DMD Fast Forward. Published on February 10, 2006 as doi:10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

DMD #8722

Journal: **DMD**

Full title

The canine CYP1A2 deficiency polymorphism dramatically affects the pharmacokinetics of YM-64227, a phosphodiesterase type 4 inhibitor

Author names

Daisuke Tenmizu, Kiyoshi Noguchi, Hidetaka Kamimura, Hisakazu Ohtani and Yasufumi Sawada

Affiliations

Drug Metabolism Research Laboratories, Astellas Pharmaceutical Inc., Tokyo, Japan (DT, KN, and HK) and Graduate School of Pharmaceutical Sciences, Tokyo University, Tokyo, Japan (HO and YS)

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

a) The canine CYP1A2 polymorphism in the pharmacokinetics of YM-64227

b) Corresponding author: Daisuke Tenmizu

Drug Metabolism Research Laboratories, Astellas Pharmaceutical Inc, 1-8, Azusawa 1-Chome, Itabashi-ku, Tokyo 174-8511, Japan

Tel: +81-3-5916-2159

Fax: +81-3-3960-6220

E-mail: daisuke.tenmizu@jp.astellas.com

c) The number of text pages: 40

The number of tables: 4

The number of figures: 6

The number of references: 31

The number of words in ***Abstract***: 234

The number of words in ***Introduction***: 463

The number of words in ***Discussion***: 1250

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

d) A list of nonstandard abbreviations

AIC: Akaike's information criterion

AUC_{iv}: area under the plasma concentration-time curve after intravenous administration

AUC_{oral}: area under the plasma concentration-time curve after oral administration

$CL_{int,\ in\ vitro}$: overall intrinsic metabolic clearance estimated from the *in vitro* study

$CL_{int,\ in\ vivo}$: overall intrinsic hepatic clearance calculated based on the *in vivo* pharmacokinetic information

$CL_{int,\ ns}$: intrinsic metabolic clearance for the non-saturable component

$CL_{int,\ s}$: intrinsic metabolic clearance for the saturable component

CL_{oral}: oral clearance ($= CL_{tot}/F_{oral}$)

$CL_{tot,\ blood}$: $= CL_{tot}/R_B$

F_a: the fraction absorbed from the intestinal tract

F_{oral}: absolute bioavailability

f_p: unbound fraction in plasma

Q_h: hepatic blood flow rate

R_B: blood-to-plasma concentration ratio

SNP: single nucleotide polymorphism

YM-64227: 4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1*H*)-one

Abstract

In a previous study, it was shown that the novel canine SNP CYP1A2 1117C>T yields an inactive enzyme. In this study, the effect that this SNP has on the pharmacokinetics of 4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1*H*)-one (YM-64227) was investigated. Plasma concentrations of the unchanged drug and five of its metabolites (MM-1 to MM-5) were determined after either intravenous or oral administration of YM-64227 to genotyped dogs (C/C, C/T, and T/T groups). Liver microsomes were prepared from these dogs to determine the *in vitro* metabolic clearance of YM-64227. After a single oral administration, the maximum plasma concentration and absolute bioavailability of YM-64227 in the T/T group were 17.1 times and 27.2 times higher than those in the C/C group, respectively, while the pharmacokinetics of YM-64227 after intravenous administration were not affected by genotype. The metabolic profiles in the T/T group were quite distinct from the others, *i.e.*, the main metabolite was MM-2 in the C/C group, while MM-1 and MM-5 were main metabolites in the T/T group. The formation clearances of MM-2 and MM-3 in the microsomes derived from T/T type dogs were significantly lower, whereas those of MM-1, MM-4, and MM-5 were not affected. A statistically significant correlation was observed between the *in vivo* and *in vitro* metabolic intrinsic clearances ($r = 0.82$, $p < 0.001$). The canine CYP1A2 1117C>T

SNP proved to be responsible for a substantial portion of the inter-individual variability in the pharmacokinetics of YM-64227.

Introduction

Cytochrome P450 (CYP) is a superfamily of enzymes that plays an important role in the oxidative metabolism of a wide variety of xenobiotics as well as endogenous compounds (Nelson *et al.*, 1996). The metabolic activity of CYP is affected by several factors such as inhibition by concomitant drugs, induction, and genetic polymorphism (Pelkonen *et al.*, 1998). The CYP polymorphisms have been investigated extensively in clinical settings, and it is well known that mutated alleles of CYP2C9, CYP2C19, and CYP2D6 cause altered plasma concentrations, which might in turn cause unforeseeable adverse reactions (Sullivan-Klose *et al.*, 1996; Furuta *et al.*, 1999; Kagimoto *et al.*, 1990).

A large number of CYP cDNA clones have been isolated, sequenced, and extensively studied in humans, mice, and rats (http://drnelson.utmem.edu/CytochromeP450.html). On the other hand, only a few studies on canine CYP cDNAs have been conducted, even though dogs are used extensively in pharmacological research and drug safety assessment studies. At present, a variety of canine CYP enzymes such as CYP1A1, CYP1A2 (Uchida *et al.*, 1990; Tenmizu *et al.*, 2004a), CYP2B11 (Graves *et al.*, 1990), CYP2C21, CYP2C41 (Uchida *et al.*, 1990; Blaisdell *et al.*, 1998), CYP2D15 (Sakamoto

et al., 1995), CYP2E1 (Lankford *et al.*, 2000), CYP3A12, and CYP3A26 (Ciaccio *et al.*, 1991; Fraser *et al.*, 1997) have been cloned and sequenced. Although gene analysis has revealed that some of these CYPs have variants, no obvious change in the plasma concentration of drug associated with single nucleotide polymorphisms (SNPs) has been reported for dogs. In fact, only a few studies have shown the possibility that interindividual variations in the pharmacokinetics of some drugs in dogs may be attributable to CYP polymorphisms (Paulson *et al.*, 1999; Azuma *et al.*, 2002; Mise *et al.*, 2004).

A novel canine CYP1A2 1117 C>T SNP that yields a stop codon was reported in a previous study (Tenmizu *et al.*, 2004a). The T-allele frequency was 0.39, which suggests that 10 to 15% of dogs would not express active CYP1A2 protein. It has also been shown that a subsequent reduction in the metabolic activity of CYP1A2 is reflected in the metabolic ratio (ratio of plasma parent drug to the major metabolite, MM-2) after oral administration of 4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1*H*)-one (YM-64227), a phosphodiesterase type 4 inhibitor, to dogs. However, the detailed influence of the SNP on the pharmacokinetics of YM-64227 is yet to be investigated. In this study, the

plasma concentrations of metabolites and the unchanged drug after intravenous and oral administration to genotyped dogs were investigated in order to clarify in detail the effect of canine CYP1A2 deficient polymorphism on the metabolism of YM-64227. Additionally, in order to compare intrinsic *in vivo* and *in vitro* metabolic clearances, the *in vitro* metabolic clearance of YM-64227 was determined using liver microsomes prepared from dogs that were used in the *in vivo* pharmacokinetics study.

Materials and Methods

Chemicals

YM-64227, its five metabolites (MM-1 to MM-5) (Figure 1), and the internal standard, 4-(3-chlorophenyl)-1-ethyl-7-(1-hydroxyethyl)pyrido[2,3-d]pyrimidin-2(1*H*)-one, were synthesized in the Chemistry Research Laboratories of Astellas Pharmaceutical Inc (Ibaraki, Japan). NADPH generating system solution A and B were purchased from BD (San Jose, CA, USA). Purified water from a Milli-Q system (Millipore Co., Bedford, MA, USA) was used throughout the study. All other chemicals used were commercially available and of the highest purity.

Animals

Male beagle dogs (body weight: 8.2 to 12.5 kg) were supplied by Covance Research Product (Kalamazoo, MI, USA). They were kept in climate-controlled rooms (temperature: 23 ± 1ºC, relative humidity: 55 ± 1%) with a light/dark cycle of 13 h/11 h. They had free access to both pellet diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water during the studies. This study was approved by the animal ethical committee of the Astellas Pharmaceutical Inc. Dogs were genotyped as C/C, C/T, or T/T at the 1117 nucleotide site of canine CYP1A2 by ASP-PCR and direct sequence analysis, as

previously described (Tenmizu *et al.*, 2004a).

Pharmacokinetics study of YM-64227 in genotyped dogs

YM-64227 solution prepared in a vehicle of 2% ethanol, 2% benzyl alcohol, 4% cremophor, and 92% water for injection was administered intravenously at 0.1 mg/kg to dogs (n = 5 per each group genotyped as C/C, C/T, or T/T) following overnight fasting. Blood (ca. 5 mL/time point) was collected from the forelimb vein using a heparinized syringe after 0, 0.1, 0.25, 0.5, 1, 2, 4, and 8 h. In another study, YM-64227 suspended in 0.5% methylcellulose solution was administered orally at 0.3 mg/kg to the same dogs (n = 5 per each group genotyped as C/C, C/T, or T/T) following an overnight fasting period. Blood (ca. 5 mL/time point) was collected from the forelimb vein using a heparinized syringe after 0, 0.25, 0.5, 1, 2, 4, and 8 h. After at least 1 week of washout period, YM-64227 suspended in 0.5% methylcellulose solution was administered orally at 0.3 mg/kg to the same dogs following an overnight fasting. Plasma was collected by centrifuging the blood. Plasma was stored frozen at -20ºC until it was analyzed to determine the concentrations of YM-64227 and its metabolites. Concentrations of YM-64227 and its metabolites were quantified using HPLC (Tenmizu *et al.*, 2004b). In brief, sample preparation used the liquid-liquid extraction using tert-butyl methyl ether.

Separation was achieved on a COSMOSIL-packed phenyl ethyl column (5 μm, 250 mm x 4.6 mm i.d., Nacalai Tesque Inc., Kyoto, Japan). The mobile phase consisting of (A) 80% 50 mM acetic acid / 20% acetonitrile and (B) 20% 50 mM acetic acid / 80% acetonitrile were used for gradient elution. YM-64227 and its metabolites were detected fluorimetrically at 400 nm with emission at 330 nm.

The pharmacokinetic parameters for YM-64227 and its metabolites were calculated using non-compartmental modeling and WinNonlin Professional version 3.3 (Pharsight, Mountain View, CA, USA). The pharmacokinetic parameters of C/C, C/T, and T/T dogs were compared using the *Tukey* test and SAS system version 8.2 (SAS Institute, Cary, NC, USA).

Protein binding to YM-64227 in dog plasma

Aliquots (0.04 mL) of 100 mM Na/K-phosphate buffer containing YM-64227 were added to 2-mL aliquots of dog plasma to yield concentrations of 2 and 2000 ng/mL. After incubation for 10 min at 37ºC, a 0.5-mL aliquot was taken from each sample to measure the total concentration, and the remainder was transferred to an ultrafiltration tube (VIVASPIN2, Sartorius AG, Goettingen, Germany). These tubes were centrifuged

at 1870 x g for 15 min at 37ºC, and then a 0.5-mL aliquot of filtrate was removed and used to measure the unbound plasma concentration. The YM-64227 concentrations in the plasma and filtrate samples were quantified using HPLC.

Blood-to-plasma concentration ratio (R_B) of YM-64227 in dogs

The R_B of YM-64227 was determined using heparinized whole blood (Lin *et al.*, 1982). Aliquots (0.05-mL) of 100 mM Na/K-phosphate buffer containing YM-64227 were added to 5-mL aliquots of dog blood preincubated at 37ºC to yield concentrations of 1, 10, and 100 ng/mL. After incubation for 5 min at 37ºC, the blood samples were centrifuged for 5 min at 1870 x g to obtain the plasma fraction. Concentrations of YM-64227 were quantified using HPLC.

Calculation of $CL_{int,\ in\ vivo}$

CL_{oral} was calculated by dividing CL_{tot} by F_{oral}. CL_h was then calculated from equation (1), using a Q_h value of 1620 mL/h/kg (Bischoff *et al.*, 1971; Dedrick *et al.*, 1973; Montandon *et al.*, 1975) and assuming that F_a was 1.0 and CL_r was 0.0. The R_B value of YM-64227 was 0.66 in dogs (estimated from this study).

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

$$CL_h = (CL_{oral} \bullet F_a / R_B - CL_r / R_B) / (1 + CL_{oral} \bullet F_a / Q_h / R_B) \quad (1)$$

Then, $CL_{int,\ in\ vivo}$ was calculated from the following equations using the dispersion model (Roberts and Rowland, 1986a; Iwatsubo *et al.*, 1997).

$$CL_h = Q_h (1 - F_h) \quad (2)$$

$$F_h = \frac{4a}{(1+a)^2 \exp\{(a-1)/2D_N\} - (1-a)^2 \exp\{-(a+1)/2D_N\}} \quad (3)$$

$$a = (1 + 4R_N \bullet D_N)^{1/2} \quad (4)$$

$$R_N = (f_p / R_B) \bullet CL_{int,\ in\ vivo} / Q_h \quad (5)$$

A D_N of 0.17 (Roberts and Rowland, 1986b; Iwatsubo *et al.*, 1996) was used to calculate $CL_{int,\ in\ vivo}$. The f_p value of YM-64227 used for 0.036 in dogs (estimated from this study).

YM-64227 metabolism in genotyped canine liver microsomes

Canine liver microsome preparation and the CYP1A2 1117 C>T SNP genotyping method was performed as described by Tenmizu *et al*. (2004a). YM-64227 was incubated in a reaction mixture (0.5 mL) consisting of 0.1 mg liver microsome protein

in the presence of an NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phoshate, 0.1 U/mL glucose-6-phosphate dehydrogenase, and 6 mM $MgCl_2$) and 100 mM Na/K-phosphate buffer. The YM-64227 concentrations used to estimate the kinetic parameters were 0.5 to 100 μM. Experiments were performed in duplicate. Linearity of metabolic activities for incubation time and microsome protein concentration was confirmed. Enzyme reactions were initiated by adding 30 μL of the NADPH generating system. After incubation for 10 min at 37ºC in a shaking water-bath, the reaction was terminated by adding 4 mL of tert-butyl methyl ether. After stopping the metabolic reaction, metabolite concentrations were quantified using HPLC.

Protein binding to YM-64227 in liver microsomes

Aliquots (0.04 mL) of 100 mM Na/K-phosphate buffer containing YM-64227 were added to 2-mL aliquots of a mixture consisting of 0.1 mg/mL liver microsomes and 100 mM Na/K-phosphate buffer to yield concentrations of 1, 10, and 100 μM. Other procedures were the same as described in “Protein binding to YM-64227 in dog plasma”.

Calculation of $CL_{int,\ in\ vitro}$

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

The clearance of each metabolite was calculated from the formation velocities of metabolites MM-1 to MM-5, and then the total was defined as the metabolic clearance of YM-64227 in its entirety. Equations (6) and (7) were fitted to the kinetic data for YM-64227 metabolism obtained with liver microsomes using SAS system version 8.2 to estimate K_m, V_{max}, and $CL_{int,\ ns}$.

$$v = V_{max} \bullet S/(K_m + S) \quad (6)$$

$$v = V_{max} \bullet S/(K_m + S) + CL_{int,\ ns} \bullet S \quad (7)$$

The method for evaluation of statistical fit [t1]was chosen using Akaike's information criteria (AIC) value as an index (Akaike, 1969). The $CL_{int,\ in\ vitro}$ values under linear conditions were calculated from the kinetic parameters obtained *in vitro* using equations (8) and (9), respectively.

$$CL_{int,\ in\ vitro} = V_{max}/K_m \quad (8)$$

$$CL_{int,\ in\ vitro} = V_{max}/K_m + CL_{int,\ ns} \quad (9)$$

The $CL_{int,\ in\ vitro}$ values expressed per mg microsome of protein calculated from the *in*

vitro metabolism study were expressed per gram of liver by taking the microsome protein content per gram of liver. The scaling factor value for the dogs used for the conversion of $CL_{int,\ in\ vitro}$ per mg of microsome protein into that per gram of liver was 77.9 mg microsome protein per gram liver (Knaak *et al.*, 1993). The liver weights and animal weights used were the individual values obtained in the study. Because the binding of YM-64227 on the microsome was negligible, no correction for protein binding was performed.

Results

Pharmacokinetics study of YM-64227 in genotyped dogs

The plasma concentration-time profiles of YM-64227 after single intravenous and oral administrations are shown in Figure 2. The pharmacokinetic parameters of YM-64227 obtained from the *in vivo* studies in dogs are summarized in Table 1. No significant changes were observed in the YM-64227 pharmacokinetic parameters among the C/C homo, C/T hetero, or T/T homo groups after the administration of a single intravenous dose of YM-64227 (dose: 0.1 mg/kg). The $CL_{tot, blood}$ values for the C/C, C/T, and T/T groups were 2120, 1787, and 2374 mL/h/kg, respectively, which were slightly higher than the canine hepatic blood flow rate (1620 mL/h/kg, Dedrick *et al.*, 1973). The C_{max} after a single oral administration (dose: 0.3 mg/kg) and absolute bioavailability in the T/T group were 17.1 times and 27.2 times higher than those for the C/C group, respectively. The $CL_{int, \textit{in vivo}}$ values for the C/C, C/T, and T/T groups were 299.7, 221.0, and 116.0 L/h/kg, respectively. The $CL_{int, \textit{in vivo}}$ for the T/T group was 38.7% of that for the C/C group.

The plasma concentration-time profiles of the metabolites after a single intravenous administration are shown in Figure 3. The C_{max} and AUC_{iv} values of MM-2 in the C/C

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

and C/T groups were highest among the five metabolites (Table 2). The plasma concentrations of MM-1, MM-3, MM-4, and MM-5 in the C/C and C/T groups were low, and their C_{max} and AUC_{iv} values were almost the same. For the T/T group, however, the plasma concentrations of MM-1, MM-4, and MM-5 were higher than those for the C/C group. The C_{max} and AUC_{iv} of MM-1, MM-4, and MM-5 were 10.4 and 44.6 times, 4.6 and 5.8 times, and 25.9 and 187.0 times higher than those for the C/C group, respectively (Table 2). The $T_{1/2}$ for MM-1 and MM-5 in the T/T group were 4.8 and 2.8 times longer than those in the C/C group. Although the AUC_{iv} for MM-3 in the T/T group was 2.4 times higher than that in the C/C group, no significant difference was observed in the T_{max}, C_{max}, and $T_{1/2}$ for MM-3 between the C/C and T/T groups.

The plasma concentration-time profiles of metabolites after a single oral administration are shown in Figure 4. The plasma MM-2 concentrations in the C/C and C/T groups were highest among the five metabolites as well as after intravenous dosing (Table 3). In the T/T group, the plasma MM-5 concentration was highest among the metabolites. The C_{max} and AUC_{oral} for the T/T group were 32.6 times and 118.2 times higher than those for the C/C group. The C_{max} and AUC_{oral} of MM-1 in the T/T group were 3.4 times and 20.5 times higher than those in the C/C group. The C_{max} and AUC_{oral} for

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

MM-4 in the T/T group were 6.4 times and 11.4 times higher than those in the C/C group. For the T/T group, the plasma concentration of MM-2, the main metabolite in the C/C and C/T groups, was lower than that for the C/C group. The C_{max} and AUC_{oral} for MM-2 in the T/T group were 8.3% and 14.2% of those in the C/C group. The $T_{1/2}$ for MM-1, MM-2, MM-4, and MM-5 in the T/T group were 7.7, 1.6, 2.0, and 3.7 times longer than that in the C/C group, respectively. No significant differences were observed in the pharmacokinetic parameters of MM-3 between the C/C and T/T groups.

YM-64227 metabolism in genotyped canine liver microsomes

Michaelis-Menten plots and Eadie-Hofstee plots of each metabolite formation from YM-64227 in genotyped canine liver microsomes are shown in Figure 5. As the contribution of multiple components in some of the metabolic pathways was suggested by the Eadie-Hofstee plots, either the mono- or bi-phasic model was fitted to the metabolic activity of each metabolite using nonlinear least-squares regression. The best model was selected based on the AIC values. The V_{max}, K_m, $CL_{int,\ s}$, $CL_{int,\ ns}$, and $CL_{int,\ in\ vitro}$ values were calculated. Furthermore, the slope of the linear portion of the Michaelis-Menten plots was calculated using linear regression and defined as $CL_{int,\ ns}$. The kinetic parameters obtained from the *in vitro* experiments using liver microsomes

are summarized in Table 4. The MM-1 formation activities of the C/C, C/T, and T/T groups had a mono-phasic pattern with a non-saturable component. No significant differences in the $CL_{int,\ in\ vitro}$ were observed among the three groups. As for the MM-2 formation activities in all of the groups, contributions of both the saturable and non-saturable component were demonstrated. Although differences in the $CL_{int,\ ns}$ were not observable among groups, the $CL_{int,\ s}$ for the T/T group was significantly lower than that for the C/C group, at only 8.6% of the C/C group value. The contribution of both the saturable and non-saturable component was shown in the MM-3 formation activities of the C/C and C/T groups. While no difference in $CL_{int,\ ns}$ among the three groups was observed, the saturable component was absent in the T/T group. Accordingly, the $CL_{int,\ in\ vitro}$ for the T/T group was 12.6% of that for the C/C group. The contribution of both a saturable and non-saturable component was detected in the MM-4 and MM-5 formation activities of all three groups. The $CL_{int,\ in\ vitro}$ values for MM-4 and MM-5 did not differ among the three groups.

Correlation between $CL_{int,\ in\ vivo}$ and $CL_{int,\ in\ vitro}$

Although the $CL_{int,\ in\ vivo}$ values were approximately 9.8 times higher than the $CL_{int,\ in\ vitro}$ values, there was a statically significant correlation between them ($r = 0.82$, $p < 0.001$).

The $CL_{int,\ in\ vivo}$ and $CL_{int,\ in\ vitro}$ values in the C/C and C/T groups were higher than those in the T/T group.

Discussion

The CYP1A2 1117 C>T SNP caused remarkable inter-individual variability in the pharmacokinetics of YM-64227 in dogs. After intravenous administration of YM-64227, the $CL_{tot,\ blood}$ values were 1787 to 2374 mL/h/kg, which are slightly greater than the canine hepatic blood flow rate (1620 mL/h/kg, Dedrick *et al.*, 1973). In contrast, the C_{max} and AUC_{oral} values in the T/T group were significantly greater than those in the C/C group. These findings suggest that the clearance of YM64227 in dogs is "hepatic blood flow-limited" and the deficiency in CYP1A2 mainly affects first pass metabolism.

After single intravenous and oral administrations of YM-64227, MM-2 was found to be the main metabolite in the C/C and C/T groups. However, the main metabolites found in the T/T group were MM-1 and MM-5, whose concentrations were significantly higher than those in the C/C and C/T groups. In addition, the $AUC_{iv,}$ AUC_{oral}, and $T_{1/2}$ values for MM-1, MM-4, and MM-5 in the T/T group were significantly greater than those in the C/C group, except for the $T_{1/2}$ for MM-4 after intravenous administration. With regard to MM-3, a significant difference was observed only in the AUC_{iv} of the T/T group. Taken together, the relative amounts and pharmacokinetic profiles of the

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

metabolites in the C/C and C/T groups were remarkably different from those in the T/T group. It was shown that a deficiency of CYP1A2 enzyme activity leads to a decrease in MM-2, and an increase in MM-1, MM-4, and MM-5. MM-3 was slightly affected.

In an *in vitro* metabolism study using microsomes prepared from dogs used in the pharmacokinetic study, the contribution of both the saturable and non-saturable components was observed in the formation of MM-2 in all groups. The $CL_{int,\ ns}$ was not significantly different among the three groups whereas the $CL_{int,\ s}$ for the T/T group was significantly lower than that for the C/C and C/T groups. This suggests that CYP1A2 is involved in the saturable component. Accordingly, the $CL_{int,\ \textit{in vitro}}$ value, defined as the sum of $CL_{int,\ ns}$ and $CL_{int,\ s}$, for the T/T group was more than 10-fold lower than that for the C/C and C/T groups. This finding was in good agreement with the fact that the plasma concentrations of MM-2 in the T/T group were significantly lower than those in the other groups.

The contribution of one non-saturable component was observed in the formation of MM-1. The kinetics of MM-4 and MM-5 formation was bi-phasic, consisting of a saturable and non-saturable component. The $CL_{int,\ \textit{in vitro}}$ values for these metabolites

were not significantly different among the three groups, which suggest that CYP1A2 does not mediate the biotransformation of YM-64227 into these metabolites. These findings suggest that the formation of MM-1, MM-4, and MM-5 is not different in the C/C, C/T, and T/T groups, but the elimination of these metabolites the T/T group is decreased. A possible explanation for this discrepancy is that CYP1A2 mediates the subsequent metabolism of MM-1, MM-4, and MM-5.

Both the saturable and non-saturable components were observed in the formation of MM-3 in the C/C and C/T groups, while only the non-saturable component was observed in the T/T group, which suggests that CYP1A2 is fully responsible for the saturable component. Accordingly, the $CL_{int,\ in\ vitro}$ for MM-3 in the T/T group was around 10-fold lower than that in the C/C and C/T groups. Thus, plasma concentrations of MM-3 in the T/T group were expected to be lower than those in the C/C and C/T groups. However, no marked differences were observed in MM-3 plasma concentration among the three groups. A possible explanation for the conflict between the *in vivo* and *in vitro* studies is that CYP1A2 plays a significant role not only in the formation of MM-3 from YM-64227, but also the subsequent metabolism of MM-3. As a consequence, a decrease in the formation of MM-3 might be compensated by a decrease

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.008722
This article has not been copyedited and formatted. The final version may differ from this version.

in the elimination of MM-3. Furthermore, *in vitro* metabolism study, another possible explanation is that the formation of MM-3 is much faster than the elimination of MM-3. Therefore, the $CL_{int,\ in\ vitro}$ for MM-3 reflected mainly the formation of this metabolite in the C/C and C/T groups, and a CYP1A2 deficiency in the T/T group resulted in the decrease in the $CL_{int,\ in\ vitro}$ for MM-3.

A statistically significant linear relationship was observed between $CL_{int,\ in\ vivo}$ and $CL_{int,\ in\ vitro}$. ($r = 0.82$, $p < 0.001$; Figure 6), indicating that the hepatic metabolism of YM-64227 was well reflected in the pharmacokinetic profile. However, the *in vitro* study underestimated the *in vivo* intrinsic clearance by 9.8 fold. We searched for the cause of the difference between *in vivo* and *in vitro*. The binding of YM-64227 to the liver microsome and the extra-hepatic metabolic clearance in the small intestine, lung, and kidney microsomes were negligible. An *in vivo* study in rats using radio-labeled YM-64227 showed that the oral absorption of YM-64227 is almost complete and that there is no specific accumulation in the liver (in-house data). In rats, the *in vivo-in vitro* correlation factor was 7.1, as it was in dogs (in-house data). It may be difficult to quantitatively estimate the $CL_{int,\ in\ vivo}$ value from $CL_{int,\ in\ vitro}$ for drugs that undergo metabolism that is limited by blood flow (Iwatsubo *et al.*, 1997). Another explanation

for the discrepancy between the *in vitro* and *in vivo* clearances may be because one or more of the following assumptions is erroneous 1) the blood concentration and the concentration in the liver are in rapid equilibrium, 2) only unbound drug is involved in the elimination and transport across membranes, and 3) metabolic enzymes and transporters are homogeneously distributed along the blood flow pathways in the liver (Iwatsubo *et al.*, 1996; Naritomi *et al.*, 2001).

In the previous study, it was first reported that CYP1A2 1117 C>T SNP causes a stop codon[t2] (Tenmizu *et al.*, 2004a). The T-allele frequency was 0.39, which suggests that 10 to 15% of the dogs would not express the CYP1A2 protein. In this study, we reported the results from *in vivo* and *in vitro* studies using genotyped dogs and liver microsomes to clearly demonstrate the influence of the SNP. Therefore, attention should be paid to canine CYP1A2 polymorphism when using dogs to investigate the pharmacokinetics of drugs that are metabolized by CYP1A2. In addition, a considerable number of detailed studies on human CYP1A2 polymorphisms have been reported. In particular, CYP1A2*1C, *1F, *1K, and *7 showed altered enzyme activity (http://www.imm.ki.se/CYPalleles/default.htm). Phenotype study using the caffeine test demonstrated that CYP1A2 has a poor metabolization frequency of only 5 to 15%

(Tantcheva-Poor I *et al*., 1999). However, no genetic factor has been identified that would cause inter-individual variation in the caffeine test; thus, lifestyle factors, such as cigarette smoking and food choice (Pantuck EJ *et al.*, 1972; Kotake AN *et al.*, 1982), are thought to influence them. A clinically significant SNP that could confer obvious inter-individual variation has not yet been discovered for human CYP1A2.

In conclusion, the CYP1A2 deficiency polymorphism, 1117C>T, dramatically decreased the metabolic clearance of YM-64227 in dogs. The CYP1A2 polymorphism caused a remarkable increase in the plasma concentration of the unchanged drug after oral administration of YM-64227, but intravenous administration did not have the same effect because the hepatic elimination of YM-64227 is hepatic blood flow is rate limited. CYP1A2 polymorphism dramatically altered the metabolic profile of YM-64227. Attention should be paid to this canine CYP1A2 polymorphism in future studies in order to understand the impact of inter-individual variability for the disposition of drugs in dogs.

Acknowledgments

We would like to acknowledge Shigeo Suzuki for very valuable technical assistance while conducting our study.

References

Akaike H (1969) Fitting autoregressive models for prediction. *Ann Inst Stat Math* **21:** 243-247.

Azuma R, Komuro M, Kawaguchi Y, Okudaira K, Hayashi M, and Kiwada H (2002) Comparative analysis of in vitro and in vivo pharmacokinetics parameters related to individual variability of GTS-21 in canine. *Drug Metab Pharmacokinet* **17:** 75-82.

Bischoff KB, Dedrick RL, Zaharko DS, and Longstreth JA (1971) Methotrexate pharmacokinetics. *J Pharm Sci* **60:** 1128-1133.

Blaisdell J, Goldstein JA and, Bai SA (1998) Isolation of a new canine cytochrome P450 cDNA from the cytochrome P450 2C subfamily (CYP2C41) and evidence for polymorphic differences in its expression. *Drug Metab Dispos* **26:** 278-283.

Ciaccio PJ, Graves PE, Bourque DP, Glinsmann-Gibson B, and Halpert JR (1991) cDNA and deduced amino acid sequences of a dog liver cytochrome P-450 of the IIIA gene subfamily. *Biochim Biophys Acta* **1088:** 319-322.

Dedrick RL, Forrester DD, Cannon JN, El Dareer SM, and Mellett LB (1973) Pharmacokinetics of 1-[beta]--arabinofuranosylcytosine (Ara-C) deamination in several species. *Biochemical Pharmacology* **22:** 2405-2417.

Fraser DJ, Feyereisen R, Harlow GR, and Halpert JR (1997) Isolation, heterologous expression and functional characterization of a novel cytochrome P450 3A enzyme from a canine liver cDNA library. *J Pharmacol Exp Ther* **283:** 1425-1432.

Furuta T, Ohashi K, Kosuge K, Zhao XJ, Takashima M, Kimura M, Nishimoto M, Hanai H, Kaneko E, and Ishizaki T (1999) CYP2C19 genotype status and effect of omeprazole on intragastric pH in humans. *Clin Pharmacol Ther* **65:** 552-561.

Graves PE, Elhag GA, Ciaccio PJ, Bourque DP, and Halpert JR (1990) cDNA and deduced amino acid sequences of a dog hepatic cytochrome P450IIB responsible for the metabolism of 2,2',4,4',5,5'-hexachlorobiphenyl. *Arch Biochem Biophys* **281:** 106-115.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, and Sugiyama Y (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* **73:** 147-171.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, and Sugiyama Y (1996) Prediction of in vivo drug disposition from in vitro data based on physiological pharmacokinetics. *Biopharm Drug Dispos* **17:** 273-310.

Kagimoto M, Heim M, Kagimoto K, Zeugin T, and Meyer UA (1990) Multiple

mutations of the human cytochrome P450IID6 gene (CYP2D6) in poor metabolizers of debrisoquine. Study of the functional significance of individual mutations by expression of chimeric genes. *J Biol Chem* **265:** 17209-17214.

Knaak JB, al-Bayati MA, Raabe OG, and Blancato JN (1993) Development of in vitro Vmax and Km values for the metabolism of isofenphos by P-450 liver enzymes in animals and human. *Toxicol Appl Pharmacol* **120**: 106-113.

Kotake AN, Schoeller DA, Lambert GH, Baker AL, Schaffer DD and Josephs H (1982) The caffeine CO_2 breath test: dose response and route of N-demethylation in smokers and nonsmokers. *Clin Pharmacol Ther* **32**:261-269.

Lankford SM, Bai SA, and Goldstein JA (2000) Cloning of canine cytochrome P450 2E1 cDNA: identification and characterization of two variant alleles. *Drug Metab Dispos* **28:** 981-986.

Lin JH, Sugiyama Y, Awazu S, and Hanano M (1982) Physiological pharmacokinetics of ethoxybenzamide based on biochemical data obtained in vitro as well as on physiological data. *J Pharmacokinet Biopharm* **10:** 649-661.

Mise M, Yadera S, Matsuda M, Hashizume T, Matsumoto S, Terauchi Y, and Fujii T (2004) Polymorphic expression of CYP1A2 leading to interindividual variability in metabolism of a novel benzodiazepine receptor partial inverse agonist in dogs.

Drug Metab Dispos **32:** 240-245.

Montandon B, Roberts RJ, and Fischer LJ (1975) Computer simulation of sulfobromophthalein kinetics in the rat using flow-limited models with extrapolation to man. *J Pharmacokinet Biopharm* **3:** 277-290.

Naritomi Y, Terashita S, Kimura S, Suzuki A, Kagayama A and Sugiyama Y (2001) Prediction of human hepatic clearance from in vivo animal experiments and in vitro metabolic studies with liver microsomes from animals and humans. *Drug Metab Dispos* **29**: 1316-1324.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:** 1-42.

Pantuck EJ, Kuntzman R and Conney AH (1972) Decreased concentration of phenacetin in plasma of cigarette smokers. *Science* **175**:1248-1250.

Paulson SK, Engel L, Reitz B, Bolten S, Burton EG, Maziasz TJ, Yan B, and Schoenhard GL (1999) Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metab Dispos* **27:** 1133-1142.

Pelkonen O, Maenpaa J, Taavitsainen P, Rautio A and Raunio H (1998) Inhibition and

induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* **28:** 1203-1253.

Roberts MS and Rowland M (1986a) A dispersion model of hepatic elimination: 1. Formulation of the model and bolus considerations. *J Pharmacokinet Biopharm* **14:** 227-260.

Roberts MS and Rowland M (1986b) Correlation between in-vitro microsomal enzyme activity and whole organ hepatic elimination kinetics: analysis with a dispersion model. *J Pharm Pharmacol* **38:** 177-181.

Sakamoto K, Kirita S, Baba T, Nakamura Y, Yamazoe Y, Kato R, Takanaka A, and Matsubara T (1995) A new cytochrome P450 form belonging to the CYP2D in dog liver microsomes: purification, cDNA cloning, and enzyme characterization. *Arch Biochem Biophys* **319:** 372-382.

Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, Miners JO, Birkett DJ, and Goldstein JA (1996) The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics* **6:** 341-349.

Tantcheva-Poor I, Zaigler M, Rietbrock S and Fuhr U (1999) Estimation of cytochrome P-450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine

test. *Pharmacogenetics* **9**: 131-144.

Tenmizu D, Endo Y, Noguchi K, and Kamimura H (2004a) Identification of the novel canine CYP1A2 1117 C>T SNP causing protein deletion. *Xenobiotica* **34:** 835-846.

Tenmizu D, Fukunaga Y, Noguchi K, and Kamimura H (2004b) Simultaneous determination of YM-64227, a phosphodiesterase type 4 inhibitor, and its five metabolites in dog plasma by high-performance liquid chromatography with fluorescence detection *Biomed Chromatogr* **18:** 667-672.

Uchida T, Komori M, Kitada M, and Kamataki T (1990) Isolation of cDNAs coding for three different forms of liver microsomal cytochrome P-450 from polychlorinated biphenyl-treated beagle dogs. *Mol Pharmacol* **38:** 644-651.

Figure legends

Figure 1

Postulated metabolic pathway of YM-64227.

Figure 2

Plasma concentration-time profiles of YM-64227 after intravenous and oral administration to dogs at 0.1 and 0.3 mg/kg. Each point represents the mean ± S.D. of five animals. (■) indicates C/C type dogs, (▲) indicates C/T type dogs, and (○) indicates T/T type dogs.

Figure 3

Plasma concentration-time profiles of YM-64227 metabolites after intravenous administration to dogs at 0.1 mg/kg. Each point represents the mean ± S.D. of five animals. (■) indicates C/C type dogs, (▲) indicates C/T type dogs, and (○) indicates T/T type dogs.

Figure 4

Plasma concentration-time profiles of YM-64227 metabolites after oral administration

to dogs at 0.3 mg/kg. Each point represents the mean ± S.D. of five animals. (■) indicates C/C type dogs, (▲) indicates C/T type dogs, and (○) indicates T/T type dogs.

Figure 5

Michaelis-Menten plots and Eadie-Hofstee plots of YM-64227 metabolism in the liver microsomes prepared from dogs genotyped with respect to CYP1A2. Each point represents the mean ± S.D. of five microsomes in duplicate experiments. (■) indicates C/C type microsomes, (▲) indicates C/T type microsomes, and (○) indicates T/T type microsomes.

Figure 6

Correlation between CL_{oral}, CL_h, and $CL_{int,\ in\ vivo}$ relative to $CL_{int,\ in\ vitro}$. (■) indicates C/C type dogs (n = 4), (▲) indicates C/T type dogs (n = 5), and (○) indicates T/T type dogs (n = 5).

Table 1. Pharmacokinetic parameters of YM-64227.

Pharmacokinetic parameters		C/C homo (n = 5)	C/T hetero (n = 5)	T/T homo (n = 5)
$T_{1/2}$ [a)]	(h)	1.2 ± 0.1	1.2 ± 0.3	1.1 ± 0.4
V_{ss}	(mL/kg)	1444 ± 167.9	1115 ± 341.6	1178 ± 271.9
CL_{tot}	(mL/h/kg)	1399 ± 72.5	1179 ± 357.4	1567 ± 184.4
$CL_{tot, blood}$	(mL/h/kg)	2120 ± 109.9	1787 ± 541.5	2374 ± 279.5
AUC_{iv}	(h·ng/mL)	71.6 ± 3.9	91.3 ± 27.8	64.5 ± 7.2
AUC_{oral}	(h·ng/mL)	1.1 ± 0.6	3.5 ± 1.4	27.2 ± 20.2 *
T_{max}	(h)	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
C_{max}	(ng/mL)	1.4 ± 0.6	3.8 ± 1.7	24.0 ± 14.8 **
F_{oral}	(%)	0.5 ± 0.3	1.4 ± 0.7	13.6 ± 9.5 **

Each value represents the mean ± S.D.

Statistically significant differences from the C/C type were determined using the *Tukey* test, and are indicated as * $p < 0.05$ and ** $p < 0.01$.

[a)]: Terminal elimination half-life for the intravenous administration of YM-64227.

Table 2. Pharmacokinetic parameters for the YM-64227 metabolites after a single intravenous administration of YM-64227.

Genotype	Analyte	T_{max} (h)	C_{max} (ng/mL)	AUC_{iv} (h·ng/mL)	$T_{1/2}$ (h)
C/C homo	MM-1	0.5 ± 0.1	1.4 ± 0.5	2.8 ± 1.7	1.0 ± 0.1 [a)]
(n = 5)	MM-2	0.5 ± 0.3	17.7 ± 3.9	43.1 ± 16.0	1.0 ± 0.1
	MM-3	0.2 ± 0.2	1.4 ± 1.3	1.3 ± 0.6	0.8 ± 0.2 [a)]
	MM-4	0.3 ± 0.1	2.1 ± 1.3	2.6 ± 1.3	0.9 ± 0.4
	MM-5	0.1 ± 0.0 [b)]	1.5 ± 1.7 [b)]	0.6 ± 0.7 [b)]	0.4 ± 0.1 [c)]
C/T hetero	MM-1	0.6 ± 0.2	1.4 ± 0.7	3.2 ± 1.6	1.2 ± 0.4 [a)]
(n = 5)	MM-2	0.9 ± 0.2	18.0 ± 5.0	46.4 ± 18.3	1.0 ± 0.1
	MM-3	0.3 ± 0.2	1.1 ± 0.4	1.4 ± 0.7	0.8 ± 0.2 [b)]
	MM-4	0.4 ± 0.1	2.3 ± 1.0	3.6 ± 2.0	0.9 ± 0.3 [a)]
	MM-5	0.3 ± 0.2 [a)]	0.7 ± 0.3 [a)]	0.5 ± 0.4 [a)]	0.5 [d)]
T/T homo	MM-1	1.8 ± 0.4 **	14.6 ± 3.5 **	124.9 ± 38.6 **	4.8 ± 1.3 **
(n = 5)	MM-2	0.5 ± 0.3	2.0 ± 0.5 **	6.5 ± 3.3 **	1.7 ± 0.7
	MM-3	0.2 ± 0.1	1.4 ± 0.8	3.1 ± 1.5 *	1.7 ± 0.4
	MM-4	0.3 ± 0.4	9.6 ± 1.8 **	15.0 ± 5.9 **	0.8 ± 0.2
	MM-5	0.9 ± 0.7	38.8 ± 9.5 **	112.2 ± 50.8 *	1.1 ± 0.1 **

Each value represents the mean ± S.D.

Statistically significant differences from the C/C type were determined using the *Tukey* test, and are indicated as * $p < 0.05$ and ** $p < 0.01$.

[a)] n = 4, [b)] n = 3, [c)] n = 2, and [d)] n = 1: due to plasma concentrations being less than the lower limit of detection (0.4 ng/mL) at many blood collection time points.

Table 3. Pharmacokinetic parameters of YM-64227 metabolites after a single oral administration of YM-64227.

Genotype	Analyte	T_{max} (h)	C_{max} (ng/mL)	AUC_{oral} (h·ng/mL)	$T_{1/2}$ (h)
C/C homo	MM-1	0.5 ± 0.0	12.4 ± 5.5	17.3 ± 10.1	0.6 ± 0.1
(n = 5)	MM-2	0.5 ± 0.0	73.2 ± 23.8	111.8 ± 33.1	0.8 ± 0.3
	MM-3	0.4 ± 0.1	4.1 ± 2.1	3.2 ± 1.4	0.4 ± 0.0
	MM-4	0.4 ± 0.1	4.3 ± 2.2	3.1 ± 1.5	0.3 ± 0.1 [a)]
	MM-5	0.4 ± 0.1	4.2 ± 2.6	3.1 ± 1.8	0.3 ± 0.0 [a)]
C/T hetero	MM-1	0.6 ± 0.2	14.9 ± 6.8	23.4 ± 13.2	0.6 ± 0.1
(n = 5)	MM-2	0.6 ± 0.2	108.9 ± 54.5	186.7 ± 106.3	0.7 ± 0.1
	MM-3	0.4 ± 0.1	8.6 ± 5.9	6.9 ± 4.4	0.3 ± 0.0
	MM-4	0.4 ± 0.1	5.6 ± 2.9	4.1 ± 2.2	0.3 ± 0.1 [b)]
	MM-5	0.4 ± 0.1	10.9 ± 7.5	8.5 ± 5.5	0.3 ± 0.1 [a)]
T/T homo	MM-1	1.8 ± 0.4 **	42.0 ± 9.0 **	355.4 ± 83.3 **	4.6 ± 0.6 **
(n = 5)	MM-2	0.6 ± 0.2	6.1 ± 1.3 *	15.9 ± 5.9	1.3 ± 0.2 **
	MM-3	0.3 ± 0.1	7.9 ± 5.1	6.1 ± 4.1	0.3 ± 0.0
	MM-4	0.4 ± 0.1	27.5 ± 14.9 **	35.4 ± 19.8 **	0.6 ± 0.1 **
	MM-5	0.7 ± 0.3 *	136.8 ± 48.1 **	366.3 ± 191.5 **	1.1 ± 0.1 **

Each value represents the mean ± S.D.

Statistically significant differences from the C/C type were determined using the *Tukey* test, and are indicated as * $p < 0.05$ and ** $p < 0.01$.

[a)] n = 4 and [b)] n = 3: due to plasma concentrations being less than the lower limit of detection (0.4 ng/mL) at many blood collection time points.

Table 4. Kinetic parameters for YM-64227 metabolism in liver microsomes prepared from dogs genotyped with respect to CYP1A2.

Kinetic Parameters		MM-1	MM-2	MM-3	MM-4	MM-5	Total
V_{max}	C/C	-	0.07 ± 0.02	0.21 ± 0.04	0.15 ± 0.05	0.21 ± 0.01	
(nmol/min/mg protein)	C/T	-	0.06 ± 0.01	0.19 ± 0.07	0.09 ± 0.02	0.21 ± 0.07	
	T/T	-	0.01 ± 0.01 **	-	0.11 ± 0.09	0.24 ± 0.16	
K_m	C/C	>100	1.2 ± 0.3	5.7 ± 1.6	2.3 ± 0.2	4.6 ± 0.5	
(μM)	C/T	>100	1.0 ± 0.1	4.1 ± 1.9	2.3 ± 0.4	3.9 ± 0.4	
	T/T	>100	4.6 ± 3.9	>100	2.6 ± 0.1	4.1 ± 1.3	
$CL_{int, s}$	C/C	-	61.8 ± 17.5	40.3 ± 12.7	63.1 ± 20.5	47.4 ± 6.7	
(μL/min/mg protein)	C/T	-	63.8 ± 10.0	49.0 ± 6.8	39.8 ± 7.8	52.7 ± 17.2	
	T/T	-	4.7 ± 4.2 **	-	41.3 ± 33.7	57.2 ± 23.7	
$CL_{int, ns}$	C/C	3.5 ± 1.2 [a)]	1.0 ± 0.4	4.1 ± 1.7	1.1 ± 0.5	0.7 ± 0.2	
(μL/min/mg protein)	C/T	3.7 ± 1.5 [a)]	0.8 ± 0.2	2.8 ± 0.3	0.9 ± 0.2	0.5 ± 0.0	
	T/T	3.7 ± 1.3 [a)]	0.6 ± 0.2	5.6 ± 2.5 [a)]	0.8 ± 0.2	0.4 ± 0.2	
$CL_{int, \textit{in vitro}}$	C/C	3.5 ± 1.2	62.8 ± 17.5	44.4 ± 12.6	64.2 ± 20.6	48.0 ± 6.6	223.0 ± 37.4
(μL/min/mg protein)	C/T	3.7 ± 1.5	64.6 ± 10.1	51.8 ± 7.1	40.7 ± 7.8	53.1 ± 17.2	213.9 ± 27.1
	T/T	3.7 ± 1.3	5.4 ± 4.3 **	5.6 ± 2.5 **	42.1 ± 33.8	57.6 ± 23.9	114.4 ± 64.3 **

Each value represents the mean ± S.D.

Statistically significant differences from the C/C type were determined using the *Tukey* test, and are indicated as * $p < 0.05$ and ** $p < 0.01$.

[a)]: linear regression analysis.

$CL_{int, s}$: saturable CL_{int}, $CL_{int, ns}$: non-saturable CL_{int}, and $CL_{int, \textit{in vitro}} = CL_{int, s} + CL_{int, ns}$.

Figure. 1

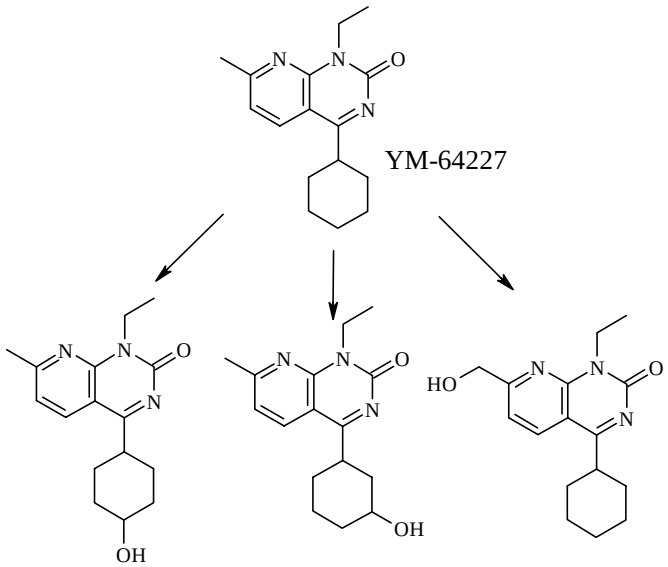

Figure. 2

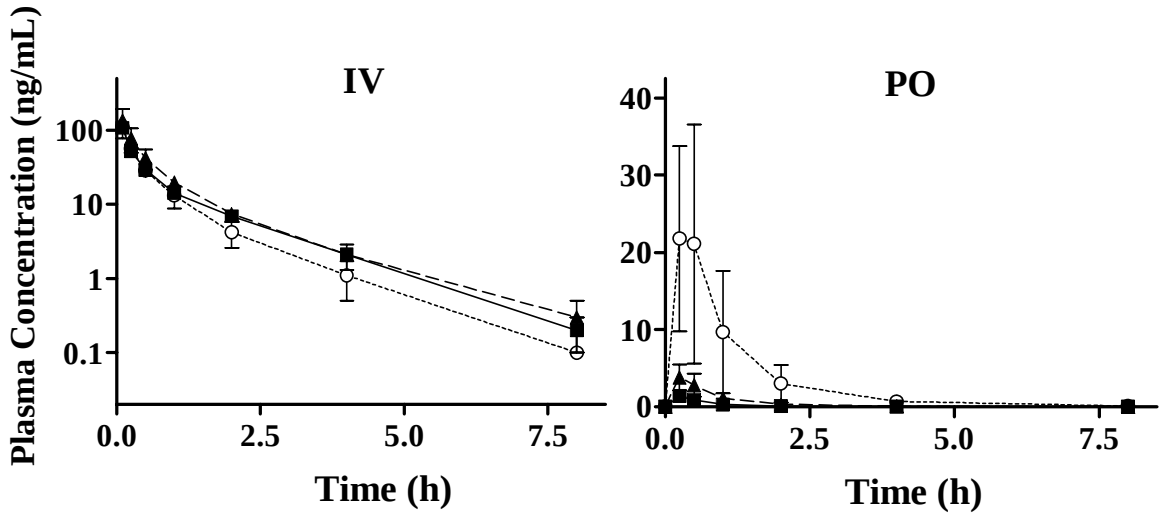

Figure. 3

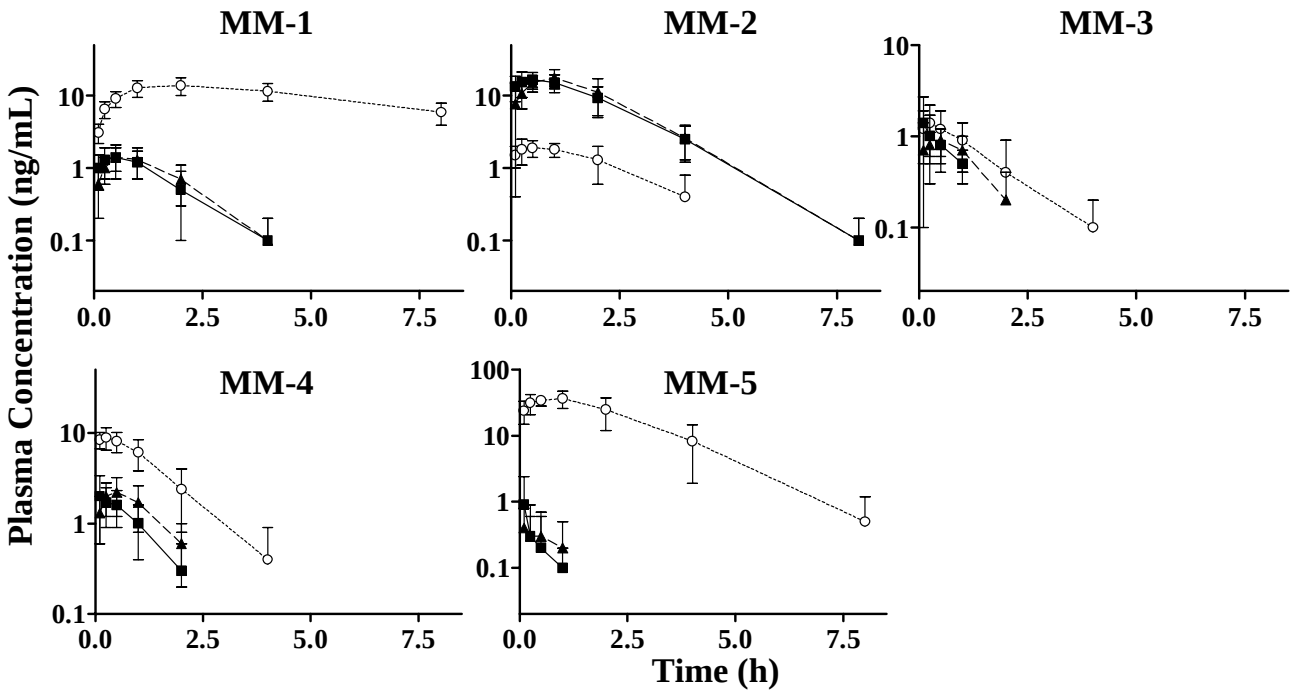

Figure. 4

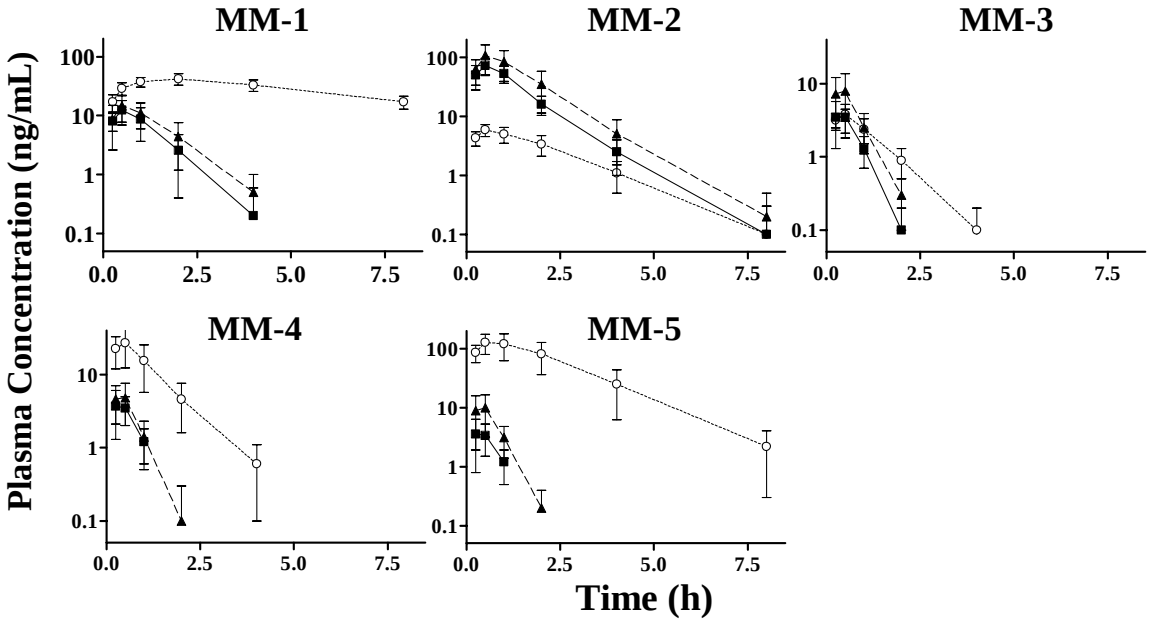

Figure. 5

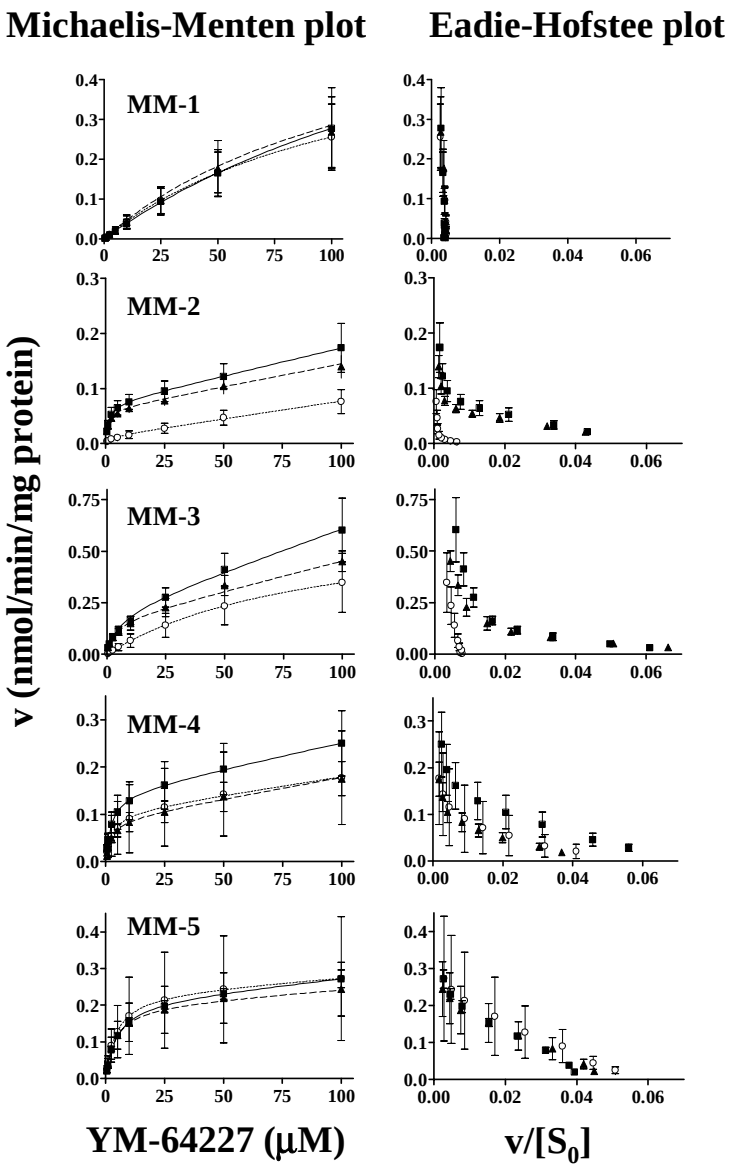

Figure. 6