Significance of reductive metabolism in human intestine and quantitative prediction of intestinal first-pass metabolism by cytosolic reductive enzymes

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Abbreviations:
AKR, aldo-keto reductase; AO, aldehyde oxidase; CBR, carbonyl reductase; $CL_{b,p}$, hepatic clearance in plasma base; $CL_{u}$, unbound intrinsic clearance; $CL_{total,p}$, total clearance in plasma base after intravenous administration; CYP, cytochrome P450; EMS, enhanced mass spectrum; $F$, bioavailability; $F_a$, fraction absorbed; $F_g$, intestinal availability; $F_h$, hepatic availability; $f_u$, unbound fractions in incubations; $f_{urine}$, the fraction of the dose excreted in urine as unchanged drug; HIC, human intestinal cytosol; HIM, human intestinal microsomes; HIS9, human intestinal S9; HLC, human liver cytosol; HLM, human liver microsomes; HLS9, human liver S9; HPLC-MS/MS, high-performance liquid chromatography with tandem mass spectrometry; HPLC-PDA/MS/MS, high-performance liquid chromatography with photodiode array detector and tandem mass spectrometry; $1\beta$-HSD, $1\beta$-hydroxysteroid dehydrogenase; $k_{el}$, elimination rate constant; m/z, mass-to-charge ratio; PAMPA, parallel
artificial membrane permeability assay; $P_{\text{app}}$, apparent permeability; $Q_h$, hepatic blood flow;
Rb, blood-to-plasma concentration ratio; SDR, short-chain dehydrogenases/reductases; UGT,
Uridine Diphosphate Glucuronosyltransferase
Abstract

The number of new drug candidates that are cleared via non-CYP enzymes has increased. However, unlike oxidation by CYP, the roles of reductive enzymes are less understood. Especially, the metabolism in intestine is not well known. The purposes of the present study were to investigate the significance of reductive metabolism in human intestine, and establish a quantitative prediction method of intestinal first-pass metabolism by cytosolic reductive enzymes, using haloperidol, mebendazole, and ziprasidone. First, we estimated the metabolic activities for these compounds in intestine and liver using subcellular fractions. Metabolic activities were detected in human intestinal cytosol (HIC) for all 3 compounds, and the intrinsic clearance values were higher than those in human liver cytosol (HLC) for haloperidol and mebendazole. These metabolic activities in HIC were NADPH- and/or NADH-dependent. Furthermore, the metabolic activities for all 3 compounds in HIC were largely inhibited by menadione, which has been used as a carbonyl reductase (CBR)-selective chemical inhibitor. Therefore, considering subcellular location, cofactor requirement, and chemical inhibition, these compounds might be metabolized by CBRs in human intestine. Subsequently, we tried to quantitatively predict intestinal availability ($F_g$) for these compounds using human intestinal S9. Our prediction model using apparent permeability of parallel artificial membrane permeability assay ($P_{app,PAMPA}$) and metabolic activities in HIS9 could predict $F_g$ in humans for the 3 compounds well. In conclusion, CBRs might have higher metabolic activities in human intestine than in human liver. Furthermore, our prediction method of human $F_g$ using HIS9 is applicable to substrates of cytosolic reductive enzymes.
Introduction

In early drug research efforts, it is commonplace for newly synthesized compounds to be tested for proclivity to high clearance by incubating with human liver microsomes and cofactors to support cytochrome P450 (CYP) activity (Obach, 2001). Compounds demonstrating high lability are either discarded or structurally modified to impart improved metabolic stability. This type of activity has now been occurring for several years, and our ability to find and design new drugs with low CYP-catalyzed lability is well established. An unintended consequence of such an effort has been that the number of new drug candidates that are cleared via non-CYP enzymes, has increased (Zientek et al., 2010).

Unlike oxidation by CYP, the roles of reductive enzymes are less well understood. Alcohol dehydrogenases, aldo-keto reductases (AKRs), short-chain dehydrogenases/reductases (SDRs), and quinone reductases (Maser, 1995; Oppermann and Maser, 2000; Parkinson, 1996) catalyze the reduction of aldehyde and ketone moieties. Most carbonyl-reducing enzymes are AKRs or SDRs and are cytosolic enzymes, except for some SDR superfamily members located in microsomes and mitochondria (Forrest and Gonzalez, 2000). Carbonyl reductases (CBRs) and 11β-hydroxysteroid dehydrogenase (11β-HSD) are SDR superfamily members. CBRs are NADPH and/or NADH-dependent cytosolic enzyme, and 11β-HSD is microsomal enzyme. Recently, it was reported that aldehyde oxidase (AO) also catalyzes reduction such as the reduction of the N-S bond in the benzisothiazole moiety of ziprasidone (Beedham et al., 2003; Prakash et al., 1997). AO is a cofactor-independent cytosolic enzyme.

Haloperidol, mebendazole, and ziprasidone undergo reductive metabolism in humans (Kudo et al., 1999; Dawson et al., 1985; Beedham et al., 2003). Haloperidol and mebendazole are carbonyl compounds. It was reported that major metabolic pathways of haloperidol in liver were reduction by CBR, and oxidative dealkylation by CYP3A4, and glucuronidation by uridine diphosphate glucuronosyltransferase (UGT) (Someya et al., 1992). Mebendazole is metabolized by hydrolysis and reduction in humans (Dawson M et al., 1985), however, the
enzyme which involve in each metabolic pathway is unknown. Ziprasidone is reductively metabolized by AO in liver cytosol (Beedham et al., 2003), and the other main metabolic pathway is oxidation by CYP3A4 (Miao et al., 2005). On the other hand, whether reductases contribute to intestinal first-pass metabolism has not been reported. Therefore, first, we estimated whether haloperidol and ziprasidone were metabolized in intestinal cytosolic fraction, because CBR and AO are cytosolic enzymes and CYP and UGT are microsomal enzymes. In addition, we investigated metabolites of mebendazole in various subcellular fractions because there is a possibility that hydrolysis is involved in the metabolic activity for mebendazole in cytosolic fraction. Furthermore, we conducted enzyme identification using chemical inhibitor. Menadione, flufenamic acid, and ondansetron was used as inhibitor for CBRs, AKRs, AO, respectively (Atalla et al., 2000; Rosemond et al., 2004; Obach et al., 2011; Atalla et al., 2000; Maser et al., 2000; Porter et al., 2000; Rosemond et al., 2004).

The pharmacological action of orally administered drugs is influenced by oral bioavailability. The fraction entering intestinal tissue, intestinal availability, and hepatic availability are involved in oral bioavailability. It is now well known that many drugs metabolized by CYP3A and UGT undergo first-pass metabolism in the intestine (Wu et al., 1995; Kato et al., 2008; Kemp et al., 2002; Cubbit et al., 2009). On the other hand, the intestinal metabolic activities by other non-CYP enzymes are not well known.

We reported a prediction method of the intestinal first-pass metabolism in humans based on in vitro data (Nishimuta et al., 2011). In this method, it is necessary to use metabolic clearance values from metabolic stability in human intestinal microsomes and permeability values from parallel artificial membrane permeability assay (PAMPA) at pH 7.4. It showed good prediction accuracy for CYP3A and UGT substrates; however, whether this method is applicable to reductive enzymes has not yet been investigated. Furthermore, there has been no report of a quantitative prediction method of intestinal first-pass metabolism by cytosolic enzymes using human intestinal cytosol or human intestinal S9.

The purposes of the present study were to investigate the significance of reductive
metabolism in human intestine, and to establish a quantitative prediction method of intestinal first-pass metabolism by cytosolic reductive enzymes, using haloperidol, mebendazole, and ziprasidone. The metabolic stabilities in intestine and liver were estimated using subcellular fractions. The reductive enzymes involved in intestinal metabolic activities for the compounds were investigated by subcellular location, cofactor requirement, and chemical inhibition. The intestinal availability (Fg) values of these compounds in humans were then quantitatively predicted using the metabolic stabilities in human intestinal S9 fraction.
Materials and Methods

Chemicals

Mebendazole, haloperidol, nitrendipine, terfenadine, (±)-verapamil hydrochloride, and flecainide acetate were purchased from Sigma Aldrich (St. Louis, MO). Midazolam and nifedipine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Buspirone hydrochloride, flufenamic acid, and ondansetron hydrochloride dihydrate were purchased from LKT Laboratories (St. Paul, MN), lovastatin and ziprasidone hydrochloride monohydrate from Toronto Research Chemicals (North York, ON, Canada), saquinavir mesylate from The United States Pharmacopeial Convention (Rockville, MD), and menadione from Enzo Life Sciences, Inc. (New York, NY). β-NADPH and β-NADH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All the other reagents and solvents were analytical grade and commercially available.

Subcellular fractions

Pooled intestinal and liver microsomes from humans [HIM and HLM; pool of 18 (male: 11, female: 7) and pool of 200 (male: 100, female: 100), respectively], pooled intestinal and liver S9 from humans [HIS9 and HLS9; pool of 18 (male: 11, female: 7) and pool of 50 (male: 24, female: 26), respectively], and pooled intestinal and liver cytosol from humans [HIC and HLC; pool of 13 (male: 7, female: 6) and pool of 50 (male: 25, female: 25), respectively] were purchased from Xenotech, LLC (Lenexa, KS, USA).

Metabolic stabilities in HIM, HLM, HIC, HLC, HIS9, and HLS9 for haloperidol, mebendazole, and ziprasidone

We incubated substrates (haloperidol, mebendazole, and ziprasidone) at 37°C in 100 μL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), cofactors (3 mM β-NADPH, 3 mM β-NADH, or distilled water), and subcellular fractions (microsomes, cytosol, or S9 from human intestine or liver). Linearity of metabolic activities for subcellular
fractions (0.2–4 mg protein/mL), substrate concentrations (50–1000 nM), and incubation times (30 min or 60 min) were confirmed, and the optimal reaction conditions were set for each compound. We adopted 200 nM as a substrate concentration because metabolic activities for haloperidol, mebendazole, and ziprasidone showed linearity in the range of 50–1000 nM. The final concentration of acetonitrile in the incubation mixture was 0.5% (v/v). After preincubation at 37°C for 5 min, the reactions were initiated by the addition of the substrates and stopped by addition of 200 μL of ice-cold methanol. Control samples were incubated using the same method under the absence of substrates; substrates were added after addition of ice-cold methanol.

The reaction mixtures were spiked with 200 μL of methanol containing the internal standard, 200 nM flecainide. The mixtures were centrifuged at 4500 rpm for 10 min to remove precipitated protein. The supernatants were then filtered using 96-well filter plates with 0.45-μm pore size (Varian Inc., Palo Alto, CA, USA). The filtrates were diluted 2-fold using distilled water and transferred to 96-well plates. A 10-μL portion was then injected into a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system.

**Metabolic route of mebendazole in HIM, HLM, HIC, HLC, HIS9, and HLS9**

We incubated 10 μM mebendazole at 37°C in 200 μL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4) and 3 mM β-NADPH with 1 mg protein/mL subcellular fractions (microsomes, cytosol, or S9 from human intestine or liver) for 60 min. The final concentration of acetonitrile in the incubation mixture was 0.5% (v/v). After preincubation at 37°C for 5 min, the reactions were initiated by the addition of mebendazole, and were later stopped by addition of 600 μL of ice-cold methanol. Control samples were incubated using the same method under the absence of mebendazole; mebendazole was added after addition of ice-cold methanol.

The mixtures were centrifuged at 4500 rpm for 10 min to remove precipitated protein.
The supernatants were then filtered using 96-well filter plates with 0.45-μm pore size (Varian Inc., Palo Alto, CA, USA). The filtrates were diluted 2-fold using distilled water and transferred to 96-well plates. A 10-μL portion was then injected into a high-performance liquid chromatography system with photodiode array detector and tandem mass spectrometry (HPLC-PDA/MS/MS).

**Metabolic stabilities in HIS9 for CYP3A substrates**

We incubated 8 CYP3A substrates (50 nM) separately at 37°C in 100 μL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), 3 mM β-NADPH, and HIS9. The incubations were conducted with the following concentrations of HIS9: 0.2 mg protein/mL for lovastatin and saquinavir; 1 mg protein/mL for other 6 compounds; and incubation times: 15 min for lovastatin and saquinavir; 30 min for other 6 compounds. Subsequently, the assay was performed as described under *Metabolic stabilities in HIM, HLM, HIC, HLC, HIS9, and HLS9 for haloperidol, mebendazole, and ziprasidone*.

**The fraction unbound in incubations**

The unbound fractions in incubations \(f_u\) for haloperidol, mebendazole, ziprasidone, and 8 CYP3A substrates were determined using high-throughput dialysis. Dialysis membranes had a 10-kDa molecular mass cutoff and were purchased from Harvard Apparatus (Holliston, MA, USA). Compounds (1 μM, final) with subcellular fractions (HIM, HLM, HIC, HLC, HIS9, or HLS9 for haloperidol, mebendazole, and ziprasidone, and HIS9 for 8 CYP3A substrates at the concentrations used in the metabolic stability studies for each compound) in 50 mM phosphate buffer (pH 7.4) were added to the acceptor chambers, and 50 mM phosphate buffer (pH 7.4) was added to the donor chamber. The dialysis plate was placed in an incubator at 37°C for 22 h on a plate rotator. 30 μL of samples from the acceptor chamber were mixed with 30 μL of 50 mM phosphate buffer (pH 7.4), and 30 μL of samples from the donor chamber were mixed with 30 μL of subcellular fractions (HIM, HIS9, HIC, HLM,
HLS9, and HLC at the concentrations used in metabolic stability studies) in 50 mM phosphate buffer (pH 7.4). These samples were then mixed with 240 μL of methanol containing the internal standard, 200 nM flecainide, and centrifuged at 4500 rpm for 10 min to remove precipitated protein. The supernatants were then filtered using 0.45-μm 96-well filter plates (Varian, Inc., Palo Alto, CA, USA) and diluted 2-fold with distilled water for HPLC-MS/MS.

Identification of metabolic enzymes of haloperidol, mebendazole, and ziprasidone in HIC using chemical inhibitors

Haloperidol, mebendazole, and ziprasidone (200 nM, final concentration) were separately incubated at 37°C in 100 μL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), HIC (1 mg protein/mL), inhibitors, and 3 mM NADPH to assess the change in percentage of substrate consumed. The incubation time was 60 min for haloperidol and ziprasidone and 30 min for mebendazole. Menadione (0, 20, 100, or 200 μM, final concentration), flufenamic acid (0, 2, 10, or 20 μM, final concentration), and ondansetron (0, 0.5, 5, or 50 μM, final concentration) was used as inhibitor for carbonyl reductase (CBR), AKRs, AO, respectively (Atalla et al., 2000; Rosemond et al., 2004; Obach et al., 2011; Atalla et al., 2000; Maser et al., 2000; Porter et al., 2000; Rosemond et al., 2004). Subsequently, the assay was carried out as described under Metabolic stabilities in HIM, HLM, HIC, HLC, HIS9, and HLS9 for haloperidol, mebendazole, and ziprasidone.

Parallel artificial membrane permeability assay for haloperidol, mebendazole, and ziprasidone

PAMPA was carried out using a PAMPA Evolution instrument from pION Inc. (Woburn, MA, USA) (Avdeef et al., 2005). The lipid solution consisted of a 20% (w/v) dodecane solution and lecithin mixture. The donor solutions consisted of test compounds dissolved in 10 mM dimethylsulfoxide diluted in pH 7.4 buffer (final concentration of 50 μM). The acceptor solution was adjusted to pH 7.4. The test plate was incubated for 240 min at room temperature in a humidity-saturated atmosphere. The concentration of each test
compound in the reference, donor, and acceptor plates was measured with a UV plate reader. The apparent permeability coefficient ($P_{app}$) was calculated using PAMPA Evolution software (pION Inc.).

**Analytical procedure**

Concentrations of compounds in samples from metabolic stability studies were measured using an HPLC-MS/MS system consisting of an API4000 mass spectrometer (Applied Biosystems, Forester City, CA, USA) with a Shimadzu 10A series HPLC system or an API3200QTrap mass spectrometer (Applied Biosystems) with a Shimadzu 20A series HPLC system. Chromatography was performed using Inertsil ODS-3 columns (3-μm particle size, 2.1×50 mm, GL Science, Tokyo, Japan) warmed to 40°C. The mobile phase consisted of 0.1% formic acid (A) and methanol (B). The flow rate was 0.2 ml/min, and the gradient conditions for elution were as follows: gradient [min, B%] = [0, 10]-[1, 90]-[4, 90]-[4.1, 10]-[7, 10] when measuring lovastatin or gradient [min, B%] = [0, 10]-[1, 90]-[3, 90]-[3.1, 10]-[6, 10] when measuring the others. Mass spectrometry detection was performed by positive ionization electrospray. The selective reaction monitoring mode was used as follows to monitor ions (m/z: precursor ion $\rightarrow$ product ion): buspirone (386.0 $\rightarrow$ 122.0), haloperidol (375.7 $\rightarrow$ 164.9), lovastatin (427.0 $\rightarrow$ 325.0), mebendazole (295.8 $\rightarrow$ 263.8), midazolam (326.0 $\rightarrow$ 291.0), nifedipine (347.0 $\rightarrow$ 254.0), nitrendipine (361.0 $\rightarrow$ 315.0), saquinavir (671.4 $\rightarrow$ 570.1), terfenadine (472.3 $\rightarrow$ 436.0), verapamil (455.2 $\rightarrow$ 165.0), ziprasidone (413.1 $\rightarrow$ 193.6), flecainide (415.1 $\rightarrow$ 398.1).

To confirm the metabolic route of mebendazole in HIM, HLM, HIC, HLC, HIS9, and HLS9, the samples described above were measured using an HPLC-PDA/MS/MS system consisting of an API5500QTrap mass spectrometer (Applied Biosystems) with a Shimadzu 20A series HPLC system. Chromatography was performed using Cadenza CD-C18 columns (3-μm particle size, 2×150 mm, Intakt Corporation, Kyoto, Japan) warmed to 40°C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid.
(B). The flow rate was 0.2 ml/min, and the gradient conditions for elution were as follows: gradient \([\text{min}, \text{B\%}] = [0, 5]-[3, 5]-[15, 35]-[30, 95]-[33, 95]-[33.1, 5]-[40, 5]\). Detection wavelength was set from 190 to 400 nm for the photodiode array detector. Mass spectrometry detection was performed by positive ionization electrospray. The mass spectra of mebendazole and its metabolites were obtained using enhanced mass spectrum (EMS) scan with the m/z range of 50-800 Da. The metabolites of mebendazole were estimated by comparing the UV spectra at 254 nm with the MS spectra from the incubated samples and control samples. The detected m/z of mebendazole, hydrolyzed mebendazole, and reduced mebendazole was 296.3, 238.3, and 298.3, respectively.

Data Analysis

The peak area ratios of test compounds to internal standards were used for calculation in experiments of the metabolic stability studies, the fraction unbound studies, and the studies with chemical inhibitors. The mean value of duplicate determinations was plotted versus incubation time on a semi-logarithmic scale, and the slope was determined by linear-regression analysis as the elimination rate constant \((k_{el} \text{ (min}^{-1})\)). The unbound intrinsic clearance values \((CL_{int,u})\) were calculated using Equation 1:

\[
CL_{int,u} \text{ (mL/min/mg protein)} = \frac{k_{el} \text{ (min}^{-1})}{\text{microsomal concentration (mg protein/mL)}} \cdot \frac{1}{f_u} \quad \text{Equation 1}
\]

Investigated \(f_u\) values were calculated using Equation 2, and the mean of duplicate determinations were calculated.

\[
f_u = \frac{\text{peak area in buffer sample/peak area internal standard}}{\text{peak area in microsomal sample/peak area internal standard}} \quad \text{Equation 2}
\]

\(F_u\) is the fraction of dose that escapes intestinal first-pass metabolism in the enterocytes.
and penetrates into blood flow of portal vein. We reported previously the $F_g$ prediction method using Equation 3 (Nishimuta et al., 2011).

\[
F_{g, \text{predicted}} = \frac{0.011 \cdot P_{\text{app, PAMPA(pH 7.4)}}}{0.011 \cdot P_{\text{app, PAMPA(pH 7.4)}} + \text{CL}_{\text{int, u, HIM}}}
\]

Equation 3

Where $P_{\text{app, PAMPA(pH 7.4)}}$ (10^{-6} \text{ cm/s}) represents the apparent permeability of PAMPA at pH 7.4, and $\text{CL}_{\text{int, u, HIM}}$ (mL/min/mg HIM) represents the unbound intrinsic clearance in HIM.

As shown in Equation 3, our $F_g$ prediction method was defined using the metabolic activity in microsomal fraction ($\text{CL}_{\text{int, u, HIM}}$ (mL/min/mg HIM)). The 0.011 value is empirical scaling factor set using compounds which are metabolized only in microsomal fraction. Therefore, in order to apply to compounds which are metabolized both in microsomal fraction and cytosolic fraction, a scaling factor from metabolic activities per S9 protein to those per microsomal protein is needed. CYP3A is expressed in microsomal fraction, but it is not expressed in cytosolic fraction. Therefore, we assumed that the difference between $\text{CL}_{\text{int, u, HIM}}$ (mL/min/mg HIM) and $\text{CL}_{\text{int, u, HIS9}}$ (mL/min/mg HIS9) for CYP3A substrates is due to the difference between protein levels in microsomes and S9 (mg HIM protein/g intestine vs mg HIS9 protein/g intestine) (Equation 4).

\[
\frac{\text{CL}_{\text{int, u, HIM}} \text{ for CYP3A substrate (mL/min/mg HIM)}}{\text{CL}_{\text{int, u, HIS9}} \text{ for CYP3A substrate (mL/min/mg HIS9)}} = \text{Scaling factor (mg HIS9/mg HIM)}
\]

Equation 4

In the present study, we investigated the relationship between metabolic activities in HIM (Nishimuta et al., 2010) and those in HIS9 for CYP3A substrates that might be metabolized only in microsomal fraction. The fitting line of the correlation was 3.35-fold higher in HIM than in HIS9 (Figure 5). Therefore, we defined that the scaling factor in Equation 4 was 3.35 although it is indirectly method.
Then, we can calculate the $F_g$ values of haloperidol, mebendazole, and ziprasidone in addition to CYP3A substrates using $CL_{\text{int}, u, \text{HIS9}}$ by Equation 6.

$$F_{\text{g, predicted}} = \frac{0.011 \cdot P_{\text{app, PAMPA(pH 7.4)}}}{0.011 \cdot P_{\text{app, PAMPA(pH 7.4)}} + 3.35 \cdot CL_{\text{int}, u, \text{HIS9}}}$$  \hspace{1cm} \text{Equation 6}$$

Where $P_{\text{app, PAMPA(pH 7.4)}}$ ($10^{-6}$ cm/s) represents the apparent permeability of PAMPA at pH 7.4, and $CL_{\text{int}, u, \text{HIS9}}$ (mL/min/mg HIS9) represents the unbound intrinsic clearance in HIS9.

**Calculation of $F_g$(observed) in humans by i.v./oral method**

$F_{g, \text{human(observed)}}$ values were obtained from a previous report (Table 3; Varma et al., 2010). They calculated hepatic clearance in plasma base ($CL_{h,p}$) using Equation 7 assuming that the nonrenal clearance is all due to hepatic clearance. Then, hepatic availability ($F_h$) calculated using Equations 7-8, as follows:

$$CL_{h,p} = CL_{\text{total, p}} \cdot (1 - f_{\text{urine}})$$  \hspace{1cm} \text{Equation 7}$$

$$F_h = 1 - \frac{CL_{h,p}}{Q_h} / R_b$$  \hspace{1cm} \text{Equation 8}$$

where $CL_{\text{total, p}}$ is total clearance in plasma base after intravenous administration, $f_{\text{urine}}$ is the fraction of the dose excreted in urine as unchanged drug, $Q_h$ (23 ml/min/kg) is hepatic blood flow in humans, and $R_b$ is blood-to-plasma concentration ratio (assumed to be 1).

Next, fraction absorbed ($F_a$) values were calculated. For mebendazole, $F_a$ was calculated using Equation 9. Mass balance excretion studies in which radiolabeled drug was administered orally and intravenously, and radioactivity excreted in the urine was compared
to estimate $F_a$:

$$F_a = \frac{A_{\text{activity excreted, urine, po}}}{A_{\text{activity excreted, urine, iv}}} \quad \text{Equation 9}$$

where $A$ is amount.

For ziprasidone, $F_a$ was calculated using Equation 10. The amount of unchanged drug in feces after oral administration was measured (assuming that all metabolites observed arise from absorbed material and not from gut microflora):

$$F_i = 1 - \frac{A_{\text{excreted, feces}}}{\text{dose}_{\text{po}}} \quad \text{Equation 10}$$

For haloperidol, Varma et al. reported that the $F_a$ value of haloperidol was 1. We could not find the data which is necessary to calculate $F_a$ and we could not know which method was used in the report. Then, we assumed that the $F_a$ value of haloperidol was 1.

Finally, observed $F_g$ values were calculated using Equation 11.

$$F_{g, \text{human(observed)}} = \frac{F}{F_a F_b} \quad \text{Equation 11}$$

where $F$ is bioavailability.
Results

$CL_{int,u}$ values in microsomes, cytosol, and S9 of human intestine and liver for haloperidol, mebendazole, and ziprasidone in the presence of NADPH

$CL_{int,u}$ values in HIM, HLM, HIC, HLC, HIS9, and HLS9 are shown in Table 1. Metabolic activities were detected in HIC for all 3 compounds, and the $CL_{int,u}$ values in HIC were higher than those in HLC for haloperidol and mebendazole. On the other hand, $CL_{int,u}$ values in HIM were lower than those in HLM for all 3 compounds. Mebendazole showed the highest $CL_{int,u}$ value in HIC among the 3 compounds, and no metabolic activity in HIM. Furthermore, $CL_{int,u}$ value in HIS9 was higher than those in HLS9 only for mebendazole.

$CL_{int,u}$ values in human intestinal S9 for haloperidol, mebendazole, and ziprasidone in the presence or absence of NADH and NADPH

$CL_{int,u}$ values in HIS9 for haloperidol, mebendazole, and ziprasidone in the presence or absence of NADH and NADPH are shown in Table 2. Metabolic activities were not detected in the absence of both NADH and NADPH for all 3 compounds. On the other hand, metabolic activities were detected in the presence of only NADPH for all 3 compounds. In the presence of only NADH, haloperidol and mebendazole were not metabolized, whereas ziprasidone was metabolized.

Metabolic route of mebendazole in microsomes, cytosol, and S9 of human intestine and liver

Metabolic routes of mebendazole in HIM, HLM, HIC, HLC, HIS9, and HLS9 are shown in Figures 1-3. The metabolites of mebendazole were estimated by comparing the UV spectra at 254 nm with the MS spectra from the incubated samples and the control samples. Reported major metabolic routes in humans were hydrolysis and reduction (Beedham et al., 2003) (Figure 1). From spectral data, the major metabolic route in HLM was hydrolysis. On the other hand, the major route in HIC, HIS9, and HLC was reduction. Both hydrolysis and reduction occurred in HLS9.
Effects of chemical inhibitors on metabolism of haloperidol, mebendazole, and ziprasidone in human intestinal cytosol

The metabolic activities for haloperidol, mebendazole, and ziprasidone in HIC were largely inhibited (by 90% at 200 μM) by menadione, which is an inhibitor of human CBRs (Figure 4A). On the other hand, the metabolic activities for haloperidol, mebendazole, and ziprasidone in HIC were not inhibited by 20 μM flufenamic acid, which is an inhibitor of human AKRs, or in 50 μM ondansetron, which is an inhibitor of human AO (Figure 4B and 4C).

Correlation of between CL_{int,u} values in human intestinal microsomes and human intestinal S9 for CYP3A substrates

In order to predict human $F_g$ for substrates of cytosolic enzymes, a scaling factor from S9 to microsomes in human intestine is needed. The relationship of the $CL_{int,u}$ values between HIM and HIS9 for CYP3A substrates is shown in Figure 5. The fitting line of the correlation for CYP3A substrates that might be metabolized only in microsomal fraction was 3.35-fold higher in HIM than in HIS9. Therefore, we calculated the $F_g$ values of haloperidol, mebendazole, and ziprasidone using Equation 6.

Calculation of $F_{g,\text{human (observed)}}$ for haloperidol, mebendazole, and ziprasidone from i.v. and oral data

$F_{g,\text{human (observed)}}$ values were calculated using Equations 7-11 (Table 3). Calculated $F_{g,\text{human (observed)}}$ for haloperidol, mebendazole, and ziprasidone was 0.903, 0.620 and 0.854, respectively.

Prediction of $F_{g,\text{human}}$

Using $P_{\text{app,PAMPA}}$ and $CL_{\text{int,u,HIM}}$ calculated from $CL_{\text{int,u,HIS9}}$ in the presence of NADH
and NADPH by equation 6, we predicted $F_{g,\text{human}}$ using equation 6 (Table 4). Using the model in the present study, $F_{g,\text{human}}$ for the 3 compounds were predicted as $F_{g,\text{observed}}$ vs $F_{g,\text{predicted}} = 0.903$ vs 0.785 for haloperidol, 0.620 vs 0.722 for mebendazole, and 0.854 vs 0.838 for ziprasidone.
Discussion

The number of new drug candidates that are cleared via non-CYP enzymes has increased in recent years. However, unlike oxidation by P450, the roles of reductive enzymes are less well understood. Especially, the intestinal metabolic activities by reductive enzymes, and a quantitative prediction method of intestinal first-pass metabolism by cytosolic enzymes are not well known. The purposes of the present study were to investigate the significance of reductive metabolism in human intestine, and to establish a quantitative prediction method for intestinal first-pass metabolism by cytosolic reductive enzymes, using haloperidol, mebendazole, and ziprasidone.

\(CL_{\text{int,u}}\) values in HIM were lower than those in HLM for all 3 compounds (Table 1). Haloperidol and ziprasidone is mainly metabolized by CYP3A4 in microsomes (Someya et al., 1992; Beedham et al., 2003). The difference between \(CL_{\text{int,u}}\) values in HLM and HIM might be due to difference of CYP3A4 expression level (von Richter et al., 2004). On the other hand, Metabolic activities were detected in HIC for all 3 compounds, and the \(CL_{\text{int,u}}\) values in HIC were higher than those in HLC for haloperidol and mebendazole (Table 1). Haloperidol and ziprasidone might be metabolized by CBR and AO in HLC, respectively (Someya et al., 1992; Beedham et al., 2003).

Carbonyl compounds, haloperidol and mebendazole, might be metabolized by intestinal cytosolic carbonyl-reducing enzymes, AKRs or CBRs (Rosemond and Walsh, 2004). In fact, human CBRs can be detected in human tissues, including small intestine, by immunohistochemical staining (Wirth and Wermuth, 1992). The highest concentrations were found in the parenchymal cells of the liver and the epithelial cells of the stomach and small intestine. Ziprasidone is reductively metabolized by AO in liver (Beedham et al., 2003). The results in the present study were consistent with the reports that AO is a cytosolic enzyme; however, they were not consistent with the reports that AO mRNA was scarcely expressed in intestine (Nishimura et al., 2006).

Mebendazole showed the highest \(CL_{\text{int,u}}\) value in HIC among the 3 compounds, and no
metabolic activity in HIM. Furthermore, the $CL_{\text{int,u}}$ value in HIS9 was higher than those in HLS9 only for mebendazole (Table 1). Reported major metabolic routes in humans were hydrolysis and reduction (Dawson et al., 1985) (Figure 1). $F_g,\text{human}$ of mebendazole is relatively low, 0.62 (Varma et al., 2010). There is a possibility that hydrolysis is involved in the metabolic activity for mebendazole in HIC. Therefore, we investigated metabolic routes of mebendazole in HIM, HLM, HIC, HLC, HIS9, and HLS9. From HPLC data, the major metabolic route in HLM was hydrolysis. On the other hand, the major metabolic route in HIC, HIS9, and HLC was reduction. Both hydrolysis and reduction occurred in HLS9 (Figures 1-3). As a result, reduction was mainly involved in the metabolic activity for mebendazole in human intestine. In human liver, hydrolysis and reduction occurred in microsomes and in cytosol, respectively. Therefore, it was suggested that reduction mainly contributes to lower $F_g$ of mebendazole.

Metabolic activities in HIS9 were not detected in the absence of both NADH and NADPH for all 3 compounds. On the other hand, metabolic activities were detected in the presence of only NADPH for all 3 compounds. In the presence of only NADH, haloperidol and mebendazole were not metabolized, whereas ziprasidone was metabolized (Table 2). Carbonyl reduction by AKRs and CBRs are NADPH and/or NADH-dependent (Rosemond and Walsh, 2004). Therefore, these results in the present study for haloperidol and mebendazole supported our speculation that these two compounds are metabolized by AKRs or CBRs. In addition, the initial metabolic pathways of haloperidol in human liver are oxidation, glucuronidation, and reduction. The oxidation is catalyzed by CYP3A4 and the reduction occurs in human liver cytosol (Kudo et al., 1999). This information supports our speculation that the metabolic activity for haloperidol in HIC might be reductively metabolized by AKRs or CBRs. On the other hand, AO is a cofactor-independent enzyme (Zientek et al., 2010). Therefore, unlike in the liver, AO might be not involved in the intestinal metabolism of ziprasidone. This might be because AO is scarcely expressed in intestine (Nishimura et al., 2006). Characterization of the metabolism of ziprasidone in human
liver preparations has suggested that two enzymes are important in the initial pathways of ziprasidone: CYP3A4 and AO (Beedham et al., 2003). Therefore, it has not been reported that ziprasidone was metabolized by a cytosolic enzyme other than AO. There is a possibility that unknown reductive metabolism of ziprasidone occurs in human intestinal cytosol.

The metabolic activities for all 3 compounds in HIC were not inhibited by 20 μM flufenamic acid or 50 μM ondansetron (Figure 4B and 4C). It has been reported that flufenamic acid has been used as a selective chemical inhibitor of AKRs, and that 20 μM flufenamic acid largely inhibited purified AKRs (Atalla et al., 2000; Rosemond et al., 2004). Ondansetron is a chemical inhibitor of AO. Obach et al. (2011) reported that 50 μM ondansetron largely inhibited the metabolic activities by AO in human liver. On the other hand, the metabolic activities for all 3 compounds in HIC were largely inhibited by 200 μM menadione (Figure 4A). Menadione has been used as a CBR-selective chemical inhibitor (Atalla et al., 2000; Maser et al., 2000; Porter et al., 2000; Rosemond et al., 2004). As a result, these data suggested that these compounds were metabolized by CBRs in human intestine.

CBR, which showed metabolic activities for haloperidol and mebendazole, might be expressed more readily in HIC than in HLC, although there has been no report regarding expression levels of CBR proteins in human liver or intestine. These results are important information, because most metabolic enzymes, including CYP3A4, show higher metabolic activities in liver than in intestine.

Our prediction method for the intestinal first-pass metabolism in humans uses metabolic stabilities in HIM (Nishimuta et al., 2011). However, we had previously not yet investigated whether this method is applicable to cytosolic enzymes using HIC or HIS9. Therefore, in order to apply to compounds which are metabolized both in microsomal fraction and cytosolic fraction, a scaling factor from S9 to microsomes is needed. Scaling factors of from S9 to microsomes or from cytosol to microsomes in human liver were reported (Houston et al., 2008); however, those in human intestine had not been reported yet. In the present study, we investigated the relationship between metabolic activities in HIM and those in HIS9 for
CYP3A substrates that might be metabolized only in microsomal fraction although it is indirectly method (Equation 4). The fitting line of the correlation was 3.35-fold higher in HIM than in HIS9 (Figure 5). Therefore, we established equation 6 using the scaling factor as 3.35.

We predicted \( F_{g,\text{human}} \) using the metabolic activities in HIS9 in order to estimate intestinal metabolism in both cytosolic and microsomal enzymes. Using \( P_{\text{app,PAMPA}} \) and \( CL_{\text{int,u,HIM}} \) calculated from \( CL_{\text{int,u,HIS9}} \) in the presence of NADH and NADPH, we predicted \( F_{g,\text{human}} \) using equation 6 (Table 4). The model in the present study could predict \( F_{g,\text{human}} \) for 3 compounds well. Our prediction method using HIS9 was applicable to the substrates of cytosolic enzymes although the prediction is only conducted with three compounds; further study with more compounds is required. To facilitate selection of candidates with high bioavailability in humans through exploratory screening, we propose that intestinal first-pass metabolism for new chemical entities that are reductively metabolized could be predicted using our prediction method of human \( F_{g} \).

It was reported that the \( K_{m} \) values for reduction of haloperidol were 250-600 μM in liver cytosol and 1200 μM in recombinant CBR (Rosemond et al., 2004). The \( K_{m} \) values were extremely higher than the concentration of haloperidol in our experiments, therefore our in vitro investigation using the substrate depletion method for haloperidol was conducted under the linear conditions. In addition, the maximum concentration of haloperidol in intestine in vivo is approximately 5 μM if it is calculated as \( C_{\text{intestine}} = F_{a} \times k_{a} \times \text{Dose}/Q_{g} \) (Dose = 20 mg in the report in Table 3, assuming \( F_{a} = 1, k_{a} \) (absorption rate constant) = 0.1 min\(^{-1} \), \( Q_{g} \) (gut blood flow) = 1100mL/min) and the value is also extremely lower than the \( K_{m} \) value. On the other hand, there is no report about \( K_{m} \) values of CBR for mebendazole and ziprasidone. The concentrations of these two compounds in our experiments were also low, and we confirmed that our experiments were conducted under the linear concentrations. Furthermore, as well as haloperidol, the \( K_{m} \) values for CBR of various compounds are tend to be high (Rosemond et al., 2004).

In conclusion, CBRs might have higher metabolic activities in human intestine than in
human liver. Furthermore, our prediction method of human \( F_g \) using HIS9 is applicable to the substrates of cytosolic reductive enzymes.
Authorship Contributions

Participated in research design: Nishimuta

Conducted experiments: Nishimuta

Performed data analysis: Nishimuta

Wrote or contributed to the writing of the manuscript: Nishimuta, Nakagawa, Nomura, and Yabuki
References


Figure Legends

Figure 1. The metabolism of mebendazole

Figure 2. Metabolic route of mebendazole in microsomes, cytosol, and S9 of human liver
UV spectra at 254 nm of mebendazole and its metabolites in the sample incubated with pooled microsomes, cytosol, and S9 in human liver (HLM, HLC, and HLS9, respectively). A and B show reductive metabolite and hydrolyzed metabolite (Figure 1).

Figure 3. Metabolic route of mebendazole in microsomes, cytosol, and S9 of human intestine
UV spectra at 254 nm of mebendazole and its metabolites in the sample incubated with pooled microsomes, cytosol, and S9 in human intestine (HIM, HIC, and HIS9, respectively). A and B show reductive metabolite and hydrolyzed metabolite (Figure 1).

Figure 4. Effects of chemical inhibitors on metabolism of haloperidol, mebendazole, and ziprasidone in human intestinal cytosol
Panel A represents the effects of menadione, which is an inhibitor of human CBRs, on metabolism of haloperidol, mebendazole, and ziprasidone. Panel B represents the effects of flufenamic acid, which is an inhibitor of human AKRs. Panel C represents the effects of ondansetron, which is an inhibitor of human AOs.

Figure 5. Correlation of between $CL_{int,u}$ values in human intestinal microsomes and human intestinal S9 for CYP3A substrates
We investigated the relationship between metabolic activities in human intestinal microsomes (HIM) (Nishimuta et al., 2010) and those in human intestinal S9 (HIS9) for CYP3A substrates that might be metabolized only in microsomal fraction. The fitting line of the correlation was
3.35-fold higher in HIM than in HIS9 (Figure 5).
Table 1. $CL_{int,u}$ values in microsomes, cytosol and S9 of human intestine and liver for haloperidol, mebendazole, and ziprasidone in the presence of NADPH

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Him</th>
<th>HLM</th>
<th>HIC</th>
<th>HLC</th>
<th>HIS9</th>
<th>HLS9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol</td>
<td>0.014</td>
<td>0.145</td>
<td>0.016</td>
<td>0.006</td>
<td>0.030</td>
<td>0.051</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>-</td>
<td>0.009</td>
<td>0.045</td>
<td>0.011</td>
<td>0.061</td>
<td>0.016</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>0.029</td>
<td>0.118</td>
<td>0.015</td>
<td>0.023</td>
<td>0.028</td>
<td>0.089</td>
</tr>
</tbody>
</table>

$CL_{int,u}$ values were determined from metabolic stabilities of haloperidol, mebendazole, and ziprasidone in pooled human intestinal and liver microsomes (HIM and HLM), cytosol (HIC and HLC), and S9 (HIS9 and HLS9) in the presence of NADPH.

a. When the remaining amount after incubation for 60 min with 0.2 mg microsomal protein/mL was >90%, $CL_{int,u}$ values were not calculated ($CL_{int} < 0.0088$ mL/min/mg protein).
Table 2. $CL_{int,u}$ values in human intestinal S9 for haloperidol, mebendazole, and ziprasidone in the presence or absence of NADH and NADPH

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$CL_{int,u}$ (mL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH(-) NADPH(-) NADH(+) NADPH(-) NADH(-) NADPH(+)</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>$^a$ $^a$ 0.030</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>$^a$ $^a$ 0.061</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>$^a$ 0.015 0.028</td>
</tr>
</tbody>
</table>

a. When the remaining amount after incubation for 60 min with 1 mg S9 protein/mL was >90%, $CL_{int,u}$ values were not calculated ($CL_{int} < 0.0018$ mL/min/mg protein).
Table 3. $CL_{h,p}$, $F_a$, $F$, $F_{g,\text{observed}}$ values for haloperidol, mebendazole, and ziprasidone from i.v. and oral data.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$CL_{h,p}$ (mL/min/kg)</th>
<th>$F_a$</th>
<th>$F$</th>
<th>$F_{g,\text{observed}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol</td>
<td>7.72</td>
<td>1(^a)</td>
<td>0.6</td>
<td>0.903</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>14.84</td>
<td>1</td>
<td>0.22</td>
<td>0.620</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>5.05</td>
<td>0.9</td>
<td>0.6</td>
<td>0.854</td>
</tr>
</tbody>
</table>

These data were obtained from a report by Varma et al., 2010.

\(^a\) The $F_a$ value of haloperidol was assumed to be 1.

$F_{g,\text{observed}}$ values were calculated using Equations 7-11.
Table 4. $\textit{CL}_{\text{int},u,HIS9}$, $\textit{CL}_{\text{int},u,HIM}$ (calculated from HIS9), $P_{\text{app,PAMPA}}$, and $F_{\text{g,predicted}}$ values for haloperidol, mebendazole, and ziprasidone.

<table>
<thead>
<tr>
<th></th>
<th>$\textit{CL}_{\text{int},u,HIS9}$ (mL/min/mg protein)</th>
<th>$\textit{CL}_{\text{int},u,HIM}$ (calculated from HIS9) (mL/min/mg protein)</th>
<th>$P_{\text{app,PAMPA}}$ ($10^{-6}$ cm/s)</th>
<th>$F_{\text{g,predicted}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol</td>
<td>0.030</td>
<td>0.102</td>
<td>33.7</td>
<td>0.785</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>0.061</td>
<td>0.205</td>
<td>48.5</td>
<td>0.722</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>0.043</td>
<td>0.143</td>
<td>67.2</td>
<td>0.838</td>
</tr>
</tbody>
</table>

$\textit{CL}_{\text{int},u,HIM}$ (calculated from HIS9) values were calculated using Equation 5.

$F_{\text{g,predicted}}$ values were calculated using Equation 6.
Figure 1

Mebendazole → Reduction → Compound A

Mebendazole → Hydrolysis → Compound B
Figure 2

HLM

HLC

HLS9

mebendazole

mebendazole

mebendazole

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Figure 3

HIM

HIC

HIS9

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Figure 4

A

Substrate consumed (%)

Menadione (μM)

B

Substrate consumed (%)

Flufenamic acid (μM)

C

Substrate consumed (%)

Ondansetron (μM)
Figure 5