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Research Paper

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Running head: Estimation of hepatic intrinsic clearance for MAO substrates

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ABSTRACT

Purpose Monoamine oxidases (MAOs) are non-CYP enzymes that contribute to systemic elimination of therapeutic agents, and localized on mitochondrial membranes. The aim of the present study was to validate quantitative estimation of metabolic clearance of MAO substrate drugs using human liver microsomes (HLMs).

Methods Three MAO substrate drugs, sumatriptan, rizatriptan and phenylephrine, as well as four CYP substrates were selected, and their disappearance during incubation with HLMs or mitochondria (HLMt) was measured. Metabolic clearance (CL) was then calculated from the disappearance curve.

Results CL obtained in HLMs for sumatriptan and a typical MAO substrate serotonin was correlated with that obtained in HLMt among ten human individual livers. Hepatic intrinsic clearance ($CL_{int,vitro}$) estimated from CL in HLMs was 14-20 and 2-5 times lower than *in vivo* hepatic intrinsic clearance ($CL_{int,vivo}$) obtained from literature for MAO and CYP substrates, respectively. Utilization of HLMs for quantitatively assessing metabolic clearance of MAO substrates was further validated by proteomics approach which has revealed that numerous proteins localized on inner and outer membranes of mitochondria were detected in both HLMs and HLMt.

Conclusion $CL_{int,vitro}$ values of MAO substrate drugs can be quantitatively estimated

with HLMs and could be used for semi-quantitative prediction of $CL_{int,vivo}$ values.

Key words: Monoamine oxidase, drug metabolism, hepatic clearance, *in vitro-in vivo* extrapolation

Abbreviations: CL, *in vitro* metabolic clearance; CL_{int}, intrinsic clearance; CYP, cytochrome P450; HLMs, human liver microsomes; HLMt, human liver mitochondria; IVIVE, *in vitro-in vivo* extrapolation; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; MAO, monoamine oxidase; NADPH, nicotinamide adenine dinucleotide phosphate

INTRODUCTION

Prediction of the pharmacokinetics of newly synthesized drug candidates in humans is essential for successful drug discovery and development. Human liver microsomes (HLMs) are the most widely used to assess metabolic clearance in humans at an early phase of drug development. Newly synthesized compounds are tested for cytochrome P450 (CYP)-mediated metabolic clearance by incubation with HLMs in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (1). Highly metabolized compounds will be then considered to be unstable in the body. However, this approach can be principally applied to CYP substrates, whereas application of HLMs to drug candidates that are metabolized by enzymes localized outside microsomes could be limited (2, 3). Nevertheless, such non-CYP enzymes also contribute to overall hepatic clearance of a certain types of drug candidates. Therefore, it is important to consider possible involvement of non-CYP enzymes in metabolism of candidate compounds for quantitative estimation of hepatic clearance.

In vitro-in vivo extrapolation (IVIVE) has been widely used to quantitatively estimate *in vivo* clearance from *in vitro* data for drugs metabolized by CYP, but relatively few studies have been done for drugs metabolized by non-CYP enzymes. There are several reports on IVIVE for drugs metabolized by UDP-glucuronyl

transferase (4, 5), which is one of the best characterized non-CYP enzymes and catalyzes conjugation reactions of many drugs. The IVIVE approach has also been applied to therapeutic agents and drug candidates metabolized by aldehyde oxidase (6). On the other hand, monoamine oxidases (MAOs) are non-CYP enzymes which catalyze oxidative deamination reactions of several therapeutic agents (7) and also play a key role in metabolism of neurotransmitters, such as serotonin, dopamine and noradrenaline, although information on IVIVE for MAO substrate drugs is quite limited. Human MAOs catalyze metabolism of all classes of amines, so any amine-containing compounds in drug development could potentially be a substrate of MAOs (7). MAOs are localized on mitochondrial membrane and expressed throughout the body in humans (8, 9). Human MAOs consist of two isoforms, MAO-A and MAO-B, which show difference in substrate recognition and sensitivity to inhibitors (10).

Kamel et al. (11) reported a good correlation between hepatic intrinsic clearance *in vitro* ($CL_{int,vitro}$), which was obtained in isolated human liver mitochondria (HLMt), and unbound oral clearance for two MAO substrates, sumatriptan and CP-409,092. However, HLMt are not commonly used in IVIVE during drug development because commercially available HLMt are quite limited. Since HLMs include the major drug metabolizing enzymes, cytochrome P450, and are more widely

used as a screening tool to assess stability of drug candidates, utilization of HLMs, rather than HLMt, may be an alternative method for the determination of $CL_{int,vitro}$ values of the drugs metabolized by MAOs. Actually, HLMs exhibit MAO activities (12-14), so it may be possible to use HLMs instead of HLMt for quantitative estimation of metabolic clearance of MAO substrates *in vivo*. In the present study, to examine the feasibility of this approach, we first examined the disappearance of several MAO substrate drugs from pooled HLMs, and compared the estimated metabolic clearance values with those obtained in HLMt. Then, the $CL_{int,vitro}$ values for MAO substrate drugs were calculated using the metabolic clearance in HLMs, and compared with *in vivo* hepatic intrinsic clearance ($CL_{int,vivo}$) values obtained from literature information. Furthemore, to validate the utilization of HLMs for estimation of MAO-mediated metabolic clearance, comprehensive quantification of mitochondrial proteins was performed in both HLMs and HLMt according to proteomics approach. Our results propose that $CL_{int,vitro}$ values of MAO substrate drugs can be quantitatively estimated with HLMs and could be used for at least semi-quantitative prediction of $CL_{int,vivo}$ values for MAO substrates during drug discovery and development.

Materials and Methods

Materials

Midazolam, rizatriptan, phenylephrine and sertraline were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sumatriptan and S-citalopram were purchased from Tokyo Chemical Industry (Tokyo, Japan). Imipramine was purchased from Nakalai Tesque (Kyoto, Japan). Eletriptan and fatty acid-free human serum albumin (HSA) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were analytical-grade products from commercial sources. Pooled HLMs (50 donors, #PLo50BA), CD-1 mouse liver microsomes, SD rat liver microsomes and beagle dog liver microsomes were purchased from Thermo Fisher Scientific (Waltham, MA). Pooled HLMt (5 donors, #1110152) was purchased from Sekisui XenoTech. (Kansas City, KS). An MAO expression system (Supersomes: MAO-A, MAO-B and control) was purchased from Corning (Corning, NY). Individual HLMs and HLMt were prepared according to the previously reported method (13). Human liver blocks were obtained from Human and Animal Bridging Research Organization (Chiba, Japan). The study protocol was approved by the ethical committees of the School of Medicine in Kanazawa University. Individual HLMs and HLMt were independently prepared to prevent loss derived from sequential purification.

Disappearance of drugs in liver microsomes, HLMt and MAO expression systems

Liver microsomes (with or without 3.9 mM NADPH), Supersomes, and HLMt were preincubated in potassium phosphate buffer (100 mM, pH 7.4) containing 5 μM HSA for 5 min at 37 ^{o}C. Reactions were initiated by adding substrate solutions prewarmed to 37 ^{o}C. At selected time points, 30 μL of incubation mixture was added to 30 μL of ice-cold acetonitrile to stop the reaction, followed by centrifugation at 15,000 rpm for 10 min at 4 ^{o}C. Fifteen μL of the supernatant was added to 135 μL of 50 % water/50 % acetonitrile for sumatriptan, rizatriptan, eletriptan, citalopram, sertraline, imipramine and midazolam; 20 μL of the supernatant was added to 80 μL of acetonitrile for phenylephrine; 20 μL of the supernatant was added to 80 μL of 50 % water/50 % acetonitrile for serotonin. The diluted solutions were centrifuged at 15,000 rpm for 10 min at 4 ^{o}C, and an aliquot of each supernatant (1 or 5 μL) was subjected to liquid chromatography-mass spectrometry (LC-MS/MS). All incubations were carried out in triplicate for each incubation time.

Equilibrium dialysis

Unbound fraction ($f_{u,incu}$) of drugs in incubation mixtures with HLMs were

determined by means of equilibrium dialysis (HTDialysis, LLC, Gales Ferry, CT). The reaction mixture of pooled HLMs was added to the donor side, and 100 mM potassium phosphate buffer (pH 7.4) was added to the acceptor side. The plate was sealed and incubated on an orbital shaker (120 rpm) at 37 ˚C for 6 hr. After the incubation, 40 µL of solution was collected from both the donor and acceptor sides, and mixed with 40 µL of ice-cold acetonitrile, followed by centrifugation at 22,000 g for 10 min at 4 ^{o}C. The supernatants were then mixed with 50 or 100% acetonitrile as described above, and again centrifuged at 22,000 g for 10 min at 4 ^{o}C. The supernatants (1 or 5 µL) were subjected to LC-MS/MS.

Measurement of blood-to-plasma concentration ratio (R_B)

Fresh human blood was purchased from Kohjin-Bio (Saitama, Japan). Test compounds dissolved in saline (10 and 100 µM) were spiked into the blood to give final concentrations of 0.1 and 1 µM, respectively, in a total volume of 150 µL, followed by incubation for 10 min at 37 ˚C. An aliquot of each blood sample (100 µL) was centrifuged to obtain plasma. The plasma and a sample of remaining blood (40 µL of each) were mixed with 50 µL of acetonitrile, followed by centrifugation at 22,000 g for 10 min at 4 ^{o}C. A 70 µL aliquot of supernatant was centrifuged, and 1 or 5 µL was

subjected to LC-MS/MS. R_B was calculated as the ratio of blood to plasma concentration.

Liquid chromatography-mass spectrometry (LC-MS/MS)

All quantification was performed by means of LC-MS/MS with a Nexera X2 LC system coupled with an LCMS-8040 (Shimadzu, Kyoto, Japan). The SRM transitions of the molecular and product ions were as follows: sumatriptan, *m/z* = 296.1 > 58.1; rizatriptan, *m/z* = 270.2 > 201.1; eletriptan, *m/z* = 383.1 > 84.1; citalopram, *m/z* = 325.1 > 109.1; sertraline, *m/z* = 306.0 > 159.0; imipramine, 280.9 > 86.2; midazolam, *m/z* = 326. > 291.2; phenylephrine, *m/z* = 168.3 > 150.0; serotonin, *m/z* = 177.0 > 160.1. For sumatriptan, rizatriptan, eletriptan, citalopram, sertraline, imipramine and midazolam, the mobile phases were (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. Gradient elution (flow rate, 0.4 mL/min) was carried out as follows: 0 to 1 min, 99% A/ 1% B; 1 to 2.5 min, 99% A/ 1% B to 70% A/ 30% B; 2.5 to 3 min, 70% A/ 30% B to 10% A/ 90% B; 3 to 3.5 min, 10% A/ 90% B; 3.5 to 3.6 min, 10% A/ 90% B to 99% A/ 1% B, on a Cosmosil C18-MS-II packed column (3 µm, 2.0 x 50 mm; Nacalai tesque). For phenylephrine, the mobile phases were (A) water containing 10 mM ammonium acetate and 0.1% formic acid and (B)

95% acetonitrile containing 10 mM ammonium acetate and 0.1% formic acid. Gradient elution (flow rate, 0.4 mL/min) was carried out as follows: 0 to 0.5 min, 1% A/ 99% B; 0.5 to 3 min, 1% A/ 99% B to 30% A/ 70% B; 3 to 3.1 min, 30% A/ 70% B to 60% A/ 40% B; 3.1 to 3.5 min, 60% A/ 40% B; 3.5 to 3.6 min, 60% A/ 40% B to 1% A/ 99% B, on an ACQUITY UPLC BEH Amide Column (1.7 μm, 2.1 mm×100 mm; Waters, Milford, MA). For serotonin, the mobile phases were (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. Gradient elution (flow rate, 0.4 mL/min) was carried out as follows: 0 to 0.5 min, 95% A/ 5% B; 0.5 to 2.5 min, 95% A/ 5% B to 50% A/ 50% B; 2.5 to 3 min, 50% A/ 50% B to 5% A/ 95% B; 3 to 4 min, 5% A/ 95% B; 4 to 5 min, 5% A/ 95% B to 95% A/ 5% B, on a Discovery HS-F5 column (3 μm, 2.1 x 100 mm; Sigma-Aldrich).

Calculation of $CL_{int,vitro}$

The slope of the disappearance curve (k_e) during incubation with HLMs, liver microsomes (LMs) of animals (mouse, rat, dog and monkey), HLMt and Supersomes was estimated by linear regression analysis, and *in vitro* metabolic clearances (CL_{HLMs}, CL_{LMs}, CL_{HLMt} and CL_{Sups}) were calculated using the following equation, respectively:

$$\mathrm{CL}_{\mathrm{HLMs}}, \mathrm{CL}_{\mathrm{LMs}}, \mathrm{CL}_{\mathrm{HLMt}} \text{ or } \mathrm{CL}_{\mathrm{Sups}} = k_e \times \frac{\text{incubation volume}}{\text{mg microsomal protein}} \qquad [1]$$

Then, $CL_{int,vitro}$ was calculated using the following equation (6, 15):

$$CL_{int,vitro} = \frac{CL_{HLMs}}{f_{u,incu}} \times SF_{physiological} \quad [2]$$

where $SF_{physiological}$ is a physiological scaling factor (SF), which was assumed to be 40 mg microsomal protein/g liver and 20 g liver/kg body weight (16).

Calculation of $CL_{int,vivo}$

Human pharmacokinetic data were obtained from the literature (17-25). Nonrenal clearance (CL_{NR}) was calculated by subtracting renal clearance (CL_R) from total body clearance (CL_{tot}), and then $CL_{int,vivo}$ was calculated from CL_{NR} using the following equations based on a dispersion model (26). Dispersion model was used in the present study because it gives good predictions of in vivo clearance from in vitro data especially for high-clearance drugs (26).

$$CL_{NR} = Q_H \times (1 - F_H) \quad [3]$$

$$F_H = 4a/(1+a)^2 \exp[(a-1)/2D_N] - (1-a)^2 \exp[-(a+1)/2D_N] \quad [4]$$

$$a = [1 + (4 \times f_B \times CL_{int,vivo} \times D_N / Q_H)]^{1/2} \quad [5]$$

where Q_H, f_B and D_N represent hepatic blood flow rate, blood unbound fraction and dispersion number, which was assumed to be 0.17, respectively. If intravenous data were not available, $CL_{int,vivo}$ was calculated from the following equation:

$$CL_{int,vivo} = \frac{CL_{oral}}{f_B} = \frac{Dose}{f_B \times AUC_B} \quad [6]$$

where CL_{oral} is oral clearance, and AUC_B is the area under the curve for blood concentration after oral administration.

Proteome Analysis of pooled HLMs and HLMt

The membrane proteomics was performed as previously described (27). Briefly, the pooled HLMt and HLMs were washed by 4 M urea and then dissolved in dissolution buffer (6 M urea, 0.1 M Na_2CO_3 and 0.5% sodium deoxycholate). A 20 μg protein was reduced, alkylated and diluted with 0.1 M triethylammonium bicarbonate buffer (pH 8.5) followed by digestion with trypsin. The PTS method was performed for detergent removal (28). Fractionation and desalting were performed using SDB and C18 StageTip, respectively (29). Tryptic peptides were subjected to LC-MS/MS analysis using the Q Exactive (Thermo Fisher Scientific) coupled with the Advance UHPLC (Bruker, Billerica, MA). All samples were measured in duplicate. The raw data obtained from mass spectrometer were analyzed using Proteome Discoverer 1.4 (Thermo Fisher Scientific) and Mascot 2.4 (Matrix Science, London, UK) against UniProt human database (2014_07). The label-free quantification was performed to quantitate the identified proteins with Proteome Discoverer. In this method, a peptide area was

estimated from peaks on spectrum (full scan), and a protein area was estimated as the average of top 3 peptides with the highest area (30). The quantitative value obtained as the area was normalized by total area of quantified proteins.

Statistical analysis

The statistical analyses between two groups were performed by means of Student's t-test, and multiple comparisons were performed by a one- or two-way ANOVA with Tukey’s multiple comparison test. $P<0.05$ was regarded as denoting a significant difference.

RESULTS

Metabolic clearance of a typical MAO substrate serotonin in pooled HLMs

To perform IVIVE for MAO substrate drugs using HLMs, enzyme activity of MAO in pooled HLMs was first confirmed by observing the disappearance of serotonin, a typical MAO substrate in incubation mixtures. Disappearance of serotonin in pooled HLMs incubation mixtures was observed, and the CL_{HLMs} was calculated based on Eq. [1]. The CL_{HLMs} of serotonin was minimally affected by CYP co-factor NADPH, but almost completely reduced by MAO inhibitor pargyline (Fig. 1A). Since MAOs are localized on mitochondrial membranes, native enzyme activity of MAOs in pooled HLMt mixture was also confirmed. Disappearance of serotonin in pooled HLMt mixture was observed, and the CL_{HLMt} was calculated based on Eq. [1]. The CL_{HLMt} of serotonin was also reduced in the presence of pargyline, but not affected by NADPH (Fig. 1A). The absolute values for CL_{HLMs} in pooled HLMs was almost comparable with those for CL_{HLMt} in pooled HLMt (Fig. 1A). As a control study, disappearance of a typical CYP3A4 substrate midazolam was also examined in pooled HLMs incubation mixture (Fig. 1B). The CL_{HLMs} was greatly reduced in the absence of NADPH, but minimally affected by pargyline (Fig. 1B), supporting that pargyline is a selective inhibitor for MAOs. The CL_{HLMt} in the pooled HLMt was not clearly observed even in

the presence of NADPH (Fig. 1B).

Estimation of $CL_{int,vitro}$ for MAO substrate drugs using pooled HLMs

Three MAO substrate drugs, sumatriptan, rizatriptan and phenylephrine (7, 31), were incubated with pooled HLMs, and their disappearances from the incubation mixture was examined to estimate CL_{HLMs}. The effect of NADPH on CL_{HLMs} of sumatriptan and rizatriptan was small (Fig. 2A, 2B), whereas CL_{HLMs} of phenylephrine was partially reduced in the absence of NADPH, compared with that in its presence (Fig. 2C), suggesting that it is metabolized by CYPs. CL_{HLMs} of all three MAO substrate drugs in the absence of NADPH was almost completely reduced when pargyline was added to the incubation mixture (Fig. 2A-2C) Absolute values for CL_{HLMs} of phenylephrine was much higher than those of the other two MAO substrate drugs (Fig. 2A-2C). As a control study, four CYP substrate drugs, eletriptan (32), citalopram (33), sertraline (34) and imipramine (35) were selected from structure similarities with MAO substrate drugs and their disappearances during incubation with pooled HLMs was examined to estimate CL_{HLMs}. In these cases, the absence of NADPH led to almost complete decrease in CL_{HLMs}, and pargyline did not affect CL_{HLMs} (Fig. 2D-2G).

Metabolic clearance in MAO expression system

To examine the involvement of each isoform of Human MAOs in hepatic elimination, the three MAO substrate drugs were incubated with expression systems for human MAO-A and MAO-B. The CL_{Sups} of sumatriptan and rizatriptan in the presence of MAO-A was much higher than that in the presence of MAO-B (Fig. 3A, 3B). The CL_{Sups} of sumatriptan and rizatriptan in the presence of MAO-B was negligible. On the other hand, the CL_{Sups} of phenylephrine in the presence of MAO-A and MAO-B was higher than control, the former being higher than the latter (Fig. 3C).

Comparison of CL_{HLMs} and CL_{HLMt} obtained from individual liver samples

HLMs and HLMt were prepared in the present study from the same 10 individual donor livers. CL_{HLMs} and CL_{HLMt} values were then measured for one of MAO substrate drugs, sumatriptan, and serotonin in the individual HLMs and HLMt. For sumatriptan, CL_{HLMs} values were comparable with CL_{HLMt} values among 10 individual donors, most of those values showing less than two times difference (Fig. 4A). Similar results were also observed for CL_{HLMs} and CL_{HLMt} values of serotonin (Fig. 4B). This finding was compatible with the observation in pooled HLMs and HLMt (Fig. 1), indicating comparable MAO activity between these two fractions. The CL_{HLMs} of

sumatriptan showed correlation (r = 0.917) with that of serotonin (Fig. 4C), supporting that metabolism of sumatriptan in HLMs is mainly mediated by MAOs.

IVIVE for MAO and CYP substrate drugs

The $f_{u,incu}$ values for the three MAO substrates and four CYP substrates were measured during incubation with pooled HLMs to estimate $CL_{int,vitro}$ (Table 1). On the other hand, the literature was searched to obtain systemic clearance (CL_{tot} or CL_{oral}) and renal clearance (CL_R) data for the selected seven drugs in humans *in vivo* (Table 1). In addition, R_B values of all the drugs were measured in the present study, enabling estimation of $CL_{int,vivo}$ (Table 1). Body weight-based values of $CL_{int,vivo}$ (mL/min/kg body weight) were then calculated according to Eq. (3) - (6) and compared with $CL_{int,vitro}$. $CL_{int,vitro}$ was correlated with $CL_{int,vivo}$, but was lower than $CL_{int,vivo}$ (Fig. 5). The difference between $CL_{int,vitro}$ and $CL_{int,vivo}$ was 2-5 and 14-20 times for CYP and MAO substrates, respectively (Fig. 5, Table 1). Thus, the *in vitro-in vivo* discrepancy of hepatic intrinsic clearance tended to be larger for MAO substrate drugs than for CYP substrates.

Proteome analysis for quantification of mitochondrial proteins detected in pooled

HLMt and HLMs

To quantitatively elucidate the existence of various mitochondrial proteins including MAOs, in liver microsomal fraction, proteome analysis was performed in pooled HLMs and HLMt. In this analysis, comprehensive quantification of proteins was performed in duplicate analysis for both pooled HLMs and HLMt. Among them, 2,106 proteins were detected at least in a single determination. According to mitochondrial protein database (MitoMiner 4.0, http://mitominer.mrc-mbu.cam.ac.uk/release-4.0/begin.do), 37, 209 and 246 were then classified as outer membrane, inner membrane and matrix proteins in mitochondria, respectively (MAO-A and MAO-B were classified as outer and inner membrane protein, respectively). Most of the outer and inner mitochondrial membrane proteins (97 and 84%, respectively) were detected in both pooled HLMt and HLMs, whereas none and only 14% of outer and inner mitochondrial membrane proteins, respectively, was detected in HLMt alone (Fig. 6A). On the other hand, 40% of mitochondrial matrix proteins was detected in HLMts alone, but 60% of them was found in both HLMt and HLMs (Fig. 6A). To see the distribution pattern of mitochondrial proteins to each fraction (HLMt vs HLMs), the amount of each mitochondrial protein was separately shown in Fig. 6B. Outer membrane proteins were evenly detected in both pooled HLMt

and HLMs (Fig. 6B). Most of the inner membrane proteins were also evenly detected in both fractions (Fig. 6B). The amount of MAO-A and MAO-B in pooled HLMt was almost comparable with that in pooled HLMs (Fig. 6B). This finding could be compatible with the comparable enzymatic activity for serotonin between pooled HLMt and pooled HLMs (Fig. 1A) Because the enzymatic activity for serotonin in both pooled HLMt and HLMs was almost completely reduced in the presence of pargyline (Fig. 1A), the pargyline-sensitive portion of the enzymatic activity at least for serotonin could mainly represent MAOs-mediated metabolism, although this does not exclude the possibility that pargyline nonspecifically inhibits other enzymes than MAOs. On the other hand, most of the matrix proteins were preferably detected in HLMt, and 98 out of 246 proteins were detected only in HLMt (Fig. 6B). Thus, a part of mitochondrial proteins, especially those localized in its matrix, was preferably detected in HLMt. Twelve members of CYPs including CYP1A2, 2C19, 2D6 and 3A4, and 11 members of UGTs including UGT1A1, 2B4 and 2B7, all of which are microsomal proteins, were preferably detected in HLMs, rather than HLMt (Fig. 6B). This was compatible with metabolism of midazolam in pooled HLMs, but not pooled HLMt (Fig. 1B).

Species difference in metabolic clearance of MAO substrate drugs in LMs

LMs of mouse, rat, dog and monkey were incubated with two MAO substrate drugs (sumatriptan and rizatriptan) to estimate CL_{LMs}. In LMs of mouse, rat, dog and monkey, addition of NADPH clearly increased CL_{LMs} of both sumatriptan and rizatriptan, and no metabolism was detected in the absence of NADPH in LMs of mouse, rat and dog (Fig.7A, 7B). This was in contrast to minimal NADPH-dependence in CL_{HLMs} of the two compounds (Fig. 2A, 2B). Effect of pargyline on CL_{LMs} of rizatriptan was minimal in mouse, rat, dog and monkey (Fig. 7B).

DISCUSSION

HLMs are widely used as a tool for quantitative prediction of metabolic clearance of drugs and drug candidates by means of IVIVE, focusing predominantly on CYP-mediated metabolism. On the other hand, drug research and development has recently yielded increasing numbers of compounds that are substrates of non-CYP enzymes as drug candidates (2). Therefore, information on a suitable IVIVE strategy for non-CYP enzymes is required, and pioneering studies have been conducted on UDP-glucuronosyltransferase (5, 36) and aldehyde oxidase (6, 15). The present study aimed to quantitatively estimate metabolic clearance of MAO substrate drugs using HLMs. MAOs are localized on mitochondrial membranes. Nevertheless, metabolic clearance (CL_{HLMs}) of a typical MAO substrate serotonin and three MAO substrate drugs can be estimated in pooled HLMs (Fig. 1, 2). The CL_{HLMs} for these four compounds was reduced in the presence of MAO inhibitor pargyline, but minimally affected by NADPH (Fig. 1, 2). Similar effect of pargyline, but not NADPH on metabolic clearance of serotonin was observed in pooled HLMt (Fig. 1). In addition, CL_{HLMs} values for sumatriptan and serotonin were comparable with CL_{HLMt} values among 10 individual human livers, most of those values showing less than two times difference (Fig. 4A, 4B). Thus, metabolic clearance of MAO substrate drugs can be

quantitatively estimated with HLMs. Furthermore, $CL_{int,vitro}$ values for MAO substrates obtained from CL_{HLMs}, together with $SF_{physiological}$ and $f_{u,incu}$, were correlated with the $CL_{int,vivo}$ values (Fig. 5). The discrepancy between $CL_{int,vitro}$ and $CL_{int,vivo}$ for MAO substrate drugs was considerably larger than that for CYP substrate drugs (Fig. 5). Nevertheless, the ratio of $CL_{int,vivo}$ to $CL_{int,vitro}$ which represents so-called empirical scaling factor, was 14-20 for all three MAO substrates (Table 1). Therefore, at least semi-quantitative prediction of $CL_{int,vivo}$ may be feasible from data obtained in HLMs if an appropriate reference compound(s) metabolized by MAO is used to compensate this *in vitro-in vivo* discrepancy. However, here we investigated only three MAO substrate drugs due to the limited number of compounds currently available. Thus, further studies are required to examine more quantitative prediction ability of this approach. The larger discrepancy between $CL_{int,vitro}$ and $CL_{int,vivo}$ for MAO substrates (Fig. 5, Table 1) could be critical in drug development since such discrepancy may lead to underestimation of systemic clearance and overestimation of systemic exposure of drug candidates. The present finding may thus propose possible risk in metabolic stability of any amine-containing compound that could potentially be a substrate of MAO.

Liver mitochondria are usually prepared from precipitation with 7,500 g centrifugation of homogenates, whereas microsomal fraction is obtained from pellet

after centrifugation with 105,000 g. Therefore, microsomal fraction may contain mitochondrial proteins. Actually, according to the proteomics approach in the present study, a lot of mitochondrial proteins were identified in HLMs (Fig. 6A). In fact, most of the mitochondrial membrane proteins were evenly detected in both pooled HLMt and HLMs, whereas most of the mitochondrial matrix proteins were preferably detected in HLMt (Fig. 6B). One possible reason for the difference in the distribution between membrane and matrix proteins (Fig. 6B) is that mitochondria might be partially damaged during the liver homogenization process, resulting in separation of membrane and matrix proteins. Membrane proteins in the damaged mitochondria including MAOs, can be precipitated with microsomal proteins, whereas matrix proteins would be leaked from the broken mitochondria and cannot be precipitated with microsomal proteins.

The large discrepancy between $CL_{int,vitro}$ and $CL_{int,vivo}$ for MAO substrate drugs (Fig. 5, Table 1) can be at least partially explained if we consider that not all the liver MAOs are recovered in HLMs fraction. Actually, quantitative proteome analysis has revealed that almost equal amount of MAOs are present between HLMs and HLMt (Fig. 6B). This finding may imply that MAOs are not perfectly precipitated even with 9,000 g centrifugation and then recovered in microsomal fraction by further centrifugation with 105,000 g. However, we have also estimated $CL_{int,vitro}$ values of three MAO substrate

drugs based on metabolic clearance observed in the pooled HLMt and pooled human hepatocytes, but the $CL_{int,vitro}$ values still exhibited large discrepancy from $CL_{int,vivo}$ (data not shown). Therefore, another possible reason for the large *in vitro-in vivo* discrepancy (Fig. 5, Table 1) may be the contribution of MAO activities in extrahepatic organs to the systemic elimination of MAO substrates. MAOs are expressed throughout the body (8, 9), and extrahepatic MAOs may also contribute to overall elimination of their substrates in the body.

Prediction of hepatic intrinsic clearance has also been performed based on allometric scaling techniques (37). However, this approach could be difficult to be applied for MAO substrate drugs because of the large species differences in the contributions of NADPH- and pargyline-dependent activity (Fig. 2, 7). For example, disappearance of sumatriptan and rizatriptan is clearly greater in the presence of NADPH than in its absence in mouse, rat, dog and monkey (Fig. 7), whereas NADPH-dependent elimination of these compounds is small in humans (Fig. 2A, 2B). In addition, disappearance of rizatriptan in the presence and absence of NADPH was almost insensitive to pargyline in mouse, rat, dog and monkey (Fig. 7), whereas pargyline had a marked effect on rizatriptan disappearance in humans (Fig. 2B). These results suggest that sumatriptan and rizatriptan may be metabolized by P450 in the

animals, whereas contribution of MAOs would be small. Thus, there are large species differences in microsomal elimination of these MAO substrate drugs. This in turn supports the validity of IVIVE approach using HLMs rather than animal scale up methods for prediction of $CL_{int,vivo}$ in humans.

In conclusion, our results suggest that metabolic clearance of MAO substrate drugs can be quantitatively estimated using HLMs, and $CL_{int,vitro}$ values obtained from the metabolic clearance in HLMs, together with physiological and empirical scaling factors, and unbound fraction in incubation mixture, could be used for semi-quantitative prediction of $CL_{int,vivo}$ values.

ACKNOWLEDGEMENT

We thank Ms Lica Ishida for technical assistance. This study was supported in part by funds from the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO) and in part by the Research on the Innovative Development and the Practical Application of New Drugs for Hepatitis B provided by the Japan Agency for Medical Research and Development (AMED).

CONFLICT OF INTEREST DISCLOSURE

We have no conflict of interest to be disclosed.

AUTHOR CONTRIBUTIONS

Y.M., S.N., A.H., K.H., N.I., and N.N. conducted experiments. S.N., A.H., and K.H. and analyzed the data. Y.M., S.N. and Yu.K. wrote the manuscript. Yo.K. and Yu.K. designed the research.

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FIGURE LEGENDS

Figure 1. Metabolic clearance of a typical MAO substrate serotonin and a CYP substrate midazolam in pooled HLMs and HLMt

Pooled HLMs or HLMt (0.2 or 0.6 mg protein/mL, respectively) were incubated with serotonin (A) and midazolam (B) with or without 10 μM pargyline. Incubation was also performed in the absence or presence of 3.9 mM NADPH. CL_{HLMs} and CL_{HLMt} were calculated from the slope of disappearance curve during incubation. Each value represents the mean ± S.E.M. (n = 3). *, $p<0.05$ compared to the incubation without pargyline. #, $p<0.05$ compared to the incubation without NADPH. N.D., not detected (< 5 μL/min/mg protein for midazolam)

Figure 2. Metabolic clearance of MAO and CYP substrates in pooled HLMs

Pooled HLMs were incubated with sumatriptan (A), rizatriptan (B), phenylephrine (C), eletriptan (D), citalopram (E), sertraline (F) and imipramine (G) with or without 10 μM pargyline. Incubation was also performed in the absence or presence of NADPH. CL_{HLMs} was calculated from the slope of disappearance curve during incubation. Each value represents the mean ± S.E.M. (n = 3). *, $p<0.05$ compared to the incubation without pargyline. #, $p<0.05$ compared to the incubation without NADPH.

Figure 3. Metabolic clearance of MAO substrate drugs in MAO expression systems

Expression systems (Supersomes) for control, human MAO-A and MAO-B (0.1 mg protein/mL) were incubated with sumatriptan (A), rizatriptan (B), and phenylephrine (C). CL_{Sups} was calculated from the slope of disappearance curve during incubation. Each value represents the mean ± S.E.M. (n = 3).

Figure 4. Comparison of CL_{HLMs} and CL_{HLMt} obtained in individual HLMs and HLMt

In-house prepared individual HLMs and HLMt (●) from the same donors, and commercially available pooled HLMs and HLMt (○) were incubated with sumatriptan or serotonin in the presence of NADPH. CL_{HLMs} and CL_{HLMt} were calculated from the slope of disappearance curve during incubation. In panels (A) and (B), correlation between CL_{HLMs} and CL_{HLMt} for sumatriptan and serotonin, respectively, was examined, and the dashed, straight and long dashed dotted line represent 1:2, 1:1 and 2:1 correlation, respectively. In panel (C), correlation of CL_{HLMs} between the two compounds was examined, and the broken lines represent correlation line (r = 0.917).

Each value represents the mean ± S.E.M. (n = 3).

Figure 5. Correlation between $CL_{int,vitro}$ and $CL_{int,vivo}$

$CL_{int,vitro}$ values estimated in pooled HLMs were plotted against $CL_{int,vivo}$ values estimated from reported *in vivo* disposition data as well as R_B and fp for MAO (●) and CYP substrates (◆). The long dashed dotted, dashed and straight lines represent 1:10, 1:3 and 1:1 correlation, respectively.

Figure 6. Proteome analysis of mitochondrial proteins detected in pooled HLMs and HLMt

(A) Pooled HLMs and HLMt were subjected to proteome analysis. In this analysis, the number of mitochondrial proteins reported to be localized in outer membranes, inner membranes and matrix of mitochondria, was determined in each fraction. The white, hatched and black boxes represent the number of mitochondrial proteins detected in pooled HLMs alone, both pooled HLMs and HLMt, and pooled HLMt alone, respectively. (B) In this analysis, the amount of mitochondrial proteins was determined in each fraction, and the ratio of the amount of mitochondrial proteins in pooled HLMt to that in pooled HLMs were shown. Each symbol represents a single protein unless the

number of proteins were written as figures inside the symbols when those proteins were detected only in pooled HLMt or HLMs. As a control, the data for CYPs and UGTs were separately shown, confirming uneven distribution to HLMs.

Figure 7. Species differences in metabolic clearance of MAO substrates in liver microsomes

Liver microsomes of mouse, rat, dog and monkey (1 mg protein/mL) were incubated with 1 μM sumatriptan (A) and rizatriptan (B) with or without NADPH in the presence or absence of 10 μM pargyline. CL_{LMs} was calculated from the slope of disappearance curve during incubation. Each value represents the mean ± S.E.M. (n = 3). *, $p<0.05$ compared to the incubation without pargyline. #, $p<0.05$ compared to the incubation without NADPH.

Table 1. Comparison of $CL_{int,vitro}$ stimated in HLMs with $CL_{int,vivo}$

Drugs	k_e[a] [$x10^{-3}$ min^{-1}]	$f_{u,incu}$	$CL_{int,vitro}$[b] [mL/min/kg]	CL_{tot}[c] or CL_{oral}[d] [mL/min/kg]	CL_R[e]	CL_{NR}[f]	R_B[g]	f_p[h]	$CL_{int,vivo}$ [mL/min/kg]
Phenylephrine	19	1	75.1	834[d]	N.A.	N.A.	1.6	0.88	1503[i]
Sumatriptan	1.7	0.97	2.31	18.0[c]	4.00	14.0	1.0	0.83	31.4[j]
Rizatriptan	1.3	1	1.73	19.1[c]	5.04	14.1	1.0	0.86	31.5[j]
Eletriptan	14	0.82	22.8	6.37[c]	0.57	5.81	1.0	0.13	55.3[j]
Citalopram	2.1	0.73	3.86	7.77[c]	0.67	7.10	1.0	0.46	19.9[j]
Sertraline	8.6	0.03	347	10.5[d]	N.A.	N.A.	2.1	0.02	1085[i]
Imipramine	1.6	0.44	47.7	7.67[c]	0.03	7.64	1.3	0.19	72.6[j]

a) HLMs concentration for phenylephrine and other drugs was set to be 0.2 and 0.6 mg protein/mL, respectively.

b) Extrapolated using $SF_{physiological}$ (40 mg microsomal protein/g liver and 20 g liver/kg body weight).

c) Total body clearance obtained from the literature.

d) Oral clearance obtained from the literature.

e) Renal clearance obtained from the literature.

f) Nonrenal clearance calculated by subtracting CL_R from CL_{tot}.

g) Blood-to-plasma concentration ratio obtained in the present study.

h) Unbound fraction in human plasma obtained from the literature.

i) Estimated from CL_{oral}.

j) Calculated using CL_{NR} based on a dispersion model.

N.A.: Not available