Title page

Title:

A proposed simple screening method to determine relative contributions of CYP3A4 and CYP3A5 to drug metabolism *in vitro*

Author names and affiliations:

Jun Matsumoto[a*], Hiroyoshi Nakamura[b,c,d], Su Nwe San[d], Hikari Sato[b], Manami Takezawa[b], Ryuto Kishi[b], Yutaro Kito[b], Junko Sugano[b], Mai Izuki[b], Nao Yanagisawa[b], Naoki Ikeda[b], Yusuke Saito[b], Yoshinori Kato[b], Harumi Yamada[b,d], Masachika Fujiyoshi[a], Noritaka Ariyoshi[a]

[a]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558, Japan

[b]Department of Pharmaceutical Sciences, International University of Health and Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi, 324-8501, Japan

[c]Division of Pharmacy, International University of Health and Welfare Mita Hospital, 1-4-3 Mita, Minato-ku, Tokyo, 108-8329, Japan

[d]Graduate School of Pharmaceutical Sciences, International University of Health and

Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi, 324-8501, Japan

*Corresponding author

Corresponding author:

Jun Matsumoto, Ph.D.

Address: 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558, Japan

Email address: matsumotoj@okayama-u.ac.jp

Declarations of interest:

none

Number of figures: 2 (Color figures: none)

Number of tables: 1

Number of supplementary figures: 2

Number of supplementary tables: 1

Number of words in the Abstract: 236/250

Number of words including references: 2059/2000

Abstract

Purpose: The cytochrome P450 (CYP) 3A family of enzymes metabolize the majority of clinically used drugs. CYP3A4 and CYP3A5 are the two major CYP3A isoforms, but exhinbit different substrate specificity. The aim of this study was to establish a simple screening method to determine the relative contributions of CYP3A4 and CYP3A5 to drug metabolism *in vitro*.

Methods: A screening method was developed based on competitive inhibition using luciferin-PPXE (L-PPXE), a luminogenic CYP3A substrate. CYP3cide, tacrolimus, and midazolam were selected as standard compounds metabolized by CYP3A4 or CYP3A5. Nine clinically-used drugs were evaluated for their abilities to inhibit luminescence resulting from L-PPXE metabolism. Appropriate reaction conditions for the screening method were determined using recombinant CYP3A4 and CYP3A5.

Results: A significant decrease in luminescence resulting from L-PPXE metabolism by CYP3A4 and CYP3A5 was observed only for drugs reported to be metabolized by CYP3As. The substrate specificities of CYP3A4 or CYP3A5 for the proposed CYP3A substrates using our screening method were consistent with those of previous reports or available drug information from pharmaceutical companies. The reaction volume for this method was 50 μL, and the time required for the entire procedure was 70 min.

Furthermore, this screening can be performed using a single tube with minimal training.

Conclusions: Through the establishment of our screening method in the present study, we are sure it is useful to determine the relative contributions of CYP3A4 and CYP3A5 to drug metabolism *in vitro*.

Key Words: CYP3A, *CYP3A5*3* allele, P450 Glo Assay system, Pharmacokinetics, Pharmacogenetics

1. Introduction

The cytochrome P450 (CYP) 3A family of enzymes is important for the metabolism of numerous clinically used drugs [1,2]. Four CYP3A isoforms have been reported in humans: CYP3A4, CYP3A5, CYP3A7, and CYP3A43 [1]. Because CYP3A7 is a predominant enzyme in fetal liver and CYP3A43 is expressed at low levels, CYP3A4 and CYP3A5 are considered to be the 2 major CYP3A enzymes responsible for drug metabolism in the adult human liver [1,3]. Recent studies have shown that CYP3A4 and CYP3A5 exhibit differences in substrate specificity despite substantial overlap [2,4].

The *CYP3A5* gene is highly polymorphic, which contributes to substantial interindividual variation in the protein expression of CYP3A5 [5]. The most common allele attributable to variable CYP3A5 expression is the *CYP3A5*3* mutant allele. Individuals homozygous for the *CYP3A5*3* allele either do not express, or express very low levels, of CYP3A5 [5]. As such, *CYP3A5* gene polymorphisms may hinder metabolism of drugs predominantly metabolized by CYP3A5 or by both CYP3A4 and CYP3A5. However, few studies have evaluated differences in substrate specificity between CYP3A4 and CYP3A5. Few clinically used drugs are known to be metabolized predominantly by CYP3A5. Thus, a simple method to estimate the relative

contributions of CYP3A4 or CYP3A5 to drug metabolism may be useful for predicting primarily metabolic fate of drugs and interpret interindividual differences in pharmacokinetics leading to prediction of drug-drug interactions in future. The aim of this study was to establish a simple method to estimate the relative contributions of CYP3A4 and CYP3A5 to drug metabolism *in vitro*.

2. Materials and methods

Luciferin-PPXE (L-PPXE), a commercially available luminogenic substrate for CYP3A4 used in the P450-Glo Assay system (Promega, Madison, WI), was used as a competitive inhibitor to screen for drug candidates metabolized predominantly by CYP3A4 or CYP3A5. Recombinant CYP3A4 and CYP3A5 co-expressed with NADPH-CYP reductase and cytochrome b_5 in microsomes of insect cells in a baculoviral system (Supersome) were purchased from Corning Gentest (Woburn, MA). Test compound solutions (12.5 μL) were mixed with recombinant CYP3A4 or CYP3A5 (12.5 μL) and incubated at 37 ºC for 10 min, followed by addition of NADPH Regeneration Solution (25.0 μL). After 40 min, Luciferin Detection Reagent (50.0 μL) was added and the samples were incubated at room temperature for 20 min. The incubation time of 40 min and recombinant CYP3A content of 1.5 pmol resulted in appropriate linearity, as shown in Supplementary Figs 1A and 1B. Luminescence was measured using a Gene Light GL-200 (Microtec Co., Chiba, Japan).

Apixaban (Chemscene, Monmouth Junction, NJ), ethosuximide (Tokyo Chemical Industry, Tokyo, Japan), lapatinib (Chemscene), midazolam (Wako, Osaka, Japan), sodium valproate (Wako), sorafenib (Chemscene), sumatriptan succinate (Chemscene), tacrolimus (Tokyo Chemical Industry), telaprevir (Chemscene),

topiramate (Chemscene), vaniprevir (Chemscene), and CYP3cide (Abcam, Cambridge, MA) were evaluated for their abilities to inhibit luminescence resulting from L-PPXE metabolism. All compounds were dissolved in dimethyl sulfoxide (DMSO), ethanol, methanol, or water. Ethanol and methanol did not affect CYP3A reactions at concentrations up to 0.95% (w/v), whereas DMSO inhibited L-PPXE metabolism by CYP3A5 and CYP3A4 at concentrations over 0.3% (w/v) and 1.0% (w/v), respectively (Supplementary Figs 1C - 1E). Therefore, solvent concentrations lower than these cutoff values were used.

Kinetic parameters were determined by nonlinear regression analysis using GraphPad Prism software, version 5.01 (GraphPad Software, La Jolla, CA). Comparisons between two groups were made using Student's *t*-test. $P < 0.05$ indicated statistical significance.

This study was approved by the recombinant DNA experiments safety committees of both Okayama University (Approval No. 18089) and International University of Health and Welfare (Approval No. D18001).

3. Results

Differences in L-PPXE metabolism between CYP3A4 and CYP3A5 are summarized in Supplementary Fig. 2A. The K_m and V_{max} values for L-PPXE as calculated by nonlinear regression analysis were 95.6 μM and 48,204 RLU for CYP3A4 and 109.9 μM and 27,335 RLU for CYP3A5, respectively. Tacrolimus and midazolam are clinical drugs known to be metabolized by both CYP3A4 and CYP3A5, but predominantly by CYP3A5 [2,6,7]. Therefore, these were used as representative CYP3A5 substrates. CYP3cide selectively inhibits CYP3A4 [8], indicating that it has specific affinity for the substrate binding site of CYP3A4. Thus, these three compounds were used to develop the screening method with L-PPXE. Differences in inhibition of L-PPXE metabolism by CYP3A4 and CYP3A5 using these compounds were investigated (Fig. 1 and Supplementary Figs 2B - 2D). The log IC_{50} values for tacrolimus and midazolam against L-PPXE metabolism by CYP3A4 were similar to those for CYP3A5 (the log IC_{50} values of tacrolimus were -4.82 ± 0.13 for CYP3A4 and -4.80 ± 0.10 for CYP3A5, while those of midazolam were -4.63 ± 0.05 for CYP3A4 and -4.64 ± 0.07 for CYP3A5). In contrast, a significant difference ($P < 0.001$) was observed in the log IC_{50} of CYP3cide against L-PPXE metabolism by CYP3A4 (-6.39 ± 0.08) and CYP3A5 (-5.02 ± 0.05).

Nine clinically used drugs were investigated to determine whether they could inhibit L-PPXE metabolism by CYP3A4 and CYP3A5. Apixaban, lapatinib, sorafenib, telaprevir, topiramate, and vaniprevir reduced metabolism of L-PPXE by CYP3A4 and CYP3A5 (Fig. 2). These results were consistent with drug information provided by pharmaceutical companies (Supplementary Table 1). Four of these 6 drugs (lapatinib, sorafenib, telaprevir, and vaniprevir) had lower log IC_{50} values against metabolism of L-PPXE by CYP3A4 than by CYP3A5. In contrast, the log IC_{50} of apixaban and topiramate against CYP3A4 metabolism of L-PPXE were higher than those against CYP3A5. L-PPXE metabolism was not inhibited by ethosuximide, sodium valproate, or sumatriptan succinate, which have not been reported as substrates of CYP3As. The relative contributions of CYP3A4 and CYP3A5 to metabolism of the drugs evaluated with our screening method were consistent with those in available drug information from pharmaceutical companies or previous reports. However, relative contributions of CYP3A4 and CYP3A5 was not available for several drugs evaluated in this study (Table 1).

4. Discussion

In this study, we attempted to develop a simple screening method to estimate the relative contributions of CYP3A4 and CYP3A5 to drug metabolism using recombinant CYP3As. As shown in Fig. 1 and Supplementary Fig. 2, the log IC_{50} for CYP3cide against L-PPXE metabolism differed between CYP3A4 and CYP3A5. In contrast, differences in the log IC_{50} of tacrolimus and midazolam against L-PPXE metabolism by CYP3A4 or CYP3A5 were not significant. However, each of these drugs clearly inhibited luminescence resulting from L-PPXE metabolism by CYP3A4 and CYP3A5. This may have resulted from differences in L-PPXE metabolic activity between CYP3A4 and CYP3A5 (Supplementary Fig. 2A). The L-PPXE metabolic activity by CY3A4 was higher than that by CYP3A5, and this may have caused a similarity of the apparent specificity to tacrolimus and midazolam in CYP3A4 and CYP3A5 using our screening method.

Our method was able to reasonably evaluate CYP3A4 and CYP3A5 substrate specificity for drugs evaluated in this study. Our results were consistent with drug information from pharmaceutical companies and previous reports, which provided additional confidence in the accuracy of our method. Moreover, we recently reported on the relative contributions of CYP3A4 and CYP3A5 to the metabolism of the

anti-hepatitis C drug paritaprevir [12]. In the paper, L-PPXE was simply used to predict whether paritaprevir was possibly metabolized by CYP3As, and we had not compared differences in inhibition of L-PPXE metabolism by paritaprevir between CYP3A4 and CYP3A5. However, inhibition of L-PPXE metabolism by paritaprevir in CYP3A4 was greater than that in CYP3A5, and this result agreed with that in the detailed pharmacokinetic parameters of a predominant CYP3A4 contribution to paritaprevir metabolism in the paper. Thus, our screening method can be used to estimate which CYP3A isoform contributes most to metabolism of some drugs. Drugs with the ability to inhibit L-PPXE metabolism by CYP3A5 to a similar or greater extent than by CYP3A4 may be specific substrates for CYP3A5.

This novel screening method to estimate relative contributions of CYP3A4 and CYP3A5 has several advantages over other previously reported methods. The reaction volume for this method is only 50 μL, and the time required from mixing drugs with CYP3As to detection is only 70 min. The method can be used to easily characterize drug candidates and may be an appropriate initial screening method to determine whether candidate drugs are metabolized by CYP3A4 or CYP3A5. Furthermore, this screening is performed in a single tube and requires little training due to the basic nature of the procedure. However, this screening method is limited in the following ways: (i) it

cannot be used to study the mechanism of inhibition of L-PPXE metabolism, and (ii) it cannot be used to determine pharmacokinetic parameters, such as V_{max} and K_m. The screening method does not distinguish between inhibition mechanisms such as non-competitive and uncompetitive inhibition. CYP3A4 is likely to have several substrate-binding sites based on its crystal structure [13]. CYP3A5 may also have several substrate-binding sites based on significant amino acid similarity with CYP3A4, although the crystal structure of CYP3A5 has not been reported. The binding sites of CYP3A4 and CYP3A5 responsible for L-PPXE metabolism have not been identified, and it is unknown whether L-PPXE competes with drugs at the same substrate-binding site. Computational techniques such as docking modeling simulations may be able to supplement the information provided by our screening method [14]. After candidate drug screening is performed using our screening method, detailed pharmacokinetic parameters should be obtained.

5. Conclusions

A novel, simple screening method was developed to estimate the relative contributions of CYP3A4 and CYP3A5 to metabolism of drugs *in vitro*. Findings from the present study may accelerate the study of differences in substrate specificity between CYP3A4 and CYP3A5 for clinically used drugs.

Acknowledgements

We would like to thank Drs. Yasuyuki Momose (International University of Health and Welfare), Kayoko Maezawa (International University of Health and Welfare), Yasunari Mano (Tokyo University of Science), Iori Hirosawa (Showa Pharmaceutical University), Masataka Tajima (Showa University), Takeshi Ito (International University of Health and Welfare), and Natsuko Sugiyama (International University of Health and Welfare) for providing useful suggestions. We thank Editage (www.editage.jp) for English language editing.

This study was supported by the JSPS KAKENHI (Grant-in-Aid for Young Scientists (B), Grant Number: 16K18955) and a Grant from the International University of Health and Welfare.

References

[1] Daly AK. Significance of the minor cytochrome P450 3A isoforms. *Clin Pharmacokinet* 2006; 45: 13-31.

[2] Niwa T, Murayama N, Emoto C, et al. Comparison of kinetic parameters for drug oxidation rates and substrate inhibition potential mediated by cytochrome P450 3A4 and 3A5. *Curr Drug Metab* 2008; 9: 20-33.

[3] Westlind A, Malmebo S, Johansson I, et al. Cloning and tissue distribution of a novel human cytochrome p450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun* 2001; 281: 1349-55.

[4] Williams JA, Ring BJ, Cantrell VE, et al. Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos* 2002; 30: 883-91.

[5] Kuehl P, Zhang J, Lin Y, et al. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 2001; 27: 383-91.

[6] Wong M, Balleine RL, Collins M, et al. *CYP3A5* genotype and midazolam clearance in Australian patients receiving chemotherapy. *Clin Pharmacol Ther* 2004; 75: 529-38.

[7] Passey C, Birnbaum AK, Brundage RC, et al. Dosing equation for tacrolimus using

genetic variants and clinical factors. *Br J Clin Pharmacol* 2011; 72: 948-57.

[8] Walsky RL, Obach RS, Hyland R, et al. Selective mechanism-based inactivation of CYP3A4 by CYP3cide (PF-04981517) and its utility as an in vitro tool for delineating the relative roles of CYP3A4 versus CYP3A5 in the metabolism of drugs. *Drug Metab Dispos* 2012; 40: 1686-97.

[9] Ueshima S, Hira D, Fujii R, et al. Impact of *ABCB1*, *ABCG2*, and *CYP3A5* polymorphisms on plasma trough concentrations of apixaban in Japanese patients with atrial fibrillation. *Pharmacogenet Genomics* 2017; 27: 329-36.

[10] Towles JK, Clark RN, Wahlin MD, et al. Cytochrome P450 3A4 and CYP3A5-Catalyzed Bioactivation of Lapatinib. *Drug Metab Dispos* 2016; 44: 1584-97.

[11] Ghassabian S, Rawling T, Zhou F, et al. Role of human CYP3A4 in the biotransformation of sorafenib to its major oxidized metabolites. *Biochem Pharmacol* 2012; 84: 215-23.

[12] San SN, Matsumoto J, Saito Y, et al. Minor contribution of CYP3A5 to the metabolism of hepatitis C protease inhibitor paritaprevir in vitro. *Xenobiotica* 2018; in press.

[13] Williams PA, Cosme J, Vinkovic DM, et al. Crystal structures of human

cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* 2004; 305: 683-6.

[14] Niwa T, Yasumura M, Murayama N, et al. Comparison of catalytic properties of cytochromes P450 3A4 and 3A5 by molecular docking simulation. *Drug Metab Lett* 2014; 8: 43-50.

Figure legends

Fig. 1. Differences in inhibition of CYP3A4 (A) and CYP3A5 (B) metabolism of L-PPXE by tacrolimus, midazolam, and CYP3cide. Concentrations of 10 μM for tacrolimus, 10 μM for midazolam, and 1 μM for CYP3cide were selected as the median concentrations in these experiments. Open and closed bars represent data obtained using drug concentrations at 10 times lower concentrations than the median concentrations and 10 times higher than the median concentrations, respectively. Percent changes were calculated based on control values. All values are presented as the mean ± SD. Single, double, and triple asterisks indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Fig. 2. Screening of drugs metabolized by CYP3A4 (A) and CYP3A5 (B). Concentrations near C_{max} (provided by the manufactures) were selected as the median concentrations for our experiments (1 μM for apixaban, 1 μM for ethosuximide, 10 μM for lapatinib, 10 μM for sodium valproate, 10 μM for sorafenib, 1 μM for sumatriptan, 1 μM for telaprevir, 100 μM for topiramate, and 10 μM for vaniprevir). Percent changes were calculated using control values. Open and closed bars represent data obtained using drug concentrations at 10 times lower concentrations than the median concentrations and 10 times higher than the median concentrations, respectively. All

values are presented as the mean ± SD. Single and double asterisks indicate $P < 0.05$ and $P < 0.01$, respectively.

Tables and table legends

Table 1. Summary of the expected predominance of CYP3A4 or CYP3A5 in drug metabolism.

Drug name	This study (CYP3A4 or CYP3A5)	DI or previous reports (CYP3A4 or CYP3A5)	Consistency
Apixaban [9]	CYP3A5	CYP3A5	Yes
Ethosuximide	—	—	Yes
Lapatinib [10]	CYP3A4	CYP3A4	Yes
Sodium valproate	—	—	Yes
Sorafenib [11]	CYP3A4	CYP3A4	Yes
Sumatriptan	—	—	Yes
Telaprevir	CYP3A4	NA	UD
Topiramate	CYP3A5	NA	UD
Vaniprevir	CYP3A4	NA	UD

DI, Drug information from pharmaceutical companies; —, Not metabolized by CYP3As; NA; There is no available information or reports on the predominance of CYP3A4 or CYP3A5 in drug metabolism; UD, Unable to determine.