Quantitative Prediction of Mechanism-Based Inhibition Caused by Mibefradil in Rats

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List of nonstandard abbreviations:

MBI, mechanism-based inhibition; DDI, drug-drug interaction; MDZ, midazolam; PBPK, physiologically-based pharmacokinetic; CYP, cytochrome P450; AUC, area under the concentration-time curve; IVIVE, in vitro-in vivo extrapolation; RLM, rat liver microsomes.
Abstract

It was previously demonstrated that mibefradil, which shows mechanism-based inhibition (MBI) in humans, also caused drug-drug interaction (DDI) with midazolam (MDZ) in rats. In this study, we aimed to quantitatively predict the DDI observed in rats using a physiologically-based pharmacokinetic (PBPK) model from in vitro inactivation parameters. For more precise predictions, contribution ratios of cytochrome P450 (CYP) isozymes involved in MDZ metabolism and inactivation parameters of mibefradil against each isozyme were incorporated in the predictive model. The evaluation of metabolic rate using recombinant CYPs suggested that CYP3A2 and CYP2C11 contributed to 89% and 11% of MDZ metabolism, respectively. Inactivation studies of mibefradil against the two isozymes showed that the maximum inactivation rate constants ($k_{\text{inact}}$) were considerable in both isozymes (0.231-0.565 min$^{-1}$), whereas the inhibitor concentration producing half the $k_{\text{inact}}$ ($K_{\text{I,app}}$) of CYP3A2 (0.263-0.410 μM) was a good deal lower than CYP2C11 (6.82-11.4 μM). As a result of predicting the DDI using the PBPK model, predicted increases in area under the concentration-time curves (AUCs) of MDZ with co-administration of mibefradil (284% and 510% at 6 mg/kg and 12 mg/kg of mibefradil, respectively) closely corresponded to the observed values (226% and 545%, respectively). From those results, it was thought that the construction of a predictive model for DDI using the PBPK model in detail would enable us to quantitatively predict in vivo DDI from in vitro data. This approach to predict DDI based on the
contributing isozymes would be important for predicting clinical DDIs of drugs metabolized by multiple enzymes.
Introduction

Irreversible inhibitors of metabolizing enzymes, as represented by mechanism-based inhibitors, may cause unanticipated adverse effects based on drug-drug interactions (DDIs) due to affecting the blood concentration of a co-administered drug for a long time because they continue to inhibit the enzyme until the inactivated enzyme is replaced by a newly synthesized one (Ito et al., 1998; Venkatakrishnan et al., 2003). Guidance from the Ministry of Health, Labour and Welfare in Japan (MHLW) (http://www.nihs.go.jp/mhlw/tuuchi/2001/010604-813/010604-813.pdf) as well as draft guidance from the US Food and Drug Administration (FDA) (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) and European Medicines Evaluation Agency (EMEA) (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf) all stipulate the need to evaluate mechanism-based inhibition (MBI).

In vitro-in vivo extrapolation (IVIVE) methods to predict clinical DDI from in vitro inhibition data are therefore important for drug development. Although we recently reported that the prediction method for competitive inhibitors using an in vivo inhibition constant ($K_i$) and a physiologically-based pharmacokinetic (PBPK) model was more accurate than the conventional method (Kato et al., 2008), there were inhibitors causing DDI which could not be predicted successfully in the manner of competitive inhibitors, and one of the possible reasons is that they
were mechanism-based inhibitors. For mechanism-based inhibitors, there have also been reports on methods to predict clinical DDI based on in vitro CYP inhibition data, although it is more challenging than predicting competitive inhibitors. Although a method of visually and easily categorizing the clinical DDI risk caused by MBI using in vitro inhibition data from the early stage of drug development was reported (Sekiguchi et al., 2009), for candidate drugs it is necessary to estimate the inactivation parameters and evaluate a more detailed DDI risk in clinic. Reported methods to predict the degree of DDI caused by MBI using in vitro inactivation parameters include a relatively simple method using a single inhibitor concentration (Wang et al., 2004; Venkatakrishnan and Obach, 2005) and a more complicated one using a PBPK model (Kanamitsu et al., 2000; Yamano et al., 2001). For a method using a single inhibitor concentration, it could often be the case that the unbound $C_{\text{max}}$ ($C_{\text{max,u}}$) is used as the inhibitor concentration to avoid a false negative prediction. However, there is a possibility that using $C_{\text{max,u}}$ could lead to overestimating DDI, while a method using a PBPK model could predict DDIs more precisely due to using the change in inhibitor and substrate concentrations over time. Additionally, a method using a single inhibitor concentration would be unsuited to the case of administering an inhibitor repeatedly, which could be overcome by using a PBPK model.

Ethical considerations make it difficult to investigate MBI in humans so a method of verifying it in animals would be valuable. Only a few reports investigating MBI in animals have been published
(Sekiguchi et al., 2008; Ogasawara et al., 2009). Demonstrating IVIVE in animals appears to increase confidence in predictions of clinical DDIs from in vitro data with human tissues. Recently, we reported that following the pretreatment of mibefradil, a mechanism-based inhibitor of CYP3A, at 6 mg/kg and 12 mg/kg 24 h before the oral administration of midazolam (MDZ), a probe substrate, in rats, the maximum plasma concentration (C_{\text{max}}) and the area under concentration-time curve (AUC) of MDZ were significantly elevated in comparison with the control (Sekiguchi et al., 2008). Therefore, we aimed to quantitatively predict the DDI observed in rats using a PBPK model from in vitro inactivation parameters in this study. Although it was suggested that MDZ was mainly metabolized by CYP3A in rats (Sekiguchi et al., 2008), not only CYP3A but also CYP2C isozymes were reported to produce metabolites of MDZ in rats (Chovan et al., 2007). Thereby, there is a possibility that CYP2C isozymes are also inactivated by mibefradil. How much metabolizing enzymes are involved in eliminating a probe and the potential of an inhibitor to inactivate each enzyme are very important factors in the analysis of the DDI. For more precise predictions, those parameters of the probe, MDZ, and the inhibitor, mibefradil, were evaluated in this study.

To establish and verify a predictive model of DDI caused by MBI, first, ratios of how much CYP isozymes contributing to MDZ metabolism were evaluated using recombinant microsomes. Then, inactivation parameters of mibefradil were evaluated in recombinant CYPs in which MDZ had been metabolized. Finally, a predictive model was constructed by inserting the in vitro parameters into the
PBPK model representing the plasma concentrations-time profiles of mibefradil and MDZ in rats.

and it was verified that the model could predict the DDI observed in rats.
Materials and Methods

Chemicals and reagents. MDZ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Hydroxymidazolam, 1’-hydroxymidazolam, and bufuralol hydrochloride were purchased from Ultrafine Chemicals Ltd. (Manchester, UK). Mibefradil dihydrochloride was obtained from Tocris Bioscience (Bristol, UK) and Sigma Chemical Co. (St. Louis, MO) for in vitro studies and in vivo studies, respectively. The reduced form β-nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Other reagents and solvents used were of the highest or high performance liquid chromatography grade.

Enzymes. Microsomes prepared from baculovirus-infected insect cells expressing individual rat CYP3A2 (rrCYP3A2), CYP2C11 (rrCYP2C11), and CYP2C13 (rrCYP2C13) besides cytochrome b5 and cytochrome P450 reductase, and control microsomes were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ). Pooled male rat liver microsomes (RLM) were purchased from Xenotech LLC (Lenexa, KS).

Animals. Male Sprague-Dawley rats at 8 weeks old (weighing 271–301 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animals were maintained on a 12 h/12 h, light/dark cycle with free access to CE-2 diet (CLEA Japan Inc., Tokyo, Japan) and water. The protocols for the animal experiments were approved by our institutional committee for animal care and use.

Metabolism of MDZ in recombinant enzymes. Metabolic rates of MDZ were evaluated in
rrCYP3A2, rrCYP2C11, and rrCYP2C13. The enzymes were diluted by 0.1 M potassium phosphate buffer (pH 7.4) at 20 pmol/ml of final concentration. Protein concentrations of incubation mixtures were adjusted at 0.2 mg/ml by adding control microsomes. After addition of NADPH to the enzyme solutions at 1 mM of final concentration and pre-incubation at 37°C for 5 min, the reaction was started by adding MDZ at 200 nM of final concentration (total volume of the reaction mixture: 900 μL). MDZ concentration in reaction mixtures, 200 nM, was thought to be considerably lower than \( K_m \) values of CYP3A2 and CYP2C11 on MDZ metabolism (Carr et al., 2006; Chovan et al., 2007). At 0, 4, 8, and 15 mins, the reaction was stopped by adding 150 μL of the reaction mixture to 75 μL of acetonitrile containing 200 nM of bufuralol as the internal standard (IS). Experiments were conducted in duplicate.

**Inactivation of MDZ metabolism by mibebradil.** After 5-min incubation at 37°C of a reaction mixture (45 μL) consisting of 1.11 mM of NADPH and 22.2 pmol/ml of rrCYP3A2 or 55.6 pmol/mL of rrCYP2C11 or 0.222 mg protein/ml of RLM in 0.1 M potassium phosphate buffer (pH 7.4), 5 μL of mibebradil solution at various concentrations in water (rrCYP3A2: 0.625, 1.25, 2.5, 5, 10, and 30 μM; rrCYP2C11: 3, 10, 25, 50, 100, 300, and 900 μM; RLM: 1.25, 2.5, 5, 10, and 30 μM) was added. In the case of rrCYP3A2 and RLM, after 0, 1, 2, or 5-min preincubation with mibebradil, 10 μL of reaction mixture was diluted with 188 μL of 0.1 M potassium phosphate buffer warmed at 37°C containing 1 mM of NADPH, and the reaction was started by adding 2 μL of 20
mM MDZ to the mixture immediately. In the case of rrCYP2C11, after 0, 2, 5, or 10-min preincubation with mibefradil, 20 μL of reaction mixture was diluted with 178 μL of 0.1 M potassium phosphate buffer warmed at 37°C containing 1 mM of NADPH, and the reaction was started by adding 2 μL of 20 mM MDZ to the mixture immediately. After 3 min, the reaction was stopped by adding 100 μL of acetonitrile containing 200 nM of bufuralol as the IS. Experiments were conducted in duplicate.

**In vivo study.** Mibefradil dihydrochloride was dissolved in water at 6 mg/ml or 12 mg/ml as the mibefradil concentration. Mibefradil was administered orally at 1 ml/kg to rats under a fed condition (n=4). Blood samples were collected from the jugular vein at 0.17, 0.5, 1, 2, 4, 6, 8, and 24 h after administration of mibefradil and immediately centrifuged to obtain plasma samples. The plasma samples were kept at approximately -80°C until analysis.

**Analysis of MDZ metabolites and mibafradil.** The concentrations of 1’-hydroxymidazolam, 4-hydroxymidazolam, and mibefradil were measured by high-performance liquid chromatography-electrospray tandem mass spectrometry.

For the in vitro studies, the samples with acetonitrile containing the IS added were centrifuged, and 10 μL of supernatants were injected to a Quattro II tandem mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a MAGIC2002 liquid chromatograph (Michrom BioResources, Inc., Auburn, CA) and a HTC PAL auto-injector (CTC Analytics AG, Zwingen, Switzerland). The
chromatography was performed on a Symmetry C18 column (150 × 2.1 mm i.d.; Waters Corp., Milford, MA) with 0.1% formic acid-methanol (59:41) as the mobile phase at a flow rate of 200 μl/min. The electrospray ionization was operated in the positive ion mode, and quantification was obtained using a selected reaction monitoring method (1'-hydroxymidazolam: m/z 342 > 203, 4-hydroxymidazolam: m/z 342 > 297, IS (bufuralol): m/z 262 > 188).

Plasma samples with added IS were prepared with Oasis MCX μElution plates (Waters Corp.). After loading samples and washing stepwise with 2% formic acid and methanol, analytes were eluted with 40 μl of methanol containing 2% ammonium and then 40 μl of water was added. From this solution, 10 μl portions were injected into a Quattro micro tandem mass spectrometer (Micromass Ltd) equipped with an Alliance 2795 Separation Module (Waters Corp.). The chromatography was performed on a SunFire C18 column (30 × 2.1 mm i.d., Waters Corp.) at a flow rate of 200 μl/min. The mobile phases were A (0.1% formic acid aqueous, v/v) and B (methanol containing 0.1% formic acid, v/v). The following gradient program was used: 0–3.5 min, linear change from mobile phase A/B (95:5, v/v) to A/B (5:95, v/v); 3.5–4 min, maintenance of A/B (5:95, v/v); 4.1–9 min, maintenance of A/B (95:5, v/v). The electrospray ionization was operated in the positive ion mode, and quantification was obtained by selected reaction monitoring (mibefradil: m/z 496 > 202, IS (bufuralol): same as above).

Data analysis. Contribution ratios of CYP isozymes involved in MDZ metabolism. The
contribution ratio was estimated by multiplying the metabolic rate of MDZ in each microsomes expressing the recombinant CYP by the abundance ratio of each isozyme in rat liver. The metabolic rate of MDZ was represented by the sum of the production rates of the two major metabolites, 1’-hydroxymidazolam and 4-hydroxymidazolam, to which MDZ was reported to be almost mainly metabolized in rats (Ghosal et al., 1996b). The production rate was estimated by dividing the metabolite concentration in a linear phase of metabolite concentration-incubation time plot by the enzyme concentration and incubation time. The abundance ratio of each isozyme was found in published data (Imaoka et al., 1990; Ghosal et al., 1996a; Shimada et al., 1997).

Kinetic parameters of enzyme inactivation. For incubations in recombinant enzymes and RLMs with or without mibefradil, the natural logarithm of the percentage of remaining activity involved in the production of 1’-hydroxymidazolam or 4-hydroxymidazolam was plotted against the preincubation time. The observed inactivation rate constants ($k_{\text{obs}}$) were determined from the slopes of the initial linear decline in activity (Silverman, 1995). The net value of $k_{\text{obs}}$ ($k'_{\text{obs}}$) was calculated by subtracting the $k_{\text{obs}}$ at zero of the inhibitor concentration from the $k_{\text{obs}}$ at each concentration of the inhibitor. Silverman reported that two principal constants useful in describing MBI, $k_{\text{inact}}$ and $K_{\text{I,app}}$, can be obtained experimentally (Silverman, 1995). $k_{\text{inact}}$ and $K_{\text{I,app}}$ represent the inactivation rate constant at infinite concentrations of inactivators and the inhibitor concentration when $k_{\text{obs}}$ is equal to half of $k_{\text{inact}}$, respectively. Values of $k_{\text{inact}}$ and $K_{\text{I,app}}$ were estimated by plotting $k'_{\text{obs}}$ against the
inhibitor concentration using the nonlinear regression program (WinNonlin ver. 5.2; Pharsight, Moutain View, CA) according to Equation 1 as determined by Silverman,

\[ k'_{\text{obs}} = \frac{k_{\text{inact}} \times I}{K_{I,\text{app}} + I} \]  

(1)

where \( I \) is the initial concentration of the inhibitor (Silverman, 1995). For the Equation 1, it was assumed that there is negligible change in \( I \) in the incubation period.

**Quantitative prediction of the in vivo DDI between MDZ and mibefradil.** A predictive model was constructed by inserting the in vitro parameters into the PBPK model, based on the modification of the method of Ito et al. (Ito et al., 2003). The differential equations for MDZ (\( S \)) and mibefradil (\( I \)) can be expressed as follows according to the perfusion model (Figure 1):

For MDZ:

\[ V_{\text{liver}} \times \frac{d S_{\text{liver}}}{dt} = Q_h \times S_{\text{pv}} - Q_h \times S_{\text{liver}}/K_{P,S} - f_{h,S} \times CL_{\text{int},S} \times E_{act}/E_0 \times S_{\text{liver}}/K_{P,S} \]  

(2)

\[ V_{\text{pv}} \times \frac{d S_{\text{pv}}}{dt} = Q_h \times S_{\text{sys}} + V_{\text{abs},S} - Q_h \times S_{\text{pv}} \]  

(3)

\[ V_{\text{abs},S} = k_{a,S} \times \text{Dose} \times F_{a,S} \times F_{g,S} \times e^{-k_{a,S} \times t} \]  

(4)

\[ V_{\text{sys},S} \times \frac{d S_{\text{sys}}}{dt} = Q_h \times S_{\text{liver}}/K_{P,S} - Q_h \times S_{\text{sys}} \]  

(5)

For mibefradil:

\[ V_{\text{liver}} \times \frac{d I_{\text{liver}}}{dt} = Q_h \times I_{\text{pv}} - Q_h \times I_{\text{liver}}/K_{P,I} - f_{b,I} \times CL_{\text{int},I} \times I_{\text{liver}}/K_{P,I} \]  

(6)

\[ V_{\text{pv}} \times \frac{d I_{\text{pv}}}{dt} = Q_h \times I_{\text{sys}} + V_{\text{abs},I} - Q_h \times I_{\text{pv}} \]  

(7)

\[ V_{\text{abs},I} = k_{a,I} \times \text{Dose} \times F_{a,I} \times F_{g,I} \times e^{-k_{a,I} \times t} \]  

(8)
\[ V_{\text{sys},I} \times \left( \frac{d S_{\text{sys}}}{dt} \right) = Q_h \times I_{\text{liver}} / K_{p,s} - Q_h \times I_{\text{sys}} \]  

(9)

where \( S_{\text{liver}}, S_{\text{pv}}, \) and \( S_{\text{sys}} \) represent the concentration of MDZ in the liver, portal vein, and central compartment, respectively; \( I_{\text{liver}}, I_{\text{pv}}, \) and \( I_{\text{sys}} \) represent the concentration of mibefradil in the liver, portal vein, and central compartment, respectively; \( V_{\text{liver}} \) and \( V_{\text{pv}} \) represent the volume of liver and portal vein, respectively; \( V_{\text{sys},S} \) and \( V_{\text{sys},I} \) represent the volume of distribution of MDZ and mibefradil in the central compartment, respectively; \( Q_h \) represents the blood flow rate; \( K_{p,s} \) and \( K_{p,i} \) represent the liver-to-blood concentration ratio of MDZ and mibefradil, respectively; \( f_{b,s} \) and \( f_{b,i} \) represent the unbound fraction of MDZ and mibefradil in blood, respectively; \( CL_{\text{int},S} \) and \( CL_{\text{int},I} \) represent the intrinsic metabolic clearance of MDZ and mibefradil, respectively; \( V_{\text{abs},S} \) and \( V_{\text{abs},I} \) represent the absorption velocity of MDZ and mibefradil, respectively; \( k_{a,s} \) and \( k_{a,i} \) represent the first-order absorption rate constant of MDZ and mibefradil, respectively; \( F_{a,s}, F_{a,i}, F_{g,s}, \) and \( F_{g,i} \) represent the fraction absorbed from the gastrointestinal tract of MDZ and mibefradil, and the intestinal availability of MDZ and mibefradil, respectively; \( E_{\text{act}} \) and \( E_{0} \) represent the active and total enzyme in the liver, respectively. It was assumed that mibefradil is eliminated only by the liver (Wiltshire et al., 1992). It was also assumed that non-metabolic clearance of MDZ could be negligible, because excretion into urine of unchanged MDZ administered intravenously was reported to be below 0.01% of the dosing amount in rats (Bittner et al., 2003). It was also assumed that the distribution of MDZ and mibefradil in the liver rapidly reaches equilibrium, and the unbound concentrations in the
hepatic vein are equal to those in the liver at equilibrium (well-stirred model). Also, gastrointestinal absorption is assumed to be described by a first-order rate constant. Plasma concentrations of MDZ from the literature (Sekiguchi et al., 2008) and mibefradil measured by the procedure described above when they were administered solely \( (E_{\text{act}} = E_0) \) were fitted into the PBPK model described above, and \( \text{CL}_{\text{int,5}}, \text{CL}_{\text{int,6}}, k_{\text{a,5}}, k_{\text{a,6}}, V_{\text{sys,5}}, \) and \( V_{\text{sys,6}} \) were calculated using SAAM II ver. 1.2 software (Saam Institute, Inc., Seattle, WA).

The differential equation for \( E_{\text{act}} \) can be described as follows:

\[
\frac{dE_{\text{act}}}{dt} = k_{\text{deg}}(E_0 - E_{\text{act}}) - R_1/100(k_{\text{inact,1}}E_{\text{act}}f_{b,1}1_{\text{liver}}/K_{p,1})(K_{L_{\text{app,1}}}+f_{b,1}1_{\text{liver}}/K_{p,1}) - R_2/100(k_{\text{inact,2}}E_{\text{act}}f_{b,1}1_{\text{liver}}/K_{p,1})/(K_{L_{\text{app,2}}}+f_{b,1}1_{\text{liver}}/K_{p,1})
\]

where \( k_{\text{deg}} \) represents the degradation rate constant (turnover rate constant) of enzyme; \( R_1 \) and \( R_2 \) represent the contribution ratio of CYP3A2 and CYP2C11, respectively; \( k_{\text{inact,1}} \) and \( k_{\text{inact,2}} \) represent the \( k_{\text{inact}} \) of mibefradil against CYP3A2 and CYP2C11, respectively; \( K_{L_{\text{app,1}}} \) and \( K_{L_{\text{app,2}}} \) represent the \( K_{L_{\text{app}}} \) of mibefradil against CYP3A2 and CYP2C11, respectively. In the absence of mibefradil, the enzyme content in the liver is at steady state and the degradation rate \( (k_{\text{deg}}E_0) \) is equal to the synthesis rate, which was assumed to be unaffected by mibefradil.

The physiological parameters of rat and pharmacokinetic parameters of MDZ and mibefradil used in the simulation are listed in Table 1. Using SAAM II and kinetic parameters for enzyme inactivation obtained in in vitro studies, the above differential equations were numerically solved to
simulate the time courses of the active enzyme content in the liver ($E_{\text{act}}$) and MDZ concentration in blood. According to the literature, the dosing route and regimen for the DDI study of MDZ and mibefradil were scheduled as follows. Twenty four hours after oral administration of mibefradil at 6 mg/kg or 12 mg/kg, MDZ was administered orally at 10 mg/kg (Sekiguchi et al., 2008). Simulated values of MDZ concentrations, $C_{\text{max}}$, and AUC were compared with the observed data.
Results

**Contribution ratios of CYP isozymes involved in MDZ metabolism.** Contribution ratios of CYP isozymes estimated using the production rate of MDZ metabolites in recombinant CYPs and the abundance ratios in the liver taken from publication data are shown in Table 2. CYP3A2 exists in rat liver to a lesser extent than CYP2C11, but metabolized MDZ much more rapidly, suggesting that CYP3A2 mainly contributes to MDZ metabolism, and CYP2C11 only slightly contributes.

Metabolites produced by CYP2C13 were not observed.

**Inactivation parameters of mibefradil against rrCYP3A2, rrCYP2C11, and RLM.** Effects of mibefradil concentration and preincubation time on 4- or 1’-hydroxylation of MDZ by rrCYP3A2, rrCYP2C11, and RLM are shown in Figure 2. In all enzyme sources, the production of metabolites decreased according to mibefradil concentration and preincubation time, suggesting that time-dependent inhibition was observed. $K_{\text{I,app}}$ and $k_{\text{inact}}$ values of mibefradil against each enzyme are shown in Table 3. The $k_{\text{inact}}$ in all enzymes were large values and almost similar, whereas the $K_{\text{I,app}}$ value in rrCYP2C11 was significantly larger than in rrCYP3A2 and RLM.

**Plasma concentrations of mibefradil in rats.** Figure 3 shows plasma concentration-time profiles of mibefradil after oral administration at 6 mg/kg or 12 mg/kg in rats and also shows estimated values which were obtained by fitting values into the model represented in Figure 1. Estimated plasma concentrations 24 h after administration were close to the observed values, while estimated
values 10 or 30 min after administration were lower than the observed values. The $k_a$ and $V_{sys}$ values of mibefradil estimated by the fitting were almost similar between 6 mg/kg and 12 mg/kg (Table 1). However, the $CL_{int}$ values at 6 mg/kg and 12 mg/kg were estimated to be 75.6 and 49.4 l/h, suggesting a non-linearity.

**Prediction of DDI caused by MBI.** Previously, as a result of the oral administration of mibefradil at 6 mg/kg or 12 mg/kg 24 h before oral administration of MDZ in rats, it was shown that plasma concentrations of MDZ with a pretreatment of mibefradil were elevated compared with the control (Sekiguchi et al., 2008). Prediction of the DDI was conducted using the model represented in Figure 1. The prediction showed that active content of CYP3A2 was significantly reduced by administering mibefradil, although that of CYP2C11 was reduced less than or comparable to 20% even at 12 mg/kg of mibefradil (Figure 4). Active content of the total enzymes in the liver was predicted at a time-profile similar to CYP3A2.

Observed and predicted plasma concentrations of MDZ with and without co-administration of mibefradil are shown in Figure 5, and $C_{max}$ and AUC values in those groups are shown in Table 4. The model predicted that co-administration of mibefradil would elevate MDZ concentration, and the predicted increases in MDZ concentrations, $C_{max}$, and AUC resulting from the co-administration closely corresponded to the observed values.
Discussion

We thought that when a decision on the development of candidate drugs possessing a potential for MBI is being made, an evaluation of in vivo MBI in animals coupled with an evaluation of in vitro DDI using human enzymes would increase the levels of confidence for that decision. We therefore previously verified whether an inhibitor which exhibits MBI in humans also gives rise to DDI in rats by ascertaining that pretreatment with mibefradil significantly elevated C\text{max} and AUC values of orally-administered MDZ compared to the control (Sekiguchi et al., 2008). In this study, to further increase confidence levels, we aimed to quantitatively predict the DDI observed in rats from in vitro inactivation data.

To predict DDI caused by the inhibition of metabolism, the contribution ratios of enzymes involved in the metabolism of an interacted drug are critical factors to be considered. Although it was reported by us and other laboratories that MDZ was mainly metabolized by CYP3A in rats (Kose et al., 2005; Sekiguchi et al., 2008), CYP2C isozymes were also reported to produce metabolites of MDZ (Chovan et al., 2007). CYP3A4 is the most highly-expressed CYP isozyme in human liver (Shimada et al., 1994), whereas in rat liver CYP2C11 is highly-expressed (Imaoka et al., 1990), and CYP2C13 was reported to be comparable to CYP3A2 (Imaoka et al., 1990). So, first, the contribution ratios of CYP3A2, 2C11, and 2C13 involved in MDZ metabolism were evaluated using the production rate of metabolites in recombinant CYPs and the abundance ratios in rat liver.
(Imaoka et al., 1990; Ghosal et al., 1996a; Shimada et al., 1997). Because it was reported that the major metabolites were 4-hydroxymidazolam and 1’-hydroxymidazolam in rats as well as in humans (Ghosal et al., 1996b), the production rates of these two metabolites were used for the estimation. The result showed that production rate by CYP3A2 was extremely large, but no metabolites by CYP2C13 were observed (Table 2). Compared to the results reported by Chovan, the rate by CYP3A2 was relatively more rapid in our study. However, the result that CYP3A2 and 2C11 preferentially produced 4-hydroxymidazolam and 1’-hydroxymidazolam, respectively, was identical. Due to the metabolic rate, CYP3A2 was suggested to be the major enzyme involved in MDZ metabolism despite it being less abundant in the liver.

Next, the potential of mibefradil to exert MBI on CYP3A2 and CYP2C11 was evaluated. Because mibefradil was reported to induce MBI against CYP3A4 and CYP2C8 in humans (Polasek et al., 2004), it was considered likely that isozymes other than CYP3A in rats are also inactivated by mibefradil. As a result of evaluating the inhibitory activity of mibefradil on MDZ metabolism using recombinant microsomes, effects of mibefradil concentration and preincubation time were observed against not only CYP3A2 but also CYP2C11 (Figure 2), suggesting that mibefradil induced MBI against both isozymes. Comparing the inactivation parameters of the isozymes, \( k_{\text{inact}} \) values of both isozymes were large, and no obvious difference in the values was observed (Table 3). On the other hand, \( K_{\text{I,app}} \) value of CYP3A2 was considerably lower than that of CYP2C11, thereby suggesting that
mibefradil caused MBI against CYP3A2 more intensely. Additionally, the parameters of mibefradil against RLM were very similar to those against CYP3A2. This result appears to indicate that MBI by mibefradil was mainly dependent on the inactivation of CYP3A2.

Next, to verify whether prediction of in vivo DDI from in vitro data is possible, a predictive model for MBI was constructed. For the prediction, we used a PBPK model which represents change in inhibitor and substrate concentrations over time and has the advantage of a detailed DDI analysis (Figure 1) (Ito et al., 2003). To construct the PBPK model, plasma concentrations of an inhibitor, mibefradil, administered solely were measured (Figure 3). As a result of fitting calculations of the observed concentrations into the PBPK model, CLint value at 12 mg/kg was smaller than that at 6 mg/kg, in brief non-linearity in the elimination phase was observed (Table 1). The phenomenon that plasma concentrations of mibefradil rose more than the dosage ratio had been observed in humans previously (Welker et al., 1998). According to a report on the metabolic pathway of mibefradil, oxidative metabolites were major (Wiltshire et al., 1997b). Therefore, this non-linear pharmacokinetics appeared to occur due to the auto-inactivation of metabolism of mibefradil by MBI. Because of the non-linearity, pharmacokinetic parameters of mibefradil were estimated and used for the prediction separately at each dose. Plasma concentrations of mibefradil calculated by the fitting were well consistent with the observed values except for the initial period after administration. Thus, it was judged that there was no problem with the prediction of the DDI where MDZ was
administered 24 h after mibefradil administration.

Then, we predicted the DDI by inserting the in vitro parameters into the PBPK model constructed. It was defined that CYP3A2 and CYP2C11 contribute to MDZ metabolism at the contribution ratios shown in Table 2, and inactivation of the two isozymes occurs according to the inactivation parameters shown in Table 3. In the model, mibefradil administered at 6 mg/kg or 12 mg/kg was predicted to reduce active CYP content in the liver (Figure 4), and increase plasma concentrations of MDZ (Figure 5), consequently causing MBI. Predicted increases in $C_{\text{max}}$ and AUC of MDZ with the co-administration of mibefradil closely corresponded to the observed values (Table 4). In the above prediction, it was assumed that intestinal metabolism is not affected by mibefradil administration, because it was reported that $F_{a,s} \times F_{g,s}$ of MDZ was relatively high, about 0.8 in rats (Higashikawa et al., 1999; Kuze et al., 2009). If it was assumed that intestinal metabolism was entirely inhibited by mibefradil ($F_{a,s} \times F_{g,s}$ of MDZ = 1), $C_{\text{max}}$ and AUC of MDZ co-administered with mibefradil were predicted to increase up to about 30% (196 ng/ml and 291 ng×h/ml at 6 mg/kg of mibefradil, 344 ng/ml and 524 ng×h/ml at 12 mg/kg of mibefradil, respectively) compared with prediction results without inhibition of intestinal metabolism. Although in this study the prediction in which intestinal metabolism was not inhibited was closer to the observed results, it may be desirable to predict DDI both with and without the intestinal metabolism inhibited because a methodology for predicting inactivation of intestinal enzymes has not been established. However, in
In this study, it was thought that both prediction results would almost correspond to the observed data. Additionally, in the prediction of MBI, $k_{\text{deg}}$ is a very important factor. Because rigorous values of $k_{\text{deg}}$ of rat CYP isozymes have not been clear yet, we assumed that $k_{\text{deg}}$ values of rat CYPs are the same. Under such an assumption, the prediction gave results close to the observed data. These results demonstrate that construction of a predictive model using the PBPK model in detail would enable a precise prediction of DDI caused by MBI. As shown above, verification that the results predicted from in vitro data correspond to the in vivo data in animals will increase confidence in results predicted by the same method in humans.

In this study, since two isozymes, CYP3A2 and CYP2C11, contribute to MDZ metabolism, their contribution ratios and inactivation parameters were inserted into the predictive model. As far as we know, this is the first study in which inserting inactivation of multiple isozymes into the predictive model of DDI using a PBPK model was tried. Maybe due to the difference in $K_{I,\text{app}}$ values, active CYP3A2 content was predicted to be decreased considerably by mibefradil, whereas active CYP2C11 content was predicted to decrease slightly (Figure 4). Change in active content of total enzymes covered by the two enzymes was almost similar to that of CYP3A2, suggesting that the DDI between MDZ and mibefradil in rats may be mostly accounted for by the contribution of CYP3A2. Although in this study it was predicted that the inactivation of one enzyme contributes extremely to the DDI, considering the inactivation of multiple isozymes contributing to substrate
metabolism would be meaningful for a more accurate prediction. Since there are many drugs metabolized by multiple enzymes in clinic, this approach is expected to contribute to improved prediction accuracy.

In conclusion, it was suggested that the DDI between MDZ and mibefardil in rats was caused by inactivation of CYP3A2 mainly, and CYP2C11 slightly. As a result of predicting DDI using the PBPK model inserted with in vitro inactivation parameters against both the enzymes, predicted values of the increase in MDZ concentrations closely corresponded to the observed values. This result demonstrates that the construction of a predictive model for DDI using the PBPK model in detail would enable us to predict in vivo DDI accurately. Predicting DDI based on the contributing isozymes as shown in this study is expected to be applied to the prediction of clinical DDIs of drugs metabolized by multiple enzymes.
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Authorship Contributions

Participated in research design: Sekiguchi, Kato

Conducted experiments: Sekiguchi, Kato, Takada, Watanabe

Contributed new reagents or analytic tools: None

Performed data analysis: Sekiguchi, Kato

Wrote or contributed to the writing of the manuscript: Sekiguchi, Kato, Takata, Mitsui, Aso, Ishigai
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Venkatakrishnan K and Obach RS (2005) In vitro-in vivo extrapolation of CYP2D6 inactivation by
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Metabolism in hepatic microsomes from rat, marmoset, cynomolgus monkey, rabbit and man. *Xenobiotica* **27**:539-556.

Legends for figures

Figure 1
Physiological model of the time-profiles of midazolam and mibefradil concentrations in rats.

Figure 2
Effects of mibefradil concentration and preincubation time on midazolam hydroxylation by rrCYP3A2 (A), rrCYP2C11 (B), and rat liver microsomes (C). The y-axis represents the formation rate of 4-hydroxymidazolam (A and C) or 1’-hydroxymidazolam (B). The control activities were A, 12.2 pmol/min/pmol P450; B, 2.77 pmol/min/pmol P450; C, 2.12 pmol/min/μg protein.

Figure 3
Plasma concentrations of mibefradil after oral administration at 6 mg/kg and 12 mg/kg in rats.
Closed triangles and squares represent observed values at 6 mg/kg and 12 mg/kg, respectively.
Dashed and solid lines represent simulated values at 6 mg/kg and 12 mg/kg by the fitting calculation, respectively.

Figure 4
Predicted change in active CYP contents after the administration of mibefradil at 6 mg/kg (A) and 12 mg/kg (B) in rats. Dashed, thin, and heavy lines represent the change in CYP3A2, CYP2C11 and total CYP contents, respectively.

**Figure 5**

Predicted and observed plasma concentrations of midazolam in rats with or without pretreatment of mibefradil. Open circles, triangles, and squares represent the observed values of the control, pretreatment of mibefradil at 6 mg/kg and 12 mg/kg, respectively. Dashed, thin, and heavy lines represent the predicted values of the control, pretreatment of mibefradil at 6 mg/kg and 12 mg/kg, respectively.
## Tables

### Table 1

Physiological parameters of rat and pharmacokinetic parameters of midazolam and mibefradil used in the simulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physiological parameter of rat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>250 g</td>
<td>Lin et al. (1982)</td>
</tr>
<tr>
<td>$V_h$</td>
<td>0.011 l</td>
<td>Lin et al. (1982)</td>
</tr>
<tr>
<td>$V_{pv}$</td>
<td>0.00028 l</td>
<td>assumed</td>
</tr>
<tr>
<td>$Q_h$</td>
<td>0.882 l/h</td>
<td>Lin et al. (1982)</td>
</tr>
<tr>
<td>$E_0$</td>
<td>5 mmol/g liver</td>
<td>CYP3A4 content in the liver in human Iwatsubo et al. (1997)</td>
</tr>
<tr>
<td>$k_{deg}$</td>
<td>0.03 h$^{-1}$</td>
<td>rat P450 Shiraki and Guengerich (1984)</td>
</tr>
<tr>
<td><strong>Midazolam</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>2500 μg</td>
<td>Sekiguchi et al. (2008)</td>
</tr>
<tr>
<td>$F_{a\times F_g}$</td>
<td>0.78</td>
<td>Higashikawa et al. (1999), Kuze et al. (2009)</td>
</tr>
<tr>
<td>$f_a$</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>$k_{deg}$</td>
<td>1</td>
<td>assumed</td>
</tr>
<tr>
<td>$CL_{int,S}$</td>
<td>830 l/h</td>
<td>by fitting</td>
</tr>
<tr>
<td>$CL_{int,S,1}$</td>
<td>740 l/h</td>
<td>using contribution ratio of CYP3A2</td>
</tr>
<tr>
<td>$CL_{int,S,2}$</td>
<td>90 l/h</td>
<td>using contribution ratio of CYP2C11</td>
</tr>
<tr>
<td>$k_a,S$</td>
<td>5.62 h$^{-1}$</td>
<td>by fitting</td>
</tr>
<tr>
<td>$V_{sys,S}$</td>
<td>0.949 l</td>
<td>by fitting</td>
</tr>
<tr>
<td><strong>Mibefradil</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>1500 μg</td>
<td>Sekiguchi et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>3000 μg</td>
<td>Sekiguchi et al. (2008)</td>
</tr>
<tr>
<td>$F_{a\times F_g}$</td>
<td>1</td>
<td>assumed</td>
</tr>
<tr>
<td>$f_a$</td>
<td>0.035</td>
<td>Wiltshire et al. (1997a)</td>
</tr>
<tr>
<td>$k_{deg}$</td>
<td>1</td>
<td>assumed</td>
</tr>
<tr>
<td>$CL_{int,I}$</td>
<td>6mg/kg dosed 75.6 l/h</td>
<td>by fitting</td>
</tr>
<tr>
<td></td>
<td>12mg/kg dosed 49.4 l/h</td>
<td>by fitting</td>
</tr>
<tr>
<td>$k_a,I$</td>
<td>6mg/kg dosed 0.174 h$^{-1}$</td>
<td>by fitting</td>
</tr>
<tr>
<td></td>
<td>12mg/kg dosed 0.165 h$^{-1}$</td>
<td>by fitting</td>
</tr>
<tr>
<td>$V_{sys,I}$</td>
<td>6mg/kg dosed 3.81 l</td>
<td>by fitting</td>
</tr>
<tr>
<td></td>
<td>12mg/kg dosed 3.55 l</td>
<td>by fitting</td>
</tr>
<tr>
<td>$k_{inact,1}$</td>
<td>23.1 h$^{-1}$</td>
<td>for 4-hydroxylation of MDZ by cCYP3A2</td>
</tr>
<tr>
<td>$k_{inact,2}$</td>
<td>33.9 h$^{-1}$</td>
<td>for 1'-hydroxylation of MDZ by cCYP2C11</td>
</tr>
<tr>
<td>$K_{I,app,1}$</td>
<td>130 μg/l</td>
<td>for 4-hydroxylation of MDZ by cCYP3A2</td>
</tr>
<tr>
<td>$K_{I,app,2}$</td>
<td>5630 μg/l</td>
<td>for 1'-hydroxylation of MDZ by cCYP2C11</td>
</tr>
</tbody>
</table>
Table 2

Contribution ratio of CYP isozymes involved in midazolam metabolism in rats

<table>
<thead>
<tr>
<th></th>
<th>Metabolic rate (pmol/min/pmol P450)</th>
<th>Abundance ratio</th>
<th>Contribution ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4-hydroxylation</td>
<td>1'-hydroxylation</td>
<td>Sum</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>0.620</td>
<td>0.369</td>
<td>0.989</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>0.0032</td>
<td>0.0392</td>
<td>0.0436</td>
</tr>
<tr>
<td>CYP2C13</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

N.C.: not calculated

a Abundance ratio of each enzyme in the liver was taken from literature.

b Contribution ratio was calculated using the production rate of the two metabolites and the abundance ratio.
Table 3

Inactivation parameters of mibefradil on midazolam metabolism in rrCYP3A2, rrCYP2C11, and rat liver microsomes

<table>
<thead>
<tr>
<th></th>
<th>4-Hydroxylation</th>
<th>1'-Hydroxylation</th>
<th>4-Hydroxylation</th>
<th>1'-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A2</td>
<td>0.386</td>
<td>0.263</td>
<td>0.238</td>
<td>6.82</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>0.238</td>
<td>6.82</td>
<td>0.565</td>
<td>11.4</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>0.390</td>
<td>0.596</td>
<td>0.203</td>
<td>0.737</td>
</tr>
</tbody>
</table>
Table 4

Comparison of observed and predicted $C_{\text{max}}$ and AUC values of midazolam in rats with or without pretreatment with mibefradil

Mibefradil was administered orally 24 h before midazolam administration. Predicted value was evaluated using the model and parameters in Figure 1 and Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>6mg/kg mibefradil</th>
<th>12mg/kg mibefradil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Simulated</td>
<td>Observed</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>51.9</td>
<td>55.4</td>
<td>123</td>
</tr>
<tr>
<td>ratio to the control (%)</td>
<td>100</td>
<td>100</td>
<td>237</td>
</tr>
<tr>
<td>$\text{AUC}_{0-3h}$ (ng·h/ml)</td>
<td>71.8</td>
<td>80.1</td>
<td>162</td>
</tr>
<tr>
<td>ratio to the control (%)</td>
<td>100</td>
<td>100</td>
<td>226</td>
</tr>
</tbody>
</table>
Figure 1

Liver CYP

- Production by turnover
- Degradation by turnover
- Inactivation by MBI

Mibefradil

1. Stomach
2. Systemic blood
3. Portal vein
4. Liver

- Dose
- $F_a \times F_g$
- $k_{a,I}$

MDZ

1. Stomach
2. Systemic blood
3. Portal vein
4. Liver

- Dose
- $F_a \times F_g$
- $k_{a,S}$

\[ f_{u,I} \times CL_{int,I} \quad \quad f_{u,S} \times CL_{int,S} \times E_{act}/E_0 \]
Figure 2

A) Formation rate (% of control) vs Preincubation time (min) for different concentrations of 3 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, 0.0625 μM, and 0 μM.

B) Formation rate (% of control) vs Preincubation time (min) for different concentrations of 90 μM, 30 μM, 10 μM, 5 μM, 2.5 μM, 1 μM, 0.3 μM, 0.15 μM, and 0 μM.

C) Formation rate (% of control) vs Preincubation time (min) for different concentrations of 3 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, and 0 μM.
Figure 3

![Graph showing plasma concentration over time after administration (h). The graph displays data with error bars and three distinct lines representing different conditions or groups.](image-url)
Figure 5

Plasma concentration (ng/mL) vs. Time after administration of midazolam (h)

- Solid line with square markers
- Dashed line with triangle markers
- Dot-dashed line with circle markers