Yes[70,71]	Ciclosporin,[72] troleandomycin,[70] delavirdine [73]
Ethinylestradiol	2-hydroxylation[74,75]	HPLC[74]	Gestodene[76]	Yes[53]	Ritonavir,[77] gestodene,[53] desogestrel[53]
Lidocaine (lignocaine)	*N*-deethylation and 3-hydroxylation[78-80]	HPLC [81]	Troleandomycin[78]	Yes[82-85]	Erythromycin[86]
Midazolam	1′-hydroxylation[87]	HPLC[88]	Isoniazid,[89] clarithromycin,[90,91] diltiazem,[92] fluoxetine[91]	Yes[93]	Clarithromycin[94]
Nifedipine	Oxidation[30,67,95]	HPLC[96]	Gestodene[76]	Yes[97]	Oral contraceptive steroids,[98] ritonavir[99]
Terfenadine	Hydroxylation[100-102]	HPLC[103]	Clarithromycin,[54] gestodene,[102] ritonavir,[104] fluoxetine[105]	No	
Testosterone	6β-hydroxylation[67]	HPLC,[106,107] GC-MS[108]	Irinotecan,[109] tamoxifen,[110] dihydralazine,[111,112] diltiazem,[95] mifepristone,[113] verapamil,[95] nicardipine,[95] raloxifene,[22] bergamottin,[114] erythromycin[44]	No	
Triazolam	1′-hydroxylation[6,28,115]	HPLC[116]	Delavirdine,[117] nelfinavir,[118] ritonavir,[118-120] erythromycin,[121] fluoxetine[115]	Yes[115,122]	Erythromycin,[122] clarithromycin,[122] diltiazem[123]

GC-MS = gas chromatography mass spectrometry; **HPLC** = high-performance liquid chromatography; **LC-MS** = liquid chromatography mass spectrometry.

metabolic intermediate-CYP3A4 complex), structural requirements for inactivators, providing insight into the active site of CYP3A4, optimising the design of drugs and selective inhibitors of CYP3A4, and predicting possible metabolic drug-drug interactions involving mechanism-based inactivation of CYP3A4.[19,150,153,154] The resulting data based on *in silico* approaches may be of clinical significance. For example, knowledge of the substrate specificity and regulation of the CYP is essential, as this will provide information on the possible drug-drug interaction.

An example of application of an *in silico* model to understanding CYP3A4 inactivation is the structure-activity relationship analysis of the 17α-acetylenic steroids, which indicates that the delta 15 double bond is critical, but is not in itself sufficient for the inactivation process, possibly owing to attack of CYP on the substituted acetylenic carbon leading to porphyrin *N*-alkylation.[76] In fact, a variety of different mechanism-based CYP inactivators have proven to be useful in identifying active site amino acid residues involved in substrate binding and catalysis.[19] Labelled peptides isolated from the inactivated proteins can be analysed by *N*-terminal amino acid sequencing in conjunction with mass spectrometric techniques to determine the active sites of covalent modification and amino acids involved.[19]

1.3 *In Vivo* Models

Although *in vitro* and *in silico* models may provide a quick screening tool for mechanism-based CYP inhibition, the relative simplicity of *in vitro* and *in silico* approaches provide limited information without considering many important physiological factors. Animal models (mouse and rat) have been extensively used to study mechanism-based CYP3A inhibition, providing important information on CYP inactivation. However, interspecies variations in the substrate specificity, catalytic features and amino acid sequences of CYP isoenzymes may cause difficulty in extrapolating animal data to humans.[155-157] Therefore, *in vivo* human studies are usually necessary to provide evidence of their clinical importance.

Most substrates listed in table I can be used *in vivo* in humans to evaluate inhibition and induction of CYP3A.[25,158] Midazolam is considered as one of the best *in vivo* probe drugs for the study of CYP3A4 activity,[17] for several reasons: it can be administrated both orally and intravenously, which can provide a measure of CYP3A4 activity relative to intestinal and hepatic metabolism, respectively; midazolam is not a substrate of P-glycoprotein (P-gp);[159] and midazolam metabolism at lower concentrations exhibits a regioselective difference which can be used to discriminate among individuals with or without CYP3A5, as CYP3A5 has a much higher 1′-OH : 4-OH ratio of midazolam metabolism than CYP3A4.[12,87] Several classical inducers such as rifampicin,[160-162] ritonavir[77,163] and phenobarbital can be used to study induction of hepatic and intestinal expression of CYP3A4 *in vivo* in humans.

2. Biochemical Mechanism for Drug-Induced Inactivation of CYP3A4

2.1 Formation of Reactive Metabolic Intermediates of Drugs

The first step of inactivating CYP3A4 by drugs is the bioactivation of drugs to reactive MIs, which is often via various CYP isoenzymes. The diversity of CYP isoforms means that a wide range of drugs can be bioactivated by either a single CYP or multiple CYPs. It is well known that drugs which contain several common moieties such as a tertiary amine function,[164-167] furan ring[114,168] and acetylene function[76,113] are metabolised by CYP isoenzymes and bind to the same enzyme covalently to form a CYP-metabolite complex and thereby inactivate the enzyme. Investigations of mechanism-based inactivation can lead to detailed information 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.[19]

For some CYP3A4 inactivators, the reactive MIs have been largely identified. Diltiazem, nicardipine and verapamil (all calcium channel antagonists)

contain an amine functional group and undergo *N*-dealkylation, resulting in MIs.[169,170] Macrolides such as erythromycin, clarithromycin, troleandomycin and oleandomycin with 14-membered rings can be metabolised by CYP3A4 to form reactive nitrosoalkanes via *N*-demethylation which interact with CYP to result in MI complex.[90,131,132,164,171-176] Ethinylestradiol, the major constituent of many oral contraceptives, is metabolised by CYP3A4 to one major metabolite, 2-hydroxyethinylestradiol, and at least three additional catechol metabolites,[129,177] which are believed to inactivate CYP3A4.[129] However, for most known CYP3A4 inactivators, their reactive MIs and bioactivation pathways are largely unknown. The CYP3A4-mediated metabolism of several protease inhibitors (amprenavir,[120] L 754394,[147,178,179] nelfinavir[180] and ritonavir[119,120]) results in unknown reactive metabolites which then inactivate CYP3A4. Midazolam is a potent CYP3A4 inactivator and such enzyme inactivation is suggested to be related to the 1′-hydroxylation metabolic pathway.[181] Several antiprogestogens (e.g. mifepristone, lilopristone and onapristone) are suggested to be oxidised by CYP3A4 to reactive nitroso species that complex the haem of the enzyme,[182] thereby inactivating it.

2.2 Multiple Active Sites of CYP3A4

CYP3A4 is known to metabolise a large variety of compounds varying in molecular weight (MW) from lidocaine (MW = 234d) to ciclosporin (MW = 1203d).[2,183] It is believed that CYP3A4 has a spacious hydrophobic active site capable of accommodating a diverse range of compounds. As a result, CYP3A4 binding interactions are dominated by the lipophilicity of the drug molecule involved, as indicated by a significant correlation between the octanol partition coefficient (log $D_{7.4}$) and apparent Michaelis-Menten constant (K_m) for CYP3A4.[184,185] Modelling and amino acid alignment studies have proposed that these amino acids occupy positions in the F-helix, remote from the haem iron of the CYP3A4 active site.[186] Mutagenesis studies have demonstrated that replacement of leucine 210 or leucine 211 with alanine reduced, but did not abolish, the activation effect of α-naphthoflavone on progesterone and testosterone hydroxylase activities.[187]

In deference to the large CYP3A4 active site, substrate-dependent drug-drug interactions and the atypical pharmacokinetics associated with certain CYP3A4-mediated oxidations, there is strong evidence in support of the hypothesis that there are multiple substrate binding sites in CYP3A4.[188-191] The evidence is mainly from studies using methods such as site-directed mutagenesis, homology modelling and functional analysis using substrates such as progesterone, testosterone and midazolam.[30,181,192] The most distant amino acids from the catalytic centre of CYP3A4 that have a role in substrate binding are leucine 210, leucine 211 and aspartic acid 214.[187,193]

CYP3A4 exhibits unusual substrate kinetics, including activation, autoactivation, partial inhibition and substrate inhibition often observed.[30,188-190,194] Several hypotheses involving two-site or three-site models as well as the existence of functionally distinct conformers have been proposed to explain the atypical CYP3A4 pharmacokinetics.[188-190] There is a 6-fold difference in the K_m values between triazolam α-hydroxylation and 4-hydroxylation[195] and two very distinct K_m values for the two hydroxylation pathways of midazolam,[181,196,197] suggesting the existence of two binding sites in CYP3A4. The differential stimulation/inhibition by α-naphthoflavone and testosterone[30,196] and observation of two distinct apparent inhibition constant (K_i) values for inhibition of 1′-OH and 4-OH midazolam formation by the peptide YPFP-NH_2 have provided additional evidence to support two binding sites of CYP3A4.[197] However, the possibility of the substrate binding at a single site but in two different orientations cannot be ruled out. Furthermore, molecular modelling[193] and mechanistic studies[190,197] suggest that the complex effects observed with select CYP3A4 substrates may be attributable to the binding of multiple substrates within the active site of the enzyme.

2.3 Mechanisms for the Inactivation of CYP3A4

Mechanisms of CYP inhibition by a drug can be divided into three categories: reversible, quasi-irreversible and irreversible.[198] 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 lead to chemical modification of the haem, the protein, or both as a result of covalent binding of modified haem to the protein.[21,199,200] The detailed mechanism of inactivation may be elucidated by a number of analytical and proteomic techniques, such as mass spectrometry and homology modelling. For example, mifepristone was shown to be covalently bound to the apoCYP3A4 by gel electrophoresis,[113,114] while both haem adduction and fragmentation are ruled out, as the Soret absorption of the inactivated CYP3A4 did not decrease when compared with the controls.[201] A similar result has been observed with bergamottin.[114,202,203]

Covalent labelling of the apoCYP isoforms has been shown to be the mechanism for inactivation of CYPs by terminal acetylenes such as 1-ethinylpyrene, 2-ethinylnaphthalene, and some other polycyclic arylacetylenes,[204-206] furan-containing compounds such as methoxsalen, coriandrin and bergamottin,[114,202,207] and sulphur-containing and halogenated compounds such as parathion and chloramphenicol.[208,209] CYP3A4 inactivators such as delavirdine,[126] L 754394,[147] ethinylestradiol[129] and midazolam[181,190] possibly bind covalently to the CYP apoprotein and inactivate it. Certain CYP3A4 inactivators such as macrolides (e.g. erythromycin[210]), glabridin[211] and nelfinavir[180] bind the haem and inactivate the enzyme.

The reactive intermediates of acetylenic compounds formed by several CYP isoenzymes have been known to alkylate the prosthetic haem group as well as to bind covalently to the protein.[200] Studies with CYP2B1 demonstrated that 2-ethinylnaphthalene predominantly inactivates CYP2B1 through modification of the apoprotein, whereas phenylacetylene inactivates CYP2B1 via *N*-alkylation of haem.[205,212] In the case of ethinylestradiol, the enzyme inactivation led to the destruction of approximately half the haem with the concomitant generation of modified haem and ethinylestradiol-labelled haem fragments and produced covalently radiolabelled CYP3A4 apoprotein.[114,129,199,213-215] The chemical structure(s) and the nature of adduction of these modified species remain to be established. Ethinylestradiol can modify both the haem and the apoprotein of CYP3A4, whereas it modified only the apoprotein in CYP2B1 and CYP2B6.[216] These results suggest that the metabolic activation of a single ethinyl compound can result in different reactivities towards haem versus apoprotein with different CYP isoenzymes.

3. Estimation of Inhibitory Potencies of CYP3A4 Inactivators

3.1 Estimation of Apparent K_I and k_{inact}

To get K_I and k_{inact}, the logarithm of the remaining enzymatic activity is plotted against the preincubation time. The apparent inactivation rate constant (k_{obs}) is determined from the slope of the initial linear phase. The value of k_{obs} is then plotted against the inhibitor concentration, and the parameters (k_{inact} and K_I) are obtained by the nonlinear least-squares method using the following equation (equation 1):[21,217]

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \quad \text{(Eq. 1)}$$

where k_{obs}, k_{inact}, K_I represent the apparent inactivation rate constant of the enzyme at the initial concentration of inhibitor [*I*], the maximum inactivation rate constant, and the apparent inactivation constant between the enzyme and inhibitor, respectively. Furthermore, the inactivation efficiency (E_{inact}) can be calculated by equation 2:

$$E_{inact} = \frac{k_{inact}}{K_I + [I]} \quad \text{(Eq. 2)}$$

The inactivation half-life ($t_{1/2inact}$) can be calculated by equation 3:

$$t_{½\text{inact}} = \frac{0.693}{k_{\text{inact}}}$$

(Eq. 3)

In addition, R_{max} can be estimated by equation 4:

$$r = R_{max} \times e^{(-k_{obs} \times t)}$$

(Eq. 4)

where r and R_{max} represent the nanomoles of reactive product formed per nanomole CYP at a particular time (t) and at infinity, respectively. It should be noted that partition ratio (R_{max}) is also an important potency parameter for CYP3A4 inactivation. The partition ratio of the most powerful mechanism-based inhibitor is zero (i.e. every turnover produces inactivated enzyme). CYP3A4 inactivators with a low partition ratio (e.g. 1.4 for L 754394[147,178]) are often potent mechanism-based inhibitors, while those with a high partition ratio (e.g. 50 for ethinylestradiol[129] and 41 for delavirdine[126]) have low inhibition of CYP3A4.

3.2 Reported Apparent K_I and k_{inact}

A number of drugs with different efficacy and structure have been reported to be mechanism-based inhibitors of CYP3A4 (table II). Most of these drugs are reported to be CYP substrates and reversible inhibitors of CYP3A4. These drugs can be classified into four groups based on their K_I values, reflecting their inhibitory potency: drugs with K_I ≤1.0 μmol/L (e.g. ritonavir,[119,120] troleandomycin[90,131-133] and tamoxifen[110]); drugs with K_I 1.1–5.0 μmol/L (e.g. amprenavir,[120] verapamil,[95,218] diltiazem[91,92,95,218] and mibefradil[219]); drugs with K_I 5.1–10.0 μmol/L (e.g. fluoxetine,[91] midazolam[181] and clarithromycin[90,91]); and drugs with K_I >10 μmol/L (e.g. irinotecan,[109] gestodene,[76] isoniazid[89] and erythromycin[220-222]). In addition, the metabolites of some drugs are also mechanism-based inhibitors of CYP3A4 (e.g. *N*-desmethyl diltiazem,[91] *N*-desmethyltamoxifen,[110] SN 38[109] and 6′, 7′-dihydroxybergamottin[223]). Among these drugs, both K 11777[128] and ritonavir[119,120] are the most potent CYP3A4 inactivators, with K_I of 0.06 and 0.07 μmol/L, respectively; whereas isoniazid[89] is a weak CYP3A4 inactivator with a K_I of 228 μmol/L.

Drugs in table II can also be classified in four groups based on their k_{inact} values: drugs with k_{inact} ≤0.06 min^{-1} (i.e. $t_{½inact}$ ≥11.6 min) [e.g. fluoxetine,[91] dihydralazine,[111] tamoxifen[110] and irinotecan[109]]; drugs with k_{inact} 0.061–0.10 min^{-1} (i.e. $t_{½inact}$ 6.93–11.5 min) [e.g. clarithromycin,[90,91] amprenavir[120] and ritonavir[119,120]]; drugs with k_{inact} 0.11–0.29 min^{-1} (i.e. $t_{½inact}$ 2.39–6.30 min) [e.g. diltiazem,[91,92,95,218] glabridin,[211] troleandomycin[90,131-133] and raloxifene[22]]; and drugs with k_{inact} ≥0.30 min^{-1} (i.e. $t_{½inact}$ ≥6.30 min) [e.g. gestodene,[76] mibefradil[219] and delavirdine[126]]. Both nicardipine[95] and L 754394[147,178] are the most potent inactivators of CYP3A4, with a k_{inact} of 2.0 and 1.62 min^{-1}, respectively, while fluoxetine[91] and K 11002[128] inhibited CYP3A4 to the least extent, with k_{inact} values of 0.017–0.026 min^{-1}.

3.3 Relationship between K_I and k_{inact}, K_m and K_i

There is no significant relationship between K_I and k_{inact} (r^2 = −0.31, p = 0.598) [figure 1a], indicating that both inhibitor concentration and time of exposure are independent determinants for CYP3A4 inactivation. Similarly, there is no correlation between apparent K_i and K_I (r^2 = 0.047, p = 0.3454) [figure 1b]. This may be because the apparent K_i is mainly determined by a reversible process where the parent drug molecules play a major role; whereas K_I is a dissociation constant reflecting an irreversible process where covalent binding occurs.[19] In particular, the K_i values for CYP3A4 inhibition may be substrate-dependent and less selective compared with K_I. As shown in table II, the apparent K_i values of drugs for the inhibition of CYP3A4-mediated reaction are often greater than the corresponding K_I values, exceptions being gestodene (5.6[47] vs 46 μmol/L[76]) and isoniazid (63.9[231] vs 228 μmol/L[89]).

Because of the highly possible relevance of drug bioactivation by CYP isoenzymes to metabolites that subsequently inactivate the enzymes, the apparent K_m values for bioactivation are often comparable to the K_I values. For example, the apparent K_m value[234] for the hydroxylation of the 17α-propynyl

Table II. Drugs reported to be mechanism-based cytochrome P450 (CYP) 3A4 inhibitors and their corresponding apparent K_m, K_i and K_I values

Drug	Drug class	Apparent K_m	Apparent K_i	Mechanism-based inhibition			
		(μmol/L)	(μmol/L)	k_{inact} (min^{-1})	$t_{½inact}$ (min)	K_I (μmol/L)	partition ratio
Amprenavir	Anti-HIV	25.5[127]	1.0[120,130]	0.073[120]	9.49	1.4	
Bergamottin	Herbal constituent		28[224,225]	0.30[114]	2.31	4.2	
Clarithromycin	Antibacterial	53.9[43]	7.0[54]	0.072[90,91]	9.63	5.49	
Delavirdine	Anti-HIV	6.8[117,226]	4.1[227]	0.44[126]	1.58	9.5	41
Dihydralazine	Antihypertensive			0.05[111]	13.86	35	
6′,7′-dihydroxybergamottin			2[225]	0.16[223]	4.33	59	
Diltiazem	Antihypertensive	23.0[228]	60.0[220,228]	0.11[91,92,95,218]	6.30	2.0	
Erythromycin	Antibacterial	61.0[67]	80.7[220-222]	0.08[121,133,229]	8.66	46.6	
Ethinylestradiol	Oral contraceptive steroid			0.04[129]	16.0	18	50
Fluoxetine	Antidepressant		7.0[115]	0.017[91]	40.76	5.26	
Gestodene	Oral contraceptive steroid		5.6[47]	0.40[76]	1.73	46	9
Glabridin	Herbal constituent			0.14[211]	4.95	7.0	
Irinotecan	Anticancer	28.0[230]		0.06[109]	11.55	24	
Isoniazid	Antitubercular		63.9[231]	0.08[89]	8.66	228	
K 11002	Cysteine protease inhibitor			0.026[128]	26.65	0.5	
K 11777	Cysteine protease inhibitor	16.4[128]	50.0[128]	0.054[128]	12.83	0.06	
L 754394	Anti-HIV	9.9[232]		1.62[147,178]	0.43	7.5	1.4
Mibefradil	Antihypertensive		1.0[219,233]	0.40[219]	1.73	2.3	1.7
Midazolam	Hypnosedative	5.1[30,181,196]		0.15[181]	4.62	5.8	51.4
Mifepristone	Antiprogestogen	10.6[234]	3.5[182]	0.089[113]	7.79	4.7	1.0
N-desmethyl diltiazem			2.4[228]	0.027[91]	25.67	0.77	
N-desmethyltamoxifen			13[110]	0.08[110]	8.66	2.6	
Nelfinavir	Anti-HIV	40.0[127]	4.8[180]	0.18[180]	3.85	5.6	
Nicardipine	Antihypertensive	1.7	10.0[221]	2.0[95]	0.35	0.6	
Oleuropein	Herbal constituent			0.09[235]	7.67	22.2	
Raloxifene	Estrogen receptor modulator		12[22]	0.16[22]	4.33	9.9	1.8
Resveratrol	Herbal constituent			0.20[133]	3.47	20	
Ritonavir	Anti-HIV	0.063[119]	0.15[104,130,236]	0.078[119,120]	8.88	0.07	10
SN 38	DNA topoisomerase I inhibitor			0.10[109]	6.93	26	
Tabimorelin	Growth hormone secretagogue			0.08[237]	8.66	4.7	
Tamoxifen	Estrogen receptor modulator	50[238]	13[110]	0.051[110]	13.59	0.2	
Troleandomycin	Antibacterial		0.17[221]	0.15[90,131-133]	4.62	0.18	
Verapamil	Antihypertensive	49.0[239]	2.5[221]	0.09[95,218]	7.70	1.7	

K_I = half-maximal rate of inactivation; K_i = apparent inhibition constant; k_{inact} = maximal rate constant of inactivation at saturation; K_m = Michaelis-Menten constant; $t_{½inact}$ = time required for half the CYP to be inactivated at saturation.

group of mifepristone (9.9 μmol/L) in human liver microsomes is comparable to its K_I (4.7 μmol/L).[113] Another example is delavirdine, which undergoes oxidation (desalkylation and 6′-hydroxylation) by pooled human liver microsomes or recombinant CYP3A4 with an apparent K_m of 5.4–6.8 μmol/L,[126] which is close to its K_I (9.5 ± 1.7 μmol/L) for CYP3A4 inactivation.[117] The similar binding affinities for these two independent processes imply that the orientation of the mifepristone molecule in the active site of CYP3A4 may be similar for both the oxidative bioactivation and enzyme inactivation. A relationship study of CYP3A4 inactivators indicates that there is a significant relationship between apparent K_m and K_I ($r^2 = 0.668$, $p = 0.004$) [figure 1c]. However, this positive correlation disappears if amprenavir,[120] clarithromycin,[90,91] diltiazem[228] and tamoxifen[110] are included. For these drugs, a large difference (9.8- to 250-fold) between apparent K_m and K_I are observed, which may be due to the involvement of multiple CYP isoenzymes in their metabolism and bioactivation, multiple binding sites of CYP3A4, nonspecific binding to microsomal proteins and different assay systems.

4. Clinical Considerations of Drug-Induced CYP3A4 Inactivation

4.1 Altered Drug Pharmacokinetics and/or Pharmacodynamics due to CYP3A4 Inactivation

Because of the pivotal role of CYP isoenzymes in drug metabolism, significant inactivation of these isoforms and particularly the major human hepatic and intestinal CYP3A4 could result in drug-drug interactions and adverse drug reactions. Compared with reversible inhibition of CYP3A4, mechanism-based inhibitors of CYP3A4 more frequently cause pharmacokinetic/pharmacodynamic drug-drug interactions, as the inactivated CYP3A4 has to be replaced by newly synthesised CYP3A4 protein. Pharmacokinetic interactions often occur as a result of a change in drug metabolism. For example, the 14-membered-ring macrolides increased the plasma

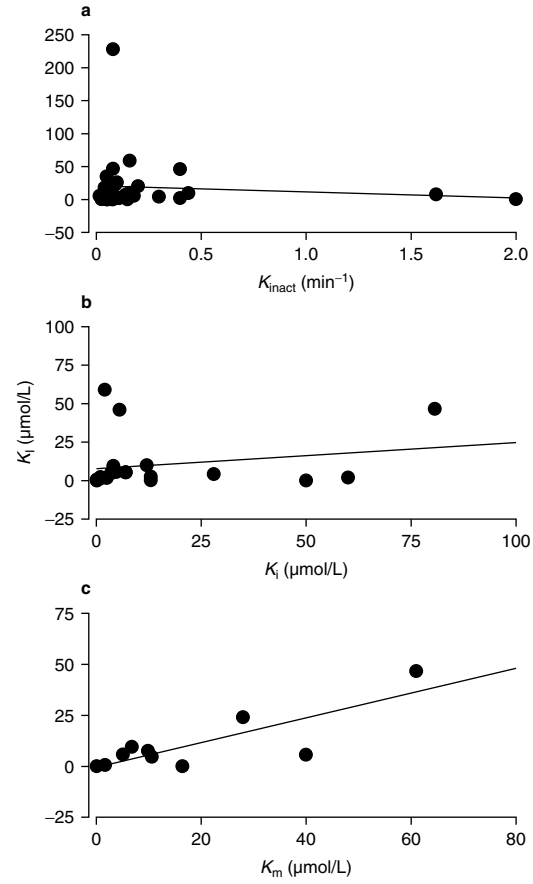

Fig. 1. Relationship between K_I and k_{inact} (**a**), K_i (**b**) and K_m (**c**). K_I = concentration required for half-maximal inactivation; K_i = apparent inhibition constant; k_{inact} = maximal rate of inactivation at saturation; K_m = Michaelis-Menten constant.

concentrations of a number of therapeutic agents that are substrates of CYP3A4 (table III). Diltiazem has been shown to potently inhibit the metabolism of a variety of coadministered drugs including carbamazepine,[240] ciclosporin,[241,242] quinidine,[243] midazolam,[244] alfentanil,[36] nifedipine[245] and lovastatin[246] (table IV). Inhibition of CYP3A by ritonavir explains, at least in part, the remarkable elevation of blood concentrations and area under the plasma concentration-time curve (AUC) of other concomitantly administered drugs that are extensively metabolised by CYP3A4 and have intermediate (10–80 L/h) to high (>80 L/h) intrinsic clearance and significant first-pass metabolism. These drugs

Table III. Examples of macrolide-drug interactions potentially involving mechanism-based cytochrome P450 3A4 inhibition

Drug combination	Study type	Participants	Altered pharmacokinetics	Altered pharmacodynamics	Comments	References
Clarithromycin +						
Carbamazepine	op	7 pts	Plasma conc ↑ 100%	Drowsiness, dizziness and ataxia	Avoid combination	255
Cisapride	r, op, co	12 HV	AUC ↑ 3.16-fold, C_{max} ↑ 2.72-fold, C_{ss} ↑ 3.20-fold	QTc elevation (↑ 32 msec), risk of torsades de pointes	Avoid combination	256
Ciclosporin	op	6 pts	C_{ss} ↑ 2-3-fold	Nephrotoxicity in one case	TDM or dose reduction	257
Indinavir	r, co	12 HV	AUC ↑ 1.19-fold, C_{max} ↑ 1.08-fold	None	No dosage adjustment required	258
Lansoprazole	r	20 pts	C_{3h} ↑ 80.8–146.5%	None	Limited clinical significance	259
Methylprednisolone	op	6 pts	CL ↓ 67%, $t_{½β}$ ↑ 2.27-fold	↑ Risk of corticosteroid-induced adverse effects	Avoid combination	260
Midazolam	r, seq	16 HV	AUC ↑ 1.42-fold, CL ↓ 64.3%	2.2-fold ↑ in sleep time	Avoid combination	94
Rifabutin	r	34 pts	AUC ↑ 1.99-fold, AUC of 25-O-desacetyl-rifabutin ↑ 3.75-fold	↑ Uveitis	Reduce dose	261
Ritonavir	r, op, co	22 HV	AUC ↑ 1.13-fold, C_{max} ↑ 1.15-fold	None	No dosage adjustment required	248
Triazolam	r, db	12 HV	CL ↓ 4.35-fold	↑ Sleep time	Avoid combination or reduce dose	122
Erythromycin +						
Alfentanil	cont, co	6 pts	CL ↓ 25.6%, $t_{½β}$ ↑ 56.4%	None	Limited clinical significance	37
Alprazolam	r, co	12 HV	AUC ↑ 1.47-fold, CL ↓ 59.8%, $t_{½β}$ ↑ 1.52-fold	None	Limited clinical significance	40
Diazepam	r, cont, co	6 HV	AUC ↑ 15%, C_{42h} ↑ 63%	None	Limited clinical significance	262
Felodipine	r, db, co	12 HV	AUC ↑ 2.49-fold, C_{max} ↑ 2.27-fold, $t_{½β}$ ↑ 1.60-fold	↑ Haemodynamic effect	Avoid combination	263
Flunitrazepam	r, db, co	5 HV	AUC ↑ 25%	None	Limited clinical significance	262
Lidocaine (lignocaine)	r, cont, co	9 HV	AUC ↑ 40%; AUC of monoethylglycinexylidide ↑ 60%			86,264
Midazolam	r, db, co	12 HV	AUC ↑ 4.42-fold, C_{max} ↑ 2.70-fold, $t_{½β}$ ↑ 1.77-fold	Enhanced sleep and altered psychomotor behaviour	Avoid combination or reduce dose by 50–75%	265
Nitrazepam	r, db, co	10 HV	AUC ↑ 25%, C_{max} ↑ 30%, t_{max} ↓ 50%	None	Limited clinical significance	266
Simvastatin	r, db, co	12 HV	AUC ↑ 6.21-fold, C_{max} ↑ 3.46-fold	↑ Risk of rhabdomyolysis	Avoid combination or reduce dose by 50–80%	263
Triazolam	r, db	12 HV	CL ↓ 2.83-fold	↑ Sleep time	Avoid combination or reduce dose	122

AUC = area under the plasma concentration-time curve; **CL** = clearance; **C_{max}** = maximum plasma concentration; **co** = crossover; **conc** = concentration; **cont** = controlled; **C_{ss}** = plasma concentration at steady state; **C_{3h}** = concentration at 3 hours; **C_{42h}** = concentration at 42 hours; **db** = double-blind; **HV** = healthy volunteers; **op** = open-label; **pts** = patients; **QTc** = corrected QT interval; **r** = randomised; **seq** = sequential; **$t_{½β}$** = elimination half-life; **TDM** = therapeutic drug monitoring; **t_{max}** = time to C_{max}; ↑ indicates increased; ↓ indicates decreased.

Table IV. Examples of diltiazem-drug interactions potentially involving mechanism-based cytochrome P450 3A4 inhibition

Interacting drug	Study type	Participants	Altered pharmacokinetics	Altered pharmacodynamics	Comment	Reference
Buspirone	r, co, pc	9 HV	AUC ↑ 5.50-fold, C_{max} ↑ 4.10-fold	↑ Adverse effects	Avoid combination	267
Ciclosporin	op, co	22 pts	C_{min} ↑ 31.2%, C_{min} of metabolite 17 ↑ 82.6%	None	No dosage adjustment required	242
Lovastatin	r, op, co	10 HV	AUC ↑ 2.57-fold, C_{max} ↑ 3.33-fold	None	Avoid combination	246
Methylprednisolone	nb, cont, co	5 HV	AUC ↑ 49.1%, CL ↓ 33.3%, $t_{1/\beta}$ ↑ 36.8%	None	Limited clinical significance	268
Midazolam	r, db, co	9 HV	AUC ↑ 275%	↑ Sedative effects	Avoid combination or reduce dose	244
Nifedipine	r, db, pc	11 pts	Plasma conc ↑ 2.06-fold	Exercise tolerance time ↑ 28.5%	No dosage adjustment required	245
Quinidine	op, co	12 HV	AUC ↑ 51.2%, $t_{1/\beta}$ ↑ 36.8%	↑ QTc and PR intervals, ↓ HR	Avoid combination	243
Triazolam	r, co	7 HV	AUC ↑ 1.28-fold, $t_{1/\beta}$ ↑ 85.4%	↑ Sedative effects	Avoid combination or reduce dose	123

AUC = area under the plasma concentration-time curve; **CL** = clearance; C_{max} = maximum plasma concentration; C_{min} = trough plasma concentration; **co** = crossover; **conc** = concentration; **cont** = controlled; **db** = double-blind; **HR** = heart rate; **HV** = healthy volunteers; **nb** = nonblind; **op** = open-label; **pc** = placebo-controlled; **pts** = patients; **QTc** = corrected QT interval; **r** = randomised; $t_{1/\beta}$ = elimination half-life; ↑ indicates increased; ↓ indicates decreased.

include rifabutin (400%),[247] clarithromycin (77%),[248] ketoconazole (350%),[163] saquinavir (5000%),[249] amprenavir (210%),[250,251] nelfinavir (152%),[163,252] lopinavir (7700%)[253] and indinavir (380%).[254]

Mechanism-based inactivation of CYP3A4 may cause severe drug toxicity due to metabolic inhibition of coadministered drugs.[269] When irreversible CYP3A4 inhibitors such as erythromycin and clarithromycin are coadministered with other drugs such as terfenadine, astemizole or pimozide, patients may experience torsades de pointes.[269-271] Terfenadine is a CYP3A4 substrate that undergoes extensive first-pass metabolism following oral administration.[54,272] In the absence of a drug interaction, the carboxylate metabolite is the principal circulating entity in plasma, whereas unchanged terfenadine, a drug known to cause torsades de pointes, is normally not present at measurable concentrations.[272,273] Rhabdomyolysis has occurred when simvastatin was combined with erythromycin or ritonavir.[274] Symptomatic hypotension may occur when mechanism-based CYP3A4 inhibitors are combined with some dihydropyridine calcium channel antagonists,[275] as well as with the phosphodiesterase inhibitor sildenafil.[276] In addition, ataxia can occur when carbamazepine is coadministered with mechanism-based CYP3A4 inhibitors such as macrolide antibacterials, isoniazid, verapamil and diltiazem.[277,278] On the other hand, beneficial drug interactions may occur as a result of CYP3A4 inactivation. Coadministration of a mechanism-based CYP3A4 inhibitor with ciclosporin may allow reduction of the dosage and cost of the immunosuppressant.[269] Certain HIV protease inhibitors (e.g. saquinavir) have low oral bioavailability that can be significantly (>50-fold) increased by the addition of ritonavir.[279,280]

In addition to toxic drug-drug interactions, the formation of drug reactive metabolite-CYP3A can also play a role in toxicity initiation. The formed adduct can induce potential immune responses, leading to production of autoantibodies against CYP3A. The formation of drug-CYP3A adducts may be nontoxic or fatal, depending on the drugs,

pharmacokinetics of adduct formation and degradation, other affected target proteins and organs, and pathological conditions of the patients.[281] The reactivity of drug intermediates and subcellular localisation of major protein targets are important determining factors in the toxicity.[282,283] Selective protein covalent binding by a drug or its metabolite(s) has been associated with target organ/tissue toxicity of drugs.[284] For example, reactive metabolites of tamoxifen are believed to bind covalently to DNA and proteins in the endometrial tissue, causing endometrial carcinoma.

4.2 *In Vitro-In Vivo* Extrapolation Involving CYP3A4 Inactivation

One of the main objectives of *in vitro* metabolic inhibition studies is the qualitative and quantitative prediction of *in vivo* drug-drug interactions, and quantitative *in vitro* metabolic data may be extrapolated reasonably well to *in vivo* situations with the application of appropriate pharmacokinetic principles.[20,285-288] It can be anticipated that the inactivation of CYP3A4 by various drugs would increase the bioavailability of coadministered drugs metabolised mainly by CYP3A4, because of intestinal and hepatic inhibition of CYP3A4. It is believed that, *in vivo*, the inhibitory effect of a mechanistic inactivator is more prominent after multiple-dose administration and lasts longer than that of a reversible inhibitor.[198] The activated species irreversibly alters the enzyme to remove it permanently from the pool of active enzyme. Thus, the time-dependent inactivation of CYP3A isoenzymes results in nonlinear pharmacokinetics, as indicated by 50–100% prolongation of the diltiazem half-life in humans after long-term administration compared with the single-dose data.[289]

The extent of a drug interaction due to CYP3A inactivation is time dependent in both onset and offset. For example, erythromycin did not significantly inhibit the clearance of alfentanil on the first day of coadministration but produced a 25% decrease after 7 days.[37] As the half-life for onset of inactivation is inversely proportional to the efficiency [$k_{inact}/(K_I + [I])$] of inactivation, the delayed onset of inhibition by erythromycin is a predictable property of a relatively weak inactivator. The delayed offset of CYP3A inhibition is expected to be independent of the inactivating drug and the extent of inhibition. This time-dependent offset may explain the serious adverse events associated with discontinuation of the irreversible inactivator, mibefradil, and immediate initiation of alternative calcium channel antagonist treatment.[219,290] A mibefradil washout period of 7–14 days was thus recommended.

Determination of *in vitro* pharmacokinetic parameters is essential for the prediction of drug metabolism by a particular CYP isoenzyme *in vivo*. The apparent K_I values for competitive inhibition determined *in vitro*, together with its relationship to unbound plasma concentrations of the inhibitor achieved with therapeutic doses *in vivo*, can be used as a rough guide to predict the possibility of a significant *in vivo* drug interaction.[20,291] Relating the *in vitro* results to *in vivo* pharmacokinetics is not straightforward. Generally, to predict the degree of interaction observed in clinical cases quantitatively, it is necessary to investigate the correlation between *in vitro* inhibitory potency of the inhibitor and *in vivo* inhibition, taking into account the distribution of the inhibitor into the liver, and extrapolation of data from animal studies.[229]

Several approaches for modelling mechanism-based enzyme inactivation have been described in the literature.[21,91,217,292-294] In a well described model of mechanism-based enzyme,[293] an inactivator can be released from the enzyme through reversible binding, converted to a product through a productive catalytic cycle, or can inactivate the enzyme by forming a complex. The active enzyme concentration at time t ($[E]t$) can be defined by equation 5:

$$[E]_t = [E]_0 \times e^{\left(\frac{-t \times [I] \times k_{inact}}{[I] + K_I}\right)} \quad \text{(Eq. 5)}$$

where $[E]_0$ is the initial enzyme concentration, $[I]$ is inactivator concentration, and k_{inact} and K_I are pa-

rameters estimable by nonlinear regression. This model assumes that:[91]

- the conditions of the well stirred model are met;
- hepatic elimination is the only pathway;
- there is complete absorption of drugs from the gastrointestinal tract;
- hepatic first-pass metabolism is influenced by an inactivator.

By using this model, it was predicted that diltiazem, clarithromycin and fluoxetine at unbound plasma concentrations of 0.1 μmol/L will increase the AUC of a coadministered CYP3A substrate by 1.4-, 2.6- and 4.7-fold, respectively.[91] These predicted results are in good qualitative agreement with reported clinical data, especially when one considers that simple competitive models completely fail to predict any interactions where a K_i of 60, 10 and 50 μmol/L for diltiazem, clarithromycin and fluoxetine, respectively,[54,220,295] and steady-state plasma concentrations of 0.3, 0.9 and 1.0 μmol/L, respectively, were used.[94,246,295]

However, the *in vitro* inhibitory potencies of mechanism-based CYP3A4 inhibitors do not necessarily translate directly into relative extents of inhibition *in vivo*. *In vivo* clinical consequences depend upon additional factors that are not easily accounted for *in vitro* and they could confound *in vitro-in vivo* extrapolation for drug-drug interactions. Such factors include:

- concentration-time course
- plasma protein binding
- atypical substrate pharmacokinetics for CYP3A4
- existence of inhibitory metabolites
- partitioning from plasma to liver
- rate-limiting transport of drug and inhibitor into the hepatocytes
- intestinal active efflux of drug and inhibitor
- extrahepatic metabolism of drugs and inhibitors.

4.3 Factors Affecting the Clinical Consequence of Mechanism-Based Inhibition of CYP3A4

4.3.1 Drug-Related Factors

The clinical importance of any drug-drug interaction due to CYP3A4 inactivation depends on factors that are associated with the administered drugs and patients, and the pharmacodynamic consequences may or may not closely follow pharmacokinetic alterations. Since many mechanism-based CYP3A4 inhibitors are also inducers of CYP isoenzymes, and enzyme inactivation is significantly affected by drug exposure time, the clearance of coadministered drugs may be increased or decreased *in vivo*, depending on the interplay between substrate, inhibitor, inducer and CYP3A4. There appear to be no common structural factors that determine whether a compound is a CYP3A4 inactivator. However, it is well known that drugs which have several common moieties such as a tertiary amine function,[164,165] furan ring[114,168] and acetylene function[76,113] are metabolised by CYP isoenzymes and bind to the same enzyme covalently to form a CYP-metabolite complex and thereby inactivate the enzyme. It appears that the chemical properties of a drug critical to CYP3A4 inactivation include formation of reactive metabolites, metabolism by CYP isoenzymes, preponderance of CYP inducers and P-gp substrate, and occurrence of clinically significant pharmacokinetic interactions with coadministered drugs.

Many CYP3A4 inactivators such as irinotecan,[296-299] some protease inhibitors[300] and mifepristone[301] are also known substrates and/or inhibitors of P-gp. P-gp encoded by the human *MDR1* is constitutively expressed in the brush border membrane of intestinal enterocytes and the canalicular membrane of hepatocytes, and it transports structurally and functionally diverse compounds.[302,303] Thus, the potential for effects of these CYP3A4 inhibitors on the bioavailability of certain drugs may be enhanced, given that P-gp is the other major determinant of the oral bioavailability of many drugs.[301,304] However, P-gp can also alter the intracellular concentration of CYP3A inhibitors and inducers and hence the magnitude of the inhibitory

and inductive response. Furthermore, 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 its substrates such as protease inhibitors in these sanctuary sites.[302] P-gp, like CYP3A, can also be induced by many drugs.[305] This would complicate the interactions between drugs, CYP3A and P-gp. It has been postulated that P-gp and CYP3A are functionally linked components of a drug detoxification cascade that limits the bioavailability of several drugs.[306] There is substantial overlap in substrate specificity between CYP3A and P-gp, and several modulators/substrates of P-gp and CYP3A have been shown to coordinately upregulate the expression of these proteins *in vitro*.[307]

4.3.2 Patient-Related Factors

The age, sex, comedications, diseases and genetic factors are all potentially important factors affecting the clinical outcomes of mechanism-based inactivation of CYP3A4. In most cases, there are remarkable interindividual variations in the mechanism-based inactivation of CYP3A4 and subsequent drug-drug interactions. This is mainly due to the significant difference of CYP3A4 content. There is a significant variation in the hepatic expression of CYP3A4 based on *in vitro* (35- to 100-fold) studies using human liver bank[11] and *in vivo* (20- to 50-fold) using probe drugs such as erythromycin[308] midazolam[93] and alfentanil.[34] Such a substantial variation is considered to be the result of a number of environmental, physiological and genetic factors.[137] Treatment of patients with rifampicin results in a 4- to 8-fold variation in induction of CYP3A4 in the enterocyte as assessed by CYP3A4 probe,[309] indicating additional interindividual variation in the CYP3A4 inductive response.

It appears that there are no marked age and sex differences in CYP3A4 expression,[310-312] although 24–36% higher activity in females than in males has been reported.[70] CYP3A4 catalytic activity is also not affected by smoking status, alcohol (ethanol) consumption or percentage ideal bodyweight.[310] Thus, it can be predicted that age and sex are not important factors determining the clinical outcome of drug interactions involving CYP3A4 inactivation. However, changes in liver blood flow, liver size, renal function, drug protein binding and distribution with aging may be significant, and thus affect drug metabolism and elimination. Further studies are required to substantiate whether there is a clinically significant sex- and age-dependent difference in CYP3A inactivation.

Inflammation and relevant cytokines are known to affect drug metabolism by downregulating or upregulating expression of several CYP isoenzymes, including the CYP3A subfamily. Cytokines such as interleukin-6 rapidly and extensively decreased the expression of both human pregnane X receptor and constitutively activated receptor mRNAs in human hepatocytes, leading to reduced expression of CYP3A4, CYP2B and CYP2C.[309] This receptor downregulation phenomenon is accompanied by reduced responsiveness of CYP3A4 to induction by both rifampicin and phenobarbital. In addition, cytokines are potent regulating factors of drug transporters,[313] indicating a complex effect on drug metabolism and transport of inflammation and cytokines.

5. Conclusion and Future Perspectives

The low substrate specificity also makes CYP3A4 susceptible to reversible or irreversible inhibition by a variety of drugs. Irreversible inhibition of CYP3A4 due to enzyme inactivation or complexation occurs when some therapeutic drugs are converted by CYP isoenzymes to reactive metabolites capable of binding covalently to CYP3A4. Clinically important mechanism-based CYP3A4 inhibitors include antibacterials (e.g. clarithromycin, erythromycin and isoniazid), anticancer agents (e.g. irinotecan and tamoxifen), anti-HIV agents (e.g. ritonavir and delavirdine), antihypertensives (e.g. verapamil and diltiazem), and sex steroids and their modulators (e.g. gestodene and mifepristone). Most of these CYP3A4 inactivators are also substrates and inducers of CYP isoenzymes (particularly CYP3A4). The drugs that inactivate CYP3A4 often possess several common moieties such as a tertiary amine function, furan ring and acetylene function.

The functional effects of CYP3A4 inactivation on drug pharmacokinetics are indistinguishable, depending on factors that are associated with drugs and patients. The pharmacodynamic consequences (efficacy and toxicity) may or may not closely follow pharmacokinetic alterations due to CYP3A4 inactivation that causes long-term effects on drug pharmacokinetics, as the inactivated CYP3A4 has to be replaced by newly synthesised CYP3A4 protein. Severe drug toxicity has been observed as a result of the inactivation of CYP3A4 by coadministered drugs. For example, when aforementioned CYP3A4 inactivators are coadministered with terfenadine, cisapride or astemizole (all CYP3A4 substrates), torsades de pointes (a life-threatening ventricular arrhythmia associated with QT prolongation) may occur.

The study of mechanism-based CYP3A4 inhibition may play an important role in drug development.[314] During drug development, early discovery of problematic drugs that inactivate CYP3A4 can save time and money. Late recognition of significant drug interactions due to CYP3A4 modulation would put patients at risk of clinical toxicity. An example of early detection of potentially toxic drug-drug interactions involving CYP3A inactivation is mibefradil, which has been withdrawn from the market. Currently, it is impossible to accurately predict which new drugs will be associated with the formation of reactive metabolites and CYP3A inactivation, leading to potentially toxic drug-drug interactions. However, by screening drug candidates for the formation of reactive metabolites and CYP3A4 inhibition nature and establishing structure-activity relationships it is possible to halt the development of problematic drugs. The application of genomic and proteomic, coupled with *in silico* methods, approaches to the study of drug-CYP interactions has the potential to lead to a more effective screen because of their high throughput capacity.

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