RISK ASSESSMENT FOR DRUG-DRUG INTERACTION CAUSED BY METABOLISM-BASED INHIBITION OF CYP3A USING AUTOMATED IN VITRO ASSAY SYSTEMS AND ITS APPLICATION IN THE EARLY DRUG DISCOVERY PROCESS

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CYP, cytochrome P450

MBI, metabolism-based inhibition

DDI, drug-drug interaction

HLM, human liver microsome

LC/MS, liquid chromatography/mass spectrometry
Abstract

CYP3A family is a major drug metabolism enzyme in humans. Metabolism-based inhibition of CYP3A might cause clinically significant drug-drug interactions. To assess the risk of DDI caused by MBI of CYP3A, we established an automated single time- and concentration-dependent inhibition assay. To create a diagram to assess DDI risk of compounds in the early discovery stage, we classified 171 marketed drugs by the possibility of the occurrence of in vivo DDI caused by MBI from the relationship between the inactivation activity determined in the MBI screening, the therapeutic blood or plasma concentration, and the in vivo DDI information. This analysis revealed that the DDI risk depends on both the MBI potential and blood concentration of a compound, and provided the criteria of the DDI risk. In the assay, 3 compounds (midazolam, nifedipine, and testosterone) were compared as CYP3A probe substrates. The results show that the evaluation for MBI does not depend on the probe substrates used in the assay. In addition, we established an automated assay to distinguish quasi-irreversible and irreversible binding to CYP3A in which the quasi-irreversible inhibitors such as diltiazem, verapamil, and nicardipine were dissociated from CYP3A by the addition of potassium ferricyanide, whereas the irreversible inhibitors such as clozapine, delavirdine, and mibebradil were not. It provides useful information related to chemical structures likely to cause MBI. By using these MBI assays supported by an extensive database of marketed compounds, a systematic MBI evaluation paradigm was established and has been incorporated into our drug discovery process.
Introduction

The cytochrome P450 (CYP) superfamily comprises many isozymes that metabolize xenobiotic chemicals, including drugs (Guengerich, 2001). CYP1A2, 2C9, 2C19, 2D6, and 3A participate in the metabolism of approximately 80% of therapeutic drugs, such that the majority of CYP-mediated drug-metabolism is mediated by the CYP3A family (Wienkers and Heath, 2005). CYP3A4 is a major isoform of CYP3A. Recently, it has been reported that CYP3A5 may also contribute to the metabolism of CYP3A4 substrates because of the overlapping substrate specificity with CYP3A4 (Huang et al., 2004). Drugs metabolized by CYPs may also inhibit the metabolism of co-administered drugs, which results in an increased blood concentration of the co-administered drugs (Bertz and Granneman, 1997).

As a result, a patient to whom 2 or more drugs are administered might suffer from adverse effects induced by such a drug-drug interaction (DDI). Drugs such as terfenadine, mibebradil, cisapride, and nefazodone have been withdrawn from the market because of CYP-related DDI (Wienkers and Heath, 2005). Consequently, pharmaceutical companies now investigate the CYP inhibitory potential of drug candidates in the early stage of development (Wienkers and Heath, 2005).

CYP inhibition can be classified into 3 categories: reversible, quasi-irreversible, and irreversible inhibition (Lin and Lu, 1998; Murray, 1997). Reversible inhibition means that 2 or more drugs compete at a CYP-active site and one drug inhibits the interaction of other drugs with the CYP. On the other hand, a drug converted to a reactive intermediate by CYP may interact with CYP.
quasi-irreversibly or irreversibly and inactivate the function of CYP. This type of inhibition is referred as metabolism-based inhibition (MBI). Such MBI is thought to cause more serious CYP-related DDI than reversible inhibition, because the inhibitory effect continues long after the initial administration until new CYP is synthesized. Many metabolism-based inhibitors are currently on the market. These include macrolide antibiotics, anticancer drugs, antidepressants, anti-HIV agents, antihypertensive agents, and steroids and their modulators. Interestingly, most of them also exhibit pharmacokinetic DDI (Zhou et al., 2004; Zhou et al., 2005b; Fontana et al., 2005). In some cases, the interaction is so serious that, as previously mentioned, the drugs must be withdrawn. For example, mibefradil, a calcium-channel blocker, is a potent metabolism-based CYP3A4 inhibitor. It was withdrawn from the US market in 1998 because of the serious adverse interactions that occurred when mibefradil and statins, antilipemic agents such as simvastatin and lovastatin, were co-administered to patients (FDA talk paper, 1998; Schmassmann-Suhijar et al., 1998; Omar and Wilson, 2002; Jacobson, 2004). Because mibefradil inactivates CYP3A4, which clears statins, the plasma concentration of statins increased. This induced rhabdomyolysis, a statin-related adverse effect.

Currently, time- and concentration-dependent inhibition assays are used to determine the MBI potential of a drug or drug candidate (Silverman, 1988; Madan et al., 2002). Many pharmaceutical companies have established MBI assay systems to detect either enzymatic activity loss at a single concentration or IC50 shift caused by preincubation. Other assays are designed to obtain MBI-related
kinetic parameters (Crespi and Stresser, 2000; Yamamoto et al., 2004; Naritomi et al., 2004; Zhao et al., 2005; Limet et al., 2005; Atkinson et al., 2005). Such systems use pooled human liver microsomes (HLMs), recombinant CYP isozymes, and human hepatocytes with CYP probe substrates and fluorescent substrates. However, a strategy to apply MBI screening to early drug discovery has not been well-established. For CYP3A4 inhibition assays, midazolam, testosterone, terfenadine, erythromycin, and nifedipine are often used as probe substrates (Yuan et al., 2002). Although several groups have reported that the reversible CYP3A4 inhibition caused by test compounds depends on the CYP3A4 probe substrate used (Kenworthy et al. 1999; Stresser et al., 2000; Wang et al., 2000), it has remained unclear whether MBI assay results are affected by CYP3A4 probe substrates.

In the present study, to establish a risk assessment system for DDI caused by MBI, an automated screening system was established to check the CYP3A MBI potential at a single preincubation time and concentration of a test compound. Then, 3 different compounds (midazolam, testosterone, and nifedipine) were compared as probe substrates. Next, 171 marketed compounds were tested using this assay. Finally, an automated screening system was established to distinguish between quasi-irreversible and irreversible binding to CYP3A. Based on these results and the additional incorporation of previously reported information, the relationship between the MBI screening results, DDI risk, and MBI reversibility was analyzed to establish a systematic MBI evaluation paradigm.
Methods

Materials. Midazolam maleate salt, testosterone, nifedipine, dextrophan tartrate, 17α-ethynylestradiol, ketoconazole, potassium ferricyanide, and glucose-6-phosphate were purchased from Sigma (St. Louis, MO, USA). 1’-Hydroxymidazolam, 6β-hydroxytestosterone, and oxidized nifedipine were purchased from Ultrafine (Manchester, UK). 0.5 M sodium phosphate buffer, MgCl₂·6H₂O, and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque (Kyoto, Japan). β-NADP⁺ and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Methanol and acetonitrile were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Formic acid was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Pooled HLMs were purchased from XenoTech LLC (Lenexa, KS, USA). All marketed compounds used as inhibitors were purchased from commercial sources.

Single Time- and Concentration-dependent Inhibition Assay. The MBI assay was automated by using a Beckman BiomekFX (Beckman Coulter Inc.; Fullerton, CA, USA). The assay was designed to run duplicates of 16 reactions: 13 test compounds, 1 MBI-positive reference (50 µM ethynylestradiol), and 1 MBI-negative reference (100 nM ketoconazole), together with 1 vehicle control (reaction mixture without an inhibitor) in a 96-well flat-bottom microplate (Nalge Nunc; Rochester, NY, USA) heated to 37°C on an aluminum plate. The preincubation solutions contained either 0.5 (for midazolam and nifedipine) or 1.0 (for testosterone) mg/ml HLM in 0.1 M sodium phosphate buffer, and the incubation solutions contained 0.1 M sodium phosphate buffer and one of
the following as a substrate: 25 µM midazolam, 200 µM testosterone, or 50 µM nifedipine. The final solvent concentration in the incubation solutions was 1% (v/v) acetonitrile. Each of the solutions were prepared in separate reagent reservoirs (Beckman Coulter Inc.; Fullerton, CA, USA). 160 µl of the preincubation solutions and 180 µl of the incubation solutions were transferred to the wells of the assay plate, and then 20 µl of the test compound solutions or control solutions (10% (v/v) DMSO) were added to the preincubation solutions. The preincubation reactions were initiated by the addition of 20 µl of an NADPH-generating system consisting of 25 mM β-NADP+, 250 mM glucose-6-phosphate, 20 units/ml glucose-6-phosphate dehydrogenase, 100 mM MgCl2·6H2O, and 0.1 M sodium phosphate buffer. For the 0-min preincubation, 20 µl of each preincubation mixture was immediately transferred into 180 µl of the incubation solution and then incubated for 10 min. For the 30-min preincubation, each preincubation mixture was diluted 10-fold with the incubation solution after 30 min. At the end of the incubation reactions, 50 µl aliquots of each incubation solution was added to 200 µl of methanol containing 200 nM dextrorphan as an internal standard in separate wells of a 96-well round-bottomed microplate (Agilent Technologies; Naerum, Denmark) cooled to 4°C on an aluminum plate. Samples were centrifuged at 2000 × g for 10 min, and the supernatants were transferred to a Millipore MultiScreen (Millipore; Molsheim, France) and filtered by centrifugation at 2000 × g for 10 min. The samples in the 96-well round-bottomed microplate were kept at -20°C until LC/MS analysis.
Multiple Time- and Concentration-dependent Inhibition Assay. The automated MBI assay described above was used with modification of the preincubation times and concentrations of the test compounds. The preincubation solutions were reacted for 0, 15, and 30 min with 5 concentrations of the test compounds.

Reversibility of MBI. The reversibility of MBI was investigated by oxidation with potassium ferricyanide. A Beckman BiomekFX was used to run 7 compounds and 1 control reactions (without an inhibitor) in a 96-well flat-bottom microplate (Nalge Nunc; Rochester, NY, USA) heated to 37°C on an aluminum plate. The compositions of the preincubation solutions and the incubation solutions were the same as those described in the single time- and concentration-dependent inhibition assay section. After a 0-min or 30-min preincubation, 50 µl of each preincubation solution was added to 50 µl of the solutions containing 0.1 M sodium phosphate buffer with or without 2 mM potassium ferricyanide in the assay plate, and then incubated for 10 min. After the 10-min reaction, each reaction mixture was diluted 5-fold with the incubation solution. At the end of the incubation reactions, samples were treated as described previously.

LC/MS Analysis. All samples were analyzed by an LC/MS system consisting of a Waters Alliance 2790 and a micromassZQ (Waters; Manchester, UK). Chromatographic separation was performed with a ChromolithTM Flash RP-18e column (4.6 mm × 25 mm; Merck KGaA, Darmstadt, Germany) coupled with a ChromolithTM Guard Cartridge RP-18e (4.6 mm × 25 mm; Merck KGaA, Darmstadt,
Germany). The mobile phase consisted of solvent A, 0.1% (v/v) formic acid in water, and solvent B, 0.1% (v/v) formic acid in acetonitrile, with an A:B gradient of 95:5 (0-0.5 min) and 65:35 (2.0-4.5 min) for 1'-OH-midazolam, 95:5 (0-0.5 min) and 70:30 (2.0-5.0 min) for 6β-hydroxytestosterone, and 95:5 (0-0.5 min) and 50/50 (0.6-4.0 min) for oxidized nifedipine with a flow rate of 0.6 mL/min. The \( m/z \) value of 1'-hydroxymidazolam is 341.9, that of 6β-hydroxytestosterone is 304.9, of oxidized nifedipine is 344.8, and of dextrorphan is 257.9.

**Data Analysis.** The concentrations of metabolites in the samples were calculated by using MassLynx V4.0 (Waters; Manchester, UK). The percentage of the metabolic activity (% of control \( (0\text{ min}) \) and % of control \( (30\text{ min}) \)) was obtained for each sample after a 0-min or 30-min preincubation with an inhibitor, compared with each control sample after a 0-min or 30-min preincubation without an inhibitor as follows.

\[
\text{% of control}_{(0\text{ min})} = \frac{V\text{-}(0\text{ min, +inhibitor})}{V\text{-}(0\text{ min, -inhibitor})} \times 100
\]

\[
\text{% of control}_{(30\text{ min})} = \frac{V\text{-}(30\text{ min, +inhibitor})}{V\text{-}(30\text{ min, -inhibitor})} \times 100
\]

\( V\text{-}(0\text{ min, +inhibitor}) \) is the metabolic activity after 0-min preincubation with (+) or without (-) an inhibitor, and \( V\text{-}(30\text{ min, +inhibitor}) \) is the metabolic activity after 30-min preincubation with (+) or without (-) an inhibitor. Using these values, the percentage of the enzymatic activity remaining (% remaining) after the 30-min preincubation relative to the 0-min preincubation was calculated as follows.

\[
\text{% remaining} = \frac{\text{% of control}_{(30\text{ min})}}{\text{% of control}_{(0\text{ min})}} \times 100
\]
The relationship between the therapeutic plasma concentrations and % remaining for CYP3A was plotted using Spotfire DecisionSite 8.0 (Spotfire KK.; Tokyo, Japan). The compounds were placed into 1 of 3 groups and marked according to the increase in AUC of co-administered drugs: (1) reported AUC ratio of co-administered drugs ≥2, (2) reported AUC ratio of co-administered drugs <2, and (3) no DDI information.

$K_{i}$ and $k_{\text{inact}}$ were determined from the results of the multiple time- and concentration-dependent MBI assay. The natural logarithm of % remaining was plotted against the preincubation times for each concentration of test compound investigated. The slope from the linear regression analysis gave the observed inactivation rate constant ($k_{\text{obs}}$) for each concentration; $k_{\text{obs}}$ and the inhibitor concentration (I) were fitted into the following expression by using GraFit Version 5.0.10 (Erithacus Software Limited; Surry, UK).

$$k_{\text{obs}} = \frac{k_{\text{inact}} \cdot I}{K_{i} + I}$$

For the reversibility of MBI, when the CYP enzymatic activity was reduced by a 30-min preincubation with a compound, and the activity could be restored more than 20% with the addition of potassium ferricyanide the compound was judged to be a quasi-irreversible inhibitor.
Results

Substrate Dependency of the CYP3A MBI Screening. To check the substrate dependency in the designed MBI assay, 3 typical probe compounds (midazolam, nifedipine, and testosterone) were used as the CYP3A substrates. Table 1 shows the interday variation in the assay for each probe substrate in the presence of 50 µM ethynylestradiol as an MBI-positive reference or 100 nM ketoconazole as an MBI-negative reference. Fig. 1 shows plots comparing the % remaining of 24 marketed compounds as determined by the screening with different substrates. Fig. 1 shows that a good relationship exists between % remaining for midazolam and nifedipine, and for midazolam and testosterone, respectively. In addition, $K_i$ and $k_{inact}$ values of 3 MBI compounds for each probe substrate were obtained from the results of the multiple time- and concentration-dependent inhibition assay shown in Fig. 2, and were listed in Table 2. Kinetic parameters of the test compounds were similar among the assays with the different probe substrates.

Risk Assessment of DDI Caused by MBI of CYP3A. To assess in vivo DDI risk from the MBI screening results, 171 marketed compounds, including compounds that have been previously reported to cause MBI (Zhou et al., 2004 & 2005b; Fontana et al., 2005), were evaluated in the established screening system. Midazolam was used as the substrate. The concentration of each test compound was initially set at 100 µM. For drugs with strong reversible inhibitory effects, the compound was diluted to a suitable concentration shown in Fig. 3. The relationship between the screening data and the therapeutic blood or plasma concentrations of these compounds (Schulz et al., 2003) was then
analyzed by combining the *in vivo* DDI information (Ito et al., 2004; Obach et al., 2006) and is shown in Fig. 3.

To begin the analysis shown in Fig. 3, the compounds were placed into 1 of 3 groups according to the criteria listed in *Methods*. Compounds reported to increase the AUC ratio of co-administered drugs more than twofold were defined as causing significant *in vivo* DDI, based on previous reports (Tucker et al., 2001; Bachmann and Lewis, 2005). Compounds for which no DDI information has been reported were regarded as causing weak or little DDI. Compounds reported to cause both MBI and DDI, such as diltiazem, verapamil, clarithromycin, erythromycin, mibebradil, and nefazodone, fell under the 80%-remaining line and in the therapeutic blood or plasma concentration range of 0.1 – 10 µM. In contrast, most compounds reported to cause neither MBI nor DDI fell over the 80%-remaining line. Thus, the 80%-remaining line was set as the threshold for the MBI screening. In the region of high therapeutic blood or plasma concentration, over 10 µM, and low % remaining, under 80%, only delavirdine, an antiviral drug, fell. Based on the analysis, the DDI risk caused by MBI could be divided into 4 zones: little DDI risk caused by MBI (zone 1), low possibility of DDI because of the low blood or plasma concentration even if the compounds have the potential of MBI (zone 2), high possibility of DDI caused by MBI (zone 3), and very low potential as a marketed drug because of high blood or plasma concentration and potent MBI (zone 4).

**Reversibility of MBI.** To distinguish between quasi-irreversible and irreversible binding to
CYP3A by MBI compounds, an automated system to assay the reversibility of MBI was established. Compounds determined to be metabolism-based inhibitors from the MBI screening data were tested in this new assay, and the results are shown in Table 3. The enzymatic activity of CYP3A inactivated after a 30-min preincubation with diltiazem, verapamil, nicardipine, amlodipine, erythromycin, clarithromycin, or troleandomycin was restored by over 20% following oxidation with potassium ferricyanide. The metabolic intermediates of compounds have been reported to make complexes with the heme of CYP3A4 (Ma et al., 2000 & Zhou et al., 2005a). In contrast, the enzymatic activity of CYP3A reduced after a 30-min preincubation with clozapine, delavirdine, mibefradil, or ethynylestradiol did not recover after oxidation with potassium ferricyanide (Zhou et al., 2005a). Reactive metabolites of these compounds are reported to bind covalently either to cellular or microsomal proteins, or to heme. In addition, prazosin, bromocriptine, bepridil, bupivacaine, and buprenorphine were shown to be irreversible inactivators.

**MBI evaluation paradigm.** Using three automated MBI assays and the diagram shown in Fig. 3, a systematic MBI evaluation paradigm was established (Fig. 4).
Discussion

To assess the risk of DDI caused by MBI of CYP3A, we established an automated screening system to evaluate CYP3A enzymatic activity remaining (% remaining) after a 30-min preincubation with a single concentration of a test compound and obtained an extensive data of marketed compounds. Because *in vivo* DDI risk depends on both the MBI potential and the blood concentration of a compound, the risk of DDI caused by MBI was classified considering these 2 factors (Fig. 3). Fluvoxamine, fluconazole, and cimetidine, which are known to cause significant *in vivo* DDI, were located in zone 1, the zone of lowest DDI risk caused by MBI. Although they are CYP3A inhibitors, they were located here because they inhibit CYP3A directly, not in a metabolism-based manner. Ethynylestradiol, which is located in zone 2, is a potent metabolism-based inhibitor, but it does not cause *in vivo* DDI because of its usually low blood concentrations of \(\leq 1\) nM (Kuhnz et al., 1996; Palovaara et al., 2000). Compounds reported to cause both MBI and significant *in vivo* DDI, such as diltiazem, verapamil, clarithromycin, erythromycin, mibefradil, and nefazodone, were mainly located in zone 3. Mibefradil and nefazodone were withdrawn from the market in 1998 and 2003, respectively, because of this CYP3A4-related DDI (Wienkers and Heath, 2005). Nicardipine, a potent metabolism-based inhibitor located in zone 3, causes mild *in vivo* DDI by increasing the AUC of co-administered drugs to about 1.5-fold.

Buprenorphine, a semi-synthetic opioid derivative is also located in zone 3. It has been reported...
that the fatal DDI between buprenorphine and benzodiazepines occurs because of their synergistic pharmacologic effect unrelated to their pharmacokinetic interaction. Buprenorphine is known to be a weak reversible CYP3A4 inhibitor. Its \( K_I \) value is large enough compared to the therapeutic concentration to allow a margin of safety (Ibrahim et al., 2000; Elkader and Sproule, 2005). However, results from this study show that buprenorphine is a potent metabolism-based inhibitor of CYP3A; consequently, the severe DDI seen with benzodiazepines might result, in part, from pharmacokinetic interactions.

The only currently marketed drug in zone 4 is delavirdine, an antiviral drug. Because it is used in anti-HIV therapy, the use of delavirdine can be regarded as exceptional. To maintain the plasma concentration of amprenavir, another antiviral drug metabolized by CYP3A4, at a sufficient level for anti-HIV therapy, amprenavir is often used in combination with delavirdine (Tran et al., 2002). Thus, this analysis shows that compounds located in zones 3 and 4 have strong \textit{in vivo} DDI potential through their actions on CYP3A.

This relationship diagram shows that in order to avoid DDI in clinical trials or after drug approval, it is important either to reduce the MBI potential of new compounds or decrease their therapeutic blood concentrations by increasing their pharmacologic activity. The diagram provides criteria to assess the DDI risk that might be useful for suggesting new synthesis directions to medicinal chemists. Moreover, in the early drug discovery process, we can estimate the therapeutic concentration of
compounds based on the *in vitro* pharmacologic data or the information of drugs that has been used already in the same therapeutic area of the clinical stage. Using the estimated therapeutic concentration, it is possible to assess the risk of DDI from the relationship diagram.

The effect of the CYP3A probe substrates used in MBI assays on the assay results was also tested. Several CYP3A4 substrates, for example, midazolam, testosterone, terfenadine, erythromycin, and nifedipine, are often used as probe substrates in enzyme inhibition assays (Yuan et al., 2002). It has been reported that the observed inhibitory effect of test compounds on CYP3A4 metabolic activity depends on the CYP3A4 probe substrate, because it is thought that CYP3A4 has multiple substrate-binding sites that complicate analysis of the interaction between CYP3A4 and its substrates (Kenworthy et al., 1999; Stresser et al., 2000; Wang et al., 2000). Consequently, to avoid under- or over-estimation of *in vivo* DDI resulting from CYP3A4 inhibition, it is recommended to use multiple probe substrates for *in vitro* enzyme inhibition assays. However, it has not been reported whether the MBI observed with a given compound depends on CYP3A4 probe substrates or not; therefore, 3 probe substrates, midazolam, nifedipine, and testosterone, were compared in the MBI assays. The % remaining data for the 24 marketed compounds obtained from the MBI screening with each probe substrate were well correlated, showing that % remaining values do not depend on the probe substrates used for the MBI screening (Fig. 1). In addition, for each probe substrate, the $K_I$ and $k_{inact}$ values of 3 compounds known to cause MBI, erythromycin, clarithromycin, and verapamil, were obtained by
using the multiple time- and concentration-dependent inhibition assay (Table 2). These parameters for
the 3 probe substrates were also similar. Thus, the MBI potential of the compounds detected using
these MBI assays appears to be independent of the probe substrates used in the assays. Based on these
results, midazolam was selected as the probe substrate for the following assays because abundant in
vivo DDI information is available on midazolam. The result is thought to be reasonable, because
reactive intermediates generated from MBI compounds can bind the apoprotein and heme of CYP
irreversibly or quasi-irreversibly, and inactivate metabolic activities, so, other compounds might not
be able to access these inactivated enzymes.

It is also said that the MBI compounds, which in turn bind covalently to cellular or microsomal
proteins, might induce hepatotoxicity in addition to DDI (Walgren et al., 2005; Zhou et al., 2005a). A
large number of compounds including methylenedioxybenzenes, alkylamines, and hydrazines form
MI complexes (Murray, 1997; Lin and Lu, 1998). Compounds containing terminal acetylenes are
converted to reactive metabolites that irreversibly alkylate the heme of CYP, and electrophilic reactive
metabolites or radical species converted by the metabolism of several compounds bind to the
apoprotein of CYP covalently (Murray, 1997; Lin and Lu, 1998). Therefore, information about MBI
reversibility might be useful for determining chemical structures likely to cause MBI. To distinguish
between quasi-irreversible and irreversible binding to CYP3A by MBI compounds, an automated
system to assess MBI reversibility was established. Quasi-irreversible MI-complexes can be
dissociated by oxidation with potassium ferricyanide; the enzymatic activity of CYP then recovers. In contrast, the enzymatic activity inhibited by irreversible binding is not restored (Lin and Lu, 1998). Compounds determined to be metabolism-based inhibitors in the MBI screening were tested by this method (Table 3). CYP3A enzymatic activity inactivated after a 30-min preincubation with quasi-irreversible inhibitors was restored more than 20% by the addition of potassium ferricyanide in the assay mixture (Ma et al., 2000; Fontana et al, 2005). On the contrary, reduced enzymatic activity resulting from a 30-min preincubation with compounds whose reactive metabolites are reported to bind irreversibly either to cellular or microsomal proteins, or to the heme of CYP, could not be restored by potassium ferricyanide treatment (Fontana et al, 2005 & Zhou et al, 2005a). It has been reported that an irreversible inhibitor, clozapine is associated with idiosyncratic toxicity, rather than MBI (Walgren et al., 2005). More studies are needed to clarify the relationship between MBI and hepatotoxicity.

In conclusion, an automated single time- and concentration-dependent inhibition assay for high-throughput screening was established. Marketed compounds tested by this assay were then categorized based on both the screening results and clinical DDI information, revealing the relationship between the MBI potential, the therapeutic blood or plasma concentrations, and the DDI risk. The results of this study also show that different CYP3A probe substrates used in MBI assays do not affect the evaluation for MBI. In addition, an automated MBI reversibility assay was established to
distinguish between quasi-irreversible and irreversible binding to CYP. Based on these observations, a systematic MBI evaluation paradigm using these MBI assays, shown in Figure 4, was established and incorporated into the drug discovery process at this company. It enables the evaluation of the MBI potential of new compounds effectively and early in their development. This system allows in vitro assessment of DDI risk without extensive and expensive in vivo testing. This authors hope that this system, and others likely to follow, will enable the speedy development of safer drugs.
Acknowledgements.

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References


Legends for figures

Fig. 1. Comparison of % remaining of marketed compounds for each CYP3A substrate. Each point was obtained from the MBI screening using midazolam, nifedipine, or testosterone as a probe substrate. The comparison between midazolam and nifedipine is shown in panel A, and that between midazolam and testosterone is shown in panel B. Each point is a mean of duplicate analyses.

Fig. 2. Multiple time- and concentration-dependent inhibition of the metabolism of midazolam (A, B, and C), nifedipine (D, E, and F), or testosterone (G, H, and I) caused by erythromycin (A, D, and G), clarithromycin (B, E, and H), or verapamil (C, F, and I). Each observed inactivation rate constant, k \text{obs}, was plotted against each inhibitor concentration. Each point is a mean of duplicate analyses.

Fig. 3. A diagram showing the relationship between % remaining and the therapeutic blood or plasma concentration of 171 marketed compounds in which \textit{in vivo} DDI information was incorporated. The % remaining data were generated from the MBI assay with midazolam as a substrate. Previously reported therapeutic blood or plasma concentrations and \textit{in vivo} DDI information for these compounds were used. The concentrations of the compounds tested are as follows: 100 \( \mu \text{M} \) (square), 50 \( \mu \text{M} \) (triangle), 10 \( \mu \text{M} \) (circle), and 1 \( \mu \text{M} \) (star). These points were also marked according to the amount of increase in the AUC of co-administered drugs: reported AUC ratio of co-administered drugs \( \geq 2 \) (red), reported
AUC ratio of co-administered drugs <2 (blue), and no DDI information (gray). The risk assessment for DDI caused by MBI can be classified into 4 zones: no DDI risk caused by MBI (zone 1), low possibility of DDI because of the low blood or plasma concentration even if the compounds have the potential of MBI (zone 2), high possibility of DDI caused by MBI (zone 3), and very low potential as a marketed drug because of high blood or plasma concentration and potent MBI (zone 4). The number added to the symbol corresponds to the compound name as follows: 1. fluconazole, 2. fluvoxamine, 3. cimetidine, 4. ethynylestradiol, 5. diltiazem, 6. clarithromycin, 7. verapamil, 8. erythromycin, 9. buprenorphine, 10. nicardipine, 11. mibefradil, 12. nefazodone, and 13. delavirdine.

Fig. 4. Systematic MBI evaluation paradigm using 3 automated MBI assays. In the single time- and concentration-dependent MBI assay, a compound under the 80%-remaining threshold is classified as having moderate to high DDI risk depending on its therapeutic concentration. Binding to CYP is then evaluated by using the MBI reversibility assay in order to determine chemical structures likely to cause MBI. If further detailed prediction of in vivo DDI is needed, values for K_i and k_inact are obtained by using the multiple time- and concentration-dependent inhibition assay.
### TABLE 1.

Interday variation of % remaining of 50 µM ethynylestradiol as a MBI-positive reference and 100 nM ketoconazole as a MBI-negative reference in the automated CYP3A4 MBI screening using each substrate.

The interday data was obtained from the average of the % remaining data from several different studies in duplicate on 3 different days.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Midazolam</th>
<th>Nifedipine</th>
<th>Testosterone</th>
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<tr>
<td>Inhibitor</td>
<td>Ethynylestradiol</td>
<td>Ketoconazole</td>
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<td>C.V.</td>
<td>12.0</td>
<td>6.2</td>
<td>7.4</td>
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</tbody>
</table>
TABLE 2.

$K_I$ and $k_{\text{inact}}$ of erythromycin, clarithromycin and verapamil obtained from the multiple time- and concentration-dependent inhibition assay with each CYP3A4 substrate.

These parameters were determined as described in *Data analysis of Methods* based on the result shown in Fig. 2.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>$K_I$ ($\mu$M)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>Midazolam</td>
<td>12.1</td>
<td>0.0215</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>11.3</td>
<td>0.0295</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>10.9</td>
<td>0.0352</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Midazolam</td>
<td>15.5</td>
<td>0.0192</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>15.9</td>
<td>0.0244</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>12.9</td>
<td>0.0324</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Midazolam</td>
<td>2.55</td>
<td>0.0277</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>3.12</td>
<td>0.0486</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>5.79</td>
<td>0.0591</td>
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</tbody>
</table>
### Table 3.

Summary of reversibility results of binding to CYP3A4 by metabolism-based inhibitors obtained from the MBI reversibility assay, and previously reported information.

Each data is a mean of duplicate analyses. Each % of control data after a 0- or 30-min preincubation followed by the incubation with (+K₃Fe(CN)₆) or without (-K₃Fe(CN)₆) potassium ferricyanide was shown below. Judgement of the reversibility was determined as described in Methods.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>% of control (0 min)</th>
<th>% of control (30 min)</th>
<th>Judgement</th>
<th>Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-K₃Fe(CN)₆</td>
<td>+K₃Fe(CN)₆</td>
<td>-K₃Fe(CN)₆</td>
<td>+K₃Fe(CN)₆</td>
<td>Information</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>100</td>
<td>85.7</td>
<td>92.5</td>
<td>62.3</td>
<td>90.7 Quasi-irreversible</td>
</tr>
<tr>
<td>Verapamil</td>
<td>10</td>
<td>71.5</td>
<td>80.1</td>
<td>41.2</td>
<td>70.1 Quasi-irreversible</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>1</td>
<td>58.3</td>
<td>75.5</td>
<td>53.3</td>
<td>96.6 Quasi-irreversible</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>100</td>
<td>68.0</td>
<td>72.1</td>
<td>49.3</td>
<td>73.2 Quasi-irreversible</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>100</td>
<td>71.4</td>
<td>88.1</td>
<td>51.0</td>
<td>73.5 Quasi-irreversible</td>
</tr>
<tr>
<td>Drug</td>
<td>Concentration</td>
<td>Affinity 1</td>
<td>Affinity 2</td>
<td>Affinity 3</td>
<td>Affinity 4</td>
</tr>
<tr>
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<td>---------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>100</td>
<td>74.8</td>
<td>89.7</td>
<td>53.7</td>
<td>76.2</td>
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<tr>
<td>Troleandomycin</td>
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<td>49.4</td>
<td>68.2</td>
<td>39.2</td>
<td>66.9</td>
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<tr>
<td>Sertraline</td>
<td>100</td>
<td>51.0</td>
<td>65.1</td>
<td>40.3</td>
<td>73.2</td>
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<tr>
<td>Clozapine</td>
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<td>76.5</td>
<td>76.4</td>
<td>62.6</td>
<td>66.3</td>
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<td>Delavirdine</td>
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<td>42.3</td>
<td>66.1</td>
<td>27.3</td>
<td>27.9</td>
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<td></td>
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<tr>
<td>Mibefradil</td>
<td>1</td>
<td>32.4</td>
<td>41.4</td>
<td>24.5</td>
<td>24.8</td>
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<tr>
<td>Ethynylestradiol</td>
<td>50</td>
<td>45.8</td>
<td>63.5</td>
<td>30.2</td>
<td>31.4</td>
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<tr>
<td>Prazosin</td>
<td>100</td>
<td>93.9</td>
<td>96.8</td>
<td>68.6</td>
<td>75.2</td>
</tr>
<tr>
<td>Bromocriptine</td>
<td>1</td>
<td>70.4</td>
<td>87.7</td>
<td>63.2</td>
<td>65.5</td>
</tr>
<tr>
<td>Drug</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
<td>Status</td>
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<tr>
<td>---------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
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<tr>
<td>Bepridil</td>
<td>63.4</td>
<td>63.5</td>
<td>48.9</td>
<td>52.0</td>
<td>Irreversible</td>
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<tr>
<td>Bupivacaine</td>
<td>88.1</td>
<td>93.0</td>
<td>57.6</td>
<td>66.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>42.1</td>
<td>52.7</td>
<td>17.8</td>
<td>19.5</td>
<td>Irreversible</td>
</tr>
</tbody>
</table>
Fig. 1

(A) y = -9.503 + 1.0185x \( R^2 = 0.79344 \)

(B) y = -9.4314 + 1.017x \( R^2 = 0.7962 \)
Fig. 3

% remaining (midazolam) vs. Therapeutic blood or plasma concentration (μM)
Estimate % remaining initially at 100 μM in single time- and concentration-dependent inhibition assay

≤80% remaining

Moderate to high DDI risk depending on the therapeutic concentration

Use MBI reversibility assay to distinguish between quasi-irreversible and irreversible binding to CYP

Quasi-irreversible

MI-complex

Irreversible

Covalent binding to protein or heme

>80% remaining

Little DDI risk

Estimate $K_i$ and $k_{inact}$ for further prediction of DDI in multiple time- and concentration-dependent inhibition assay