DMD Fast Forward. Published on March 28, 2007 as doi:10.1124/dmd.107.015016

DMD #15016

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

Akiko Watanabe, Koichi Nakamura, Noriko Okudaira, Osamu Okazaki and Ken-ichi Sudo

Drug Metabolism & Physicochemistry Research Laboratory, Daiichi Pharmaceutical Co., Ltd.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Drug-drug Interaction Risk by Metabolism-based CYP3A Inhibition

Address Correspondance to

Akiko Watanabe

16-13, Kita-Kasai 1-Chome Edogawa-ku, Tokyo 134-8630 Japan

Telephone: (81)3-3680-0151

Fax: (81)3-5696-8228

E-mail: morikhlu@daiichipharm.co.jp

Number of Text Pages: 21

Number of Tables: 3

Number of Figures: 4

Number of References: 39

Number of Abstract: 248 words

Number of Introduction: 721 words

Number of Discussion: 1345 words

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

List of nonstandard abbreviations

CYP, cytochrome P450

MBI, metabolism-based inhibition

DDI, drug-drug interaction

HLM, human liver microsome

LC/MS, liquid chromatography/mass spectrometry

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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 occurence 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 mibefradil 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.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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, mibefradil, 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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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 inhibiton 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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Materials. Midazolam maleate salt, testosterone, nifedipine, dextrorphan 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, $MgCl_2 \cdot 6H_2O$, 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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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 $MgCl_2 \cdot 6H_2O$, 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.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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,

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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 $_{(0\ min)}$ and % of control $_{(30\ min)}$) 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.

$$\%\ of\ control_{(0\,min)} = \frac{v_{(0\ min,\ +inhibitor)}}{v_{(0\ min,\ -inhibitor)}} \times 100$$

$$\%\ of\ control_{(30\,min)} = \frac{v_{(30\ min,\ +inhibitor)}}{v_{(30\ min,\ -inhibitor)}} \times 100$$

$v_{(0\ min,\ \pm inhibitor)}$ is the metabolic activity after 0-min preincubation with (+) or without (-) an inhibitor, and $v_{(30\ min,\ \pm inhibitor)}$ 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.

$$\%\ remaining = \frac{\%\ of\ control_{(30min)}}{\%\ of\ control_{(0min)}} \times 100$$

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

The relationship between thetherapeutic 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_{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_{obs}) for each concentration; k_{obs} and the inhibitor concentration (I) were fitted into the following expression by using GraFit Version 5.0.10 (Erithacus Software Limited; Surry, UK).

$$k_{obs} = \frac{k_{inact} \cdot I}{K_I + I}$$

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.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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, mibefradil, 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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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).

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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 ≤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 *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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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.

We thank Ms Takae Tanaka for her excellent technical support.

References

Atkinson A, Kenny JR and Grime K (2005) Automated assessment of time-dependent inhibition of human cytochrome P450 enzymes using liquid chromatography-tandem mass spectrometry analysis. *Drug Metab Dispos* 33:1637-47.

Bachmann KA and Lewis JD (2005) Predicting inhibitory drug-drug interactions and evaluating drug interaction reports using inhibition constants. *Ann Pharmacother* 39:1064-72.

Bertz RJ and Granneman GR (1997) Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* 32:210-58.

Crespi CL and Stresser DM (2000) Fluorometric screening for metabolism-based drug-drug interactions. *J Pharmacol Toxicol Methods* 44:325-31.

Delaforge M, Jaouen M and Mansuy D (1983) Dual effects of macrolide antibiotics on rat liver cytochrome P-450. Induction and formation of metabolite-complexes: a structure-activity relationship. *Biochem Pharmacol* 32:2309-18.

Elkader A and Sproule B (2005) Buprenorphine: clinical pharmacokinetics in the treatment of opioid dependence. *Clin Pharmacokinet* 44:661-80.

FDA talk paper. June 8, 1998. Roche Laboratories announces withdrawal of Posicor from the market [cited 2001 Sep 20]. Available from: URL: http://www.fda.gov/bbs/topics/ANSWERS/ANS00876.html

Fontana E, Dansette PM and Poli SM (2005) Cytochrome p450 enzymes mechanism based inhibitors: common sub-structures and reactivity. *Curr Drug Metab* 6:413-54.

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* 14:611-50.

Huang W, Lin YS, McConn DJ, Calamia JC, Totah RA, Isoherranen N, Glodowski M, Thummel KE (2004) Evidence of significant contribution from CYP3A5 to hepatic drug metabolism. *Drug Metab Dispos.* 32:1434-45.

Ibrahim RB, Wilson JG, Thorsby ME and Edwards DJ (2000) Effect of buprenorphine on CYP3A activity in rat and human liver microsomes. *Life Sci* 66:1293-8.

Ito K, Brown HS and Houston JB (2004) Database analyses for the prediction of in vivo drug-drug interactions from in vitro data. *Br J Clin Pharmacol* 57:473-86.

Jacobson TA (2004) Comparative pharmacokinetic interaction profiles of pravastatin, simvastatin, and atorvastatin when coadministered with cytochrome P450 inhibitors. Am J Cardiol 94:1140-6.

Kenworthy KE, Bloomer JC, Clarke SE and Houston JB (1999) CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. *Br J Clin Pharmacol* 48:716-27.

Kuhnz W, Hümpel M, Biere H and Gross D (1996) Influence of repeated oral doses of ethinyloestradiol on the metabolic disposition of [13C2]-ethinyloestradiol in young women. *Eur J Clin Pharmacol* 50:231-5.

Lim HK, Duczak N, Brougham L, Elliot M, Patel K and Chan K (2005) Automated screening with confirmation of mechanism-based inactivation of CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP1A2 in pooled human liver microsomes. *Drug Metab Dispos* 33:1211-9.

Lin JH and Lu AY (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet* 35:361-90.

Ma B, Prueksaritanont T and Lin JH (2000) Drug interactions with calcium channel blockers: possible involvement of metabolite-intermediate complexation with CYP3A. *Drug Metab Dispos* 28:125-30.

Madan A, Usuki E, Burton LA, Ogilvie BW and Parkinson A (2002) In vitro approaches for studying the inhibition of drug-metabolizing enzymes and identifying the drug-metabolizing enzymes responsible for the metabolism of drugs, in *Drug-Drug Interactions* pp 217-294, Marcel Dekker, Inc. New York.

Murray M (1997) Drug-mediated inactivation of cytochrome P450. *Clin Exp Pharmacol Physiol* 24:465-70.

Naritomi Y, Teramura Y, Terashita S and Kagayama A (2004) Utility of microtiter plate assays for human cytochrome P450 inhibition studies in drug discovery: application of simple method for detecting quasi-irreversible and irreversible inhibitors. *Drug Metab Pharmacokinet* 19:55-61.

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB and Tremaine LM (2006) The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J*

Pharmacol Exp Ther 316:336-48.

Omar MA and Wilson JP (2002) FDA adverse event reports on statin-associated rhabdomyolysis. *Ann Pharmacother* 36:288-95.

Palovaara S, Kivistö KT, Tapanainen P, Manninen P, Neuvonen PJ and Laine K (2000) Effect of an oral contraceptive preparation containing ethinylestradiol and gestodene on CYP3A4 activity as measured by midazolam 1'-hydroxylation. *Br J Clin Pharmacol* 50:333-7.

Schmassmann-Suhijar D, Bullingham R, Gasser R, Schmutz J and Haefeli WE (1998) Rhabdomyolysis due to interaction of simvastatin with mibefradil. *Lancet* 351:1929-30.

Schulz M and Schmoldt A (2003) Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. *Pharmazie* 58:447-74.

Silverman RB (1988) Mechanism-based enzyme inactivation, in *Chemistry and Enzymology Volume 1* pp 3-16, CRC Press Inc, Florida.

Stresser DM, Blanchard AP, Turner SD, Erve JC, Dandeneau AA, Miller VP and Crespi CL (2000) Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metab Dispos* 28:1440-8.

Tran JQ, Petersen C, Garrett M, Hee B and Kerr BM (2002) Pharmacokinetic interaction between amprenavir and delavirdine: evidence of induced clearance by amprenavir. *Clin Pharmacol Ther* 72:615-26.

Tucker GT, Houston JB and Huang SM (2001) Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential-toward a consensus. *Pharm Res* 18:1071-80.

Walgren JL, Mitchell MD and Thompson DC (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit Rev Toxicol* 35:325-61.

Wang RW, Newton DJ, Liu N, Atkins WM and Lu AY (2000) Human cytochrome P-450 3A4: in vitro drug-drug interaction patterns are substrate-dependent. *Drug Metab Dispos* 28:360-6.

Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* 4:825-33.

Yamamoto T, Suzuki A and Kohno Y (2004) High-throughput screening for the assessment of time-dependent inhibitions of new drug candidates on recombinant CYP2D6 and CYP3A4 using a single concentration method. *Xenobiotica* 34:87-101.

Yuan R, Madani S, Wei XX, Reynolds K and Huang SM (2002) Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 30:1311-9.

Zhao P, Kunze KL and Lee CA (2005) Evaluation of time-dependent inactivation of CYP3A in cryopreserved human hepatocytes. *Drug Metab Dispos* 33:853-61.

Zhou S, Chan E, Lim LY, Boelsterli UA, Li SC, Wang J, Zhang Q and Huang M, Xu A (2004) Therapeutic drugs that behave as mechanism-based inhibitors of cytochrome P450 3A4. *Curr Drug*

Metab 5:415-42.

Zhou S, Chan E, Duan W, Huang M and Chen YZ (2005a) Drug bioactivation, covalent binding to target proteins and toxicity relevance. *Drug Metab Rev* 37:41-213.

Zhou S, Yung Chan S, Cher Goh B, Chan E, Duan W, Huang M and McLeod HL (2005b) Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs. *Clin Pharmacokinet* 44:279-304.

DMD Fast Forward. Published on March 28, 2007 as DOI: 10.1124/dmd.107.015016
This article has not been copyedited and formatted. The final version may differ from this version.

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_{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 *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 *in vivo* DDI information for these compounds were used. The concentrations of the compounds tested are as follows: 100 μM (square), 50 μM (triangle), 10 μM (circle), and 1 μM (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 ≥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.

	% remaining					
Substrate	Midazolam		Nifedipine		Testosterone	
Inhibitor	Ethynylestradiol	Ketoconazole	Ethynylestradiol	Ketoconazole	Ethynylestradiol	Ketoconazole
Days	3	3	3	3	3	3
Mean	33.7	108.0	35.7	112.3	46.9	101.8
S.D.	4.0	6.7	2.7	6.0	5.9	5.8
C.V.	12.0	6.2	7.4	5.3	12.5	5.7

TABLE 2.

K_I and k_{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.

Inhibitor		Substrate		
		Midazolam	Nifedipine	Testosterone
Erythromycin	K_I (μM)	12.1	11.3	10.9
	k_{inact} (min^{-1})	0.0215	0.0295	0.0352
Clarithromycin	K_I (μM)	15.5	15.9	12.9
	k_{inact} (min^{-1})	0.0192	0.0244	0.0324
Verapamil	K_I (μM)	2.55	3.12	5.79
	k_{inact} (min^{-1})	0.0277	0.0486	0.0591

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 ($+K_3Fe(CN)_6$) or without ($-K_3Fe(CN)_6$) potassium ferricyanide was shown below. Judgement of the reversibility was determined as described in *Methods*.

Inhibitor	Concentration	% of control (0 min)		% of control (30 min)		Judgement	Reported
	(μM)	$-K_3Fe(CN)_6$	$+K_3Fe(CN)_6$	$-K_3Fe(CN)_6$	$+K_3Fe(CN)_6$		Information
Diltiazem	100	85.7	92.5	62.3	90.7	Quasi-irreversible	MI-complex
Verapamil	10	71.5	80.1	41.2	70.1	Quasi-irreversible	MI-complex
Nicardipine	1	58.3	75.5	53.3	96.6	Quasi-irreversible	MI-complex
Amlodipine	100	68.0	72.1	49.3	73.2	Quasi-irreversible	MI-complex
Erythromycin	100	71.4	88.1	51.0	73.5	Quasi-irreversible	MI-complex

Clarithromycin	100	74.8	89.7	53.7	76.2	Quasi-irreversible	MI-complex
Troleandomycin	100	49.4	68.2	39.2	66.9	Quasi-irreversible	MI-complex
Sertraline	100	51.0	65.1	40.3	73.2	Quasi-irreversible	
Clozapine	100	76.5	76.4	62.6	66.3	Irreversible	Covalent binding to cellular proteins and GSH
Delavirdine	10	42.3	66.1	27.3	27.9	Irreversible	Covalent binding to microsomal proteins
Mibefradil	1	32.4	41.4	24.5	24.8	Irreversible	Irreversible binding
Ethynylestradiol	50	45.8	63.5	30.2	31.4	Irreversible	Covalent binding to apoproteins and heme of CYP
Prazosin	100	93.9	96.8	68.6	75.2	Irreversible	
Bromocriptine	1	70.4	87.7	63.2	65.5	Irreversible	

Bepridil	100	63.4	63.5	48.9	52.0	Irreversible
Bupivacaine	100	88.1	93.0	57.6	66.3	Irreversible
Buprenorphine	100	42.1	52.7	17.8	19.5	Irreversible

Fig. 1

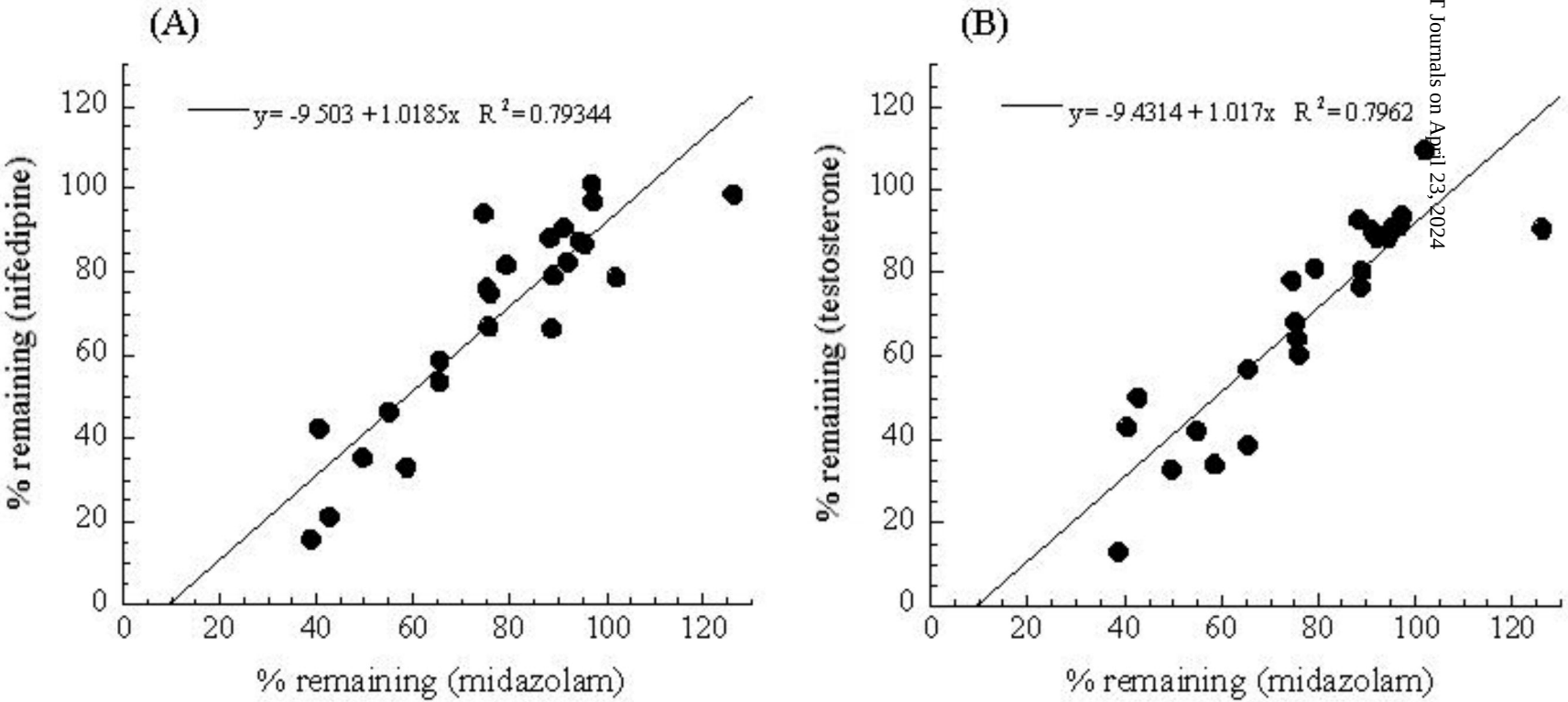

Fig. 2

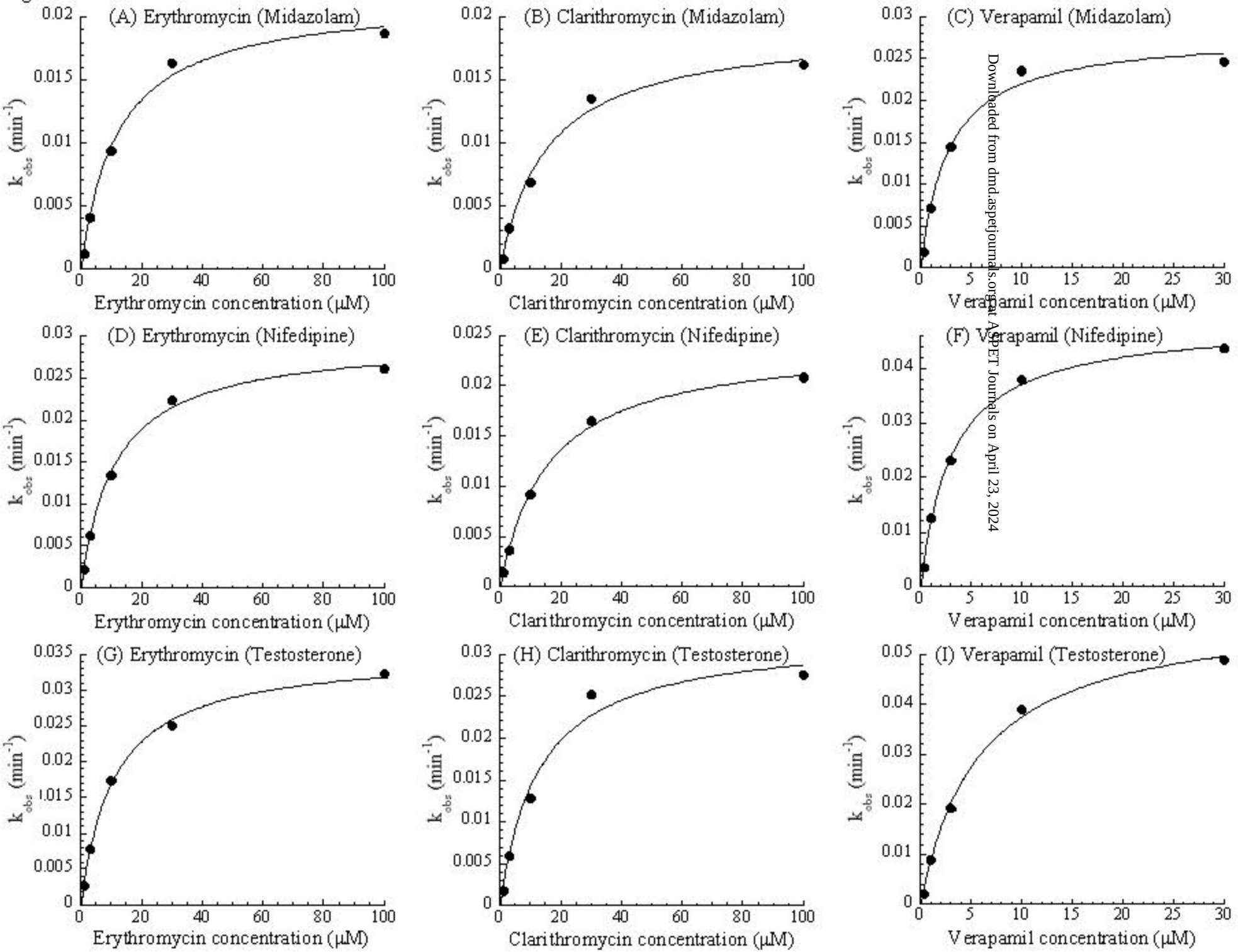

Fig. 3

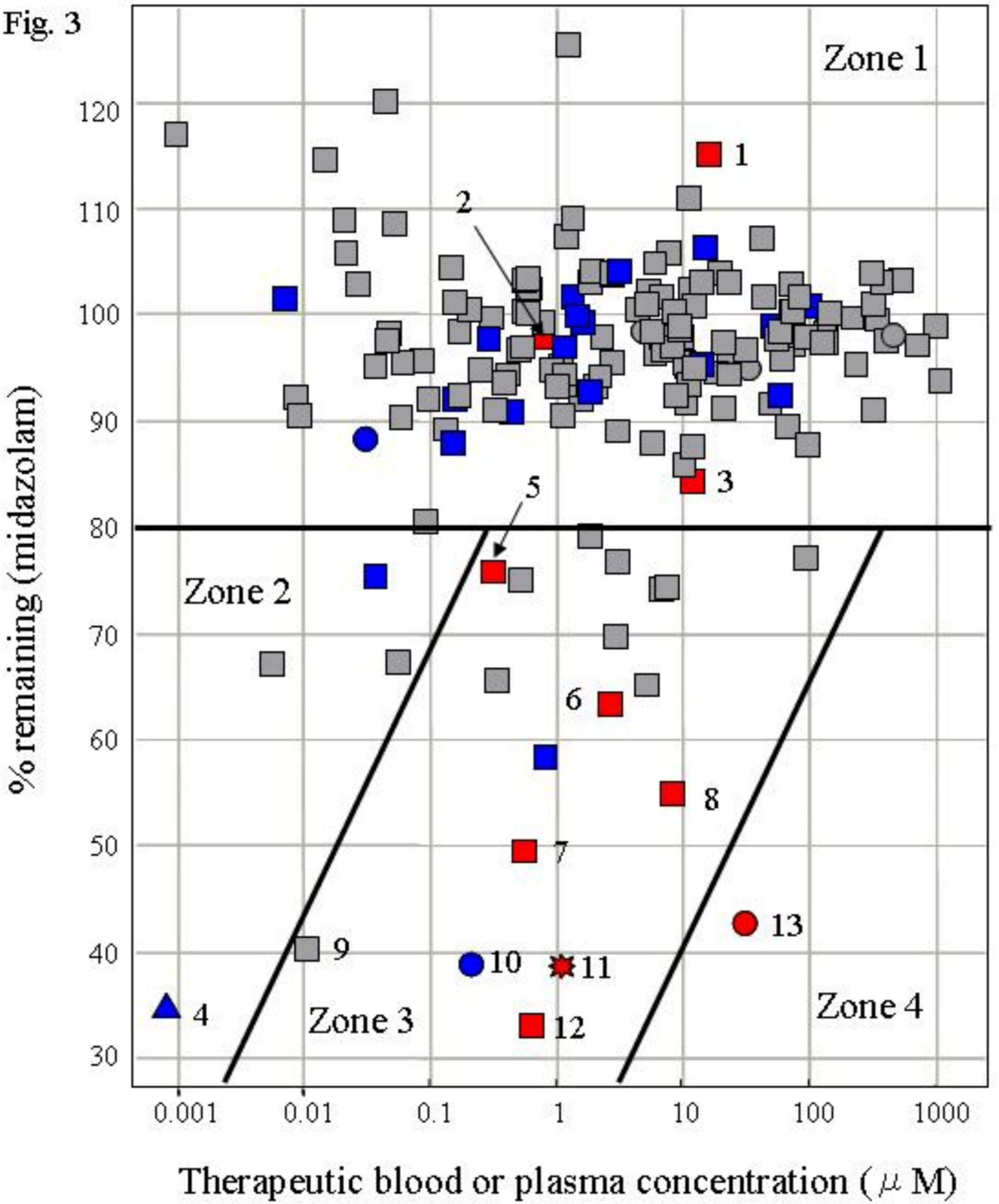

Fig. 4