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Evaluation of Approach to Predict the Contribution of Multiple Cytochrome P450s in Drug Metabolism using Relative Activity Factor: Effects of the Differences in Expression Levels of NADPH-Cytochrome P450 Reductase and Cytochrome b_5 in the Expression System and the Differences in the Marker Activities

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ABSTRACT: The concept of relative activity factor (RAF) to extrapolate data obtained with recombinant cytochrome P450(CYP)s to human liver microsomes has been proposed. To evaluate the approach to predict the contribution of multiple CYPs using RAF, we investigated the effects of the differences in the expression levels of NADPH-cytochrome P450 reductase (OR) and cytochrome b_5 (b_5) in recombinant CYPs from baculovirus-infected insect cells and the differences in the marker activities. Since we previously clarified that azelastine, an antiallergy and antiasthmatic drug, is *N*-demethylated by CYP1A2, CYP2D6 and CYP3A4 in humans, the reaction was used as a model. For calculation of RAF, three lots of recombinant CYP1A2, CYP2D6, and CYP3A4 from baculovirus-infected insect cells with different expression levels of OR and b_5 were used. The OR/CYP ratios for recombinant CYP1A2, CYP2D6 and CYP3A4 were 3.9 – 4.8, 5.1 – 8.7 and 8.0 – 11.3, respectively. The b_5/CYP ratio for recombinant CYP3A4 was 2.1 – 18.7. As marker activities, ethoxyresorufin *O*-deethylation and phenacetin *O*-deethylation for CYP1A2, bufuralol 1'-hydroxylation and debrisoquin 4-hydroxylation for CYP2D6, testosterone 6β-hydroxylation and midazolam 1'-hydroxylation for CYP3A4 were compared. Our results indicated that the differences in the expression levels of OR and b_5 co-expressed in baculovirus-infected insect cells would not be a critical factor for the quantitative prediction using RAF. In addition, we confirmed that differences in the marker activities did not significantly affect the calculation of RAF values, when the marker activities are specific for a certain CYP isoform. It was suggested that the RAF approach using recombinant CYPs from baculovirus-infected insect cells co-expressing OR (and b_5 if required) could be valuable for the prediction of the contribution of each CYP in drug metabolism.

INTRODUCTION

Cytochrome P450 (CYP) comprises a superfamily of enzymes that have been recognized as the primary enzymes responsible for human drug metabolism. Although the number of individual enzymes that have been identified and characterized is increasing,[1] the metabolism of xenobiotics in humans is catalyzed mainly by enzymes from three families: CYP1, CYP2, and CYP3.[2] Clinically relevant drug interactions are frequently the result of the effects on CYP enzymes involved in biotransformation.[3] Multiple CYP isoforms are often involved in the metabolism of a single drug. Thus, it is important to determine the relative contribution of each isoform to the drug metabolism. cDNA-expressed human recombinant CYP has become increasingly important for *in vitro* drug metabolism studies. Recently, several prediction methods using recombinant CYP for assessing the contribution of multiple CYPs to certain metabolic reactions in human liver microsomes have been reported.[4-6] Two major methods are relevant: one is based on the abundance of the CYP protein in human liver microsomes and the other is based on the relative activity factor (RAF). RAF was first proposed by Crespi[4] as the ratio of the specific activity in human liver microsomes to the activity by recombinant CYP.

In our previous study,[7] we clarified that azelastine, a long-acting antiallergy and antiasthmatic drug, is *N*-demethylated by CYP1A2, CYP2D6 and CYP3A4 in humans. Furthermore, we demonstrated that the RAF as the ratio of intrinsic clearance of specific activity using microsomes from baculovirus-infected insect cells appeared to be the most appropriate approach for estimating the contributions of CYPs involved in the metabolism of certain drugs, comparing five different prediction methods and two different expression systems.[7] However, some problems remain to be solved: (1) Does the expression level of NADPH-cytochrome P450 reductase (OR) or cytochrome b_5 (b_5) in the expression systems affect the prediction? (2) Does the kind of marker activity as an index affects the calculation

of the RAF of each CYP isoform? In the present study, we expanded our previous research to solve these problems. We evaluated the approach to predict the contribution of multiple CYPs using RAF with azelastine *N*-demethylation as a model. We compared the RAF values calculated using three different lots of microsomes from baculovirus-infected insect cells with various expression levels of OR and b_5. Furthermore, two different marker activities for each CYP isoform were compared for the calculation of RAF: ethoxyresorufin *O*-deethylation (EROD) and phenacetin *O*-deethylation (POD) for CYP1A2, bufuralol 1'-hydroxylation (BFOH) and debrisoquin 4-hydroxylation (DEBOH) for CYP2D6, and testosterone 6β-hydroxylation (TESOH) and midazolam 1'-hydroxylation (MDZOH) for CYP3A4.

Materials and Methods

Chemicals

Azelastine hydrochloride, 4-(*p*-chlorobenzyl)-2-(hexahydro-1-methyl-1*H*-azepin-4-yl)-1(2*H*)-phthalazinone hydrochloride, was kindly provided by Eisai (Tokyo, Japan). Desmethylazelastine hydrobromide, 4-(*p*-chlorobenzyl)-2-(hexahydro-1*H*-azepin-4-yl)-1(2*H*)-phthalazinone hydrobromide, was kindly provided by Degussa Japan (Tokyo, Japan). 7-Ethoxyresorufin and resorufin were from Sigma (St. Louis, MO). Phenacetin, acetaminophen, and caffeine were purchased from Wako Pure Chemical Industries (Osaka, Japan). (±)-Bufuralol hydrochloride, 1'-hydroxybufuralol maleate, debrisoquin sulphate, and (±)-4-hydroxydebrisoquin sulphate were from Ultrafine chemicals (Manchester, UK). Testosterone, 6β-hydroxytestosterone and 11β-hydroxytestosterone were obtained from Steraloids (Wilton, NH). Midazolam, 1'-hydroxymidazolam, and clonazepam were kindly provided by Yamanouchi Pharmaceutical (Tokyo, Japan). $NADP^+$, glucose-6-phosphate and

glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). Other chemicals were of the highest grade commercially available.

Enzyme Preparations

Microsomes from baculovirus-infected insect cells expressing CYP1A2, CYP2D6(Val), and CYP3A4+b_5 were obtained from Gentest (Woburn, MA). All enzymes were co-expressed with OR. Three different lots of the expression system for each isoform (lot 9, 12, and 13 for CYP1A2; lot 8, 11, and 14 for CYP2D6; lot 1, 19, and 23 for CYP3A4) were used. Human liver microsomes (HLG1, HLG4, HLG6, HLG7, HLG10, and HLG11) were also purchased from Gentest. These microsomes correspond to HG3, HG30, HG43, HG56, HG89, and HG93 by the Gentest's designation, respectively. The expression levels of total CYP, OR and b_5, and specific catalytic activities of each CYP isoform in those microsomes were provided in the data sheets by the manufacturer (Table 1). Since the NADPH-cytochrome c reductase activities were provided, the OR contents were determined based on an assumed specific activity of 3.0 μmol reduced cytochrome c/min/nmol purified human OR.[8,9] HLG 4 was surmised to be from a poor metabolizer of CYP2D6.

In the expression levels of OR and b_5 in six human liver microsomes, there were no large interindividual differences. However, there were large interindividual differences in POD, BFOH and TESOH. The differences in the expression level of OR in the expression systems were 1.7 fold for recombinant CYP1A2 and 2.1 fold for recombinant CYP2D6. The OR/CYP ratios for CYP1A2 and CYP2D6 were 3.9 – 4.8 and 5.1 – 8.7, respectively. Although the expression level of OR in lot 1 recombinant CYP3A4 was not available, the OR levels in lot 19 and lot 23 were similar. The OR/CYP ratios in lot 19 and lot 23 were 11.3 and 8.0, respectively. Recombinant CYP1A2 and CYP2D6 were not co-expressed with b_5. The difference in the expression levels of b_5 in the three lots of recombinant CYP3A4 was 2.5 fold.

The b_5/CYP ratio was 2.1 – 18.7. The specific activities of recombinant CYP1A2, CYP2D6 and CYP3A4 were closely similar between lots.

Enzyme Activities

The azelastine *N*-demethylase activity, EROD, BFOH, and TESOH activities in human liver microsomes or microsomes from baculovirus-infected insect cells were determined as described previously.[7] POD was determined by the method of Nakajima et al.[10] DEBOH was determined as described previously[11] with slight modifications. The incubation mixture (0.2 ml of total volume) contained 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system, 0.25 mg/ml microsomal protein or 8 pmol/ml recombinant CYP2D6 and debrisoquin as the substrate. The reaction was initiated by the addition of an NADPH-generating system following a 2 min pre-incubation at 37 °C. After incubation for 10 min at 37°C, the reaction was terminated by the addition of 20 μl of 60% perchloric acid. The incubation mixtures were centrifuged at 10,000 g for 5 min and a 100 μl portion of the supernatant was subjected to HPLC. The HPLC equipment was the same as described previously.[7] The analytical column was Symmetry C_{18} (4.6 x 150 mm, 4 μm, Waters) and the formed 4-hydroxydebrisoquin was detected fluorometrically (excitation; 229 nm, emission; 286 nm). The mobile phase was 12% acetonitrile containing 20 mM perchloric acid. The flow rate was 1.0 ml/min and the column temperature was 35°C.

MDZOH was determined as described previously[12] with slight modifications. The incubation mixture (0.2 ml of total volume) contained 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system, 0.08 mg/ml microsomal protein or 8 pmol/ml recombinant CYP3A4 and midazolam as the substrate. The reaction was initiated by the addition of an NADPH-generating system following a 1 min pre-incubation at 37 °C. After incubation for 5 min at 37°C, the reaction was terminated by the addition of 100 μl of ice-

cold methanol. Clonazepam (20 ng) was added as an internal standard. The incubation mixtures were centrifuged at 10,000 g for 5 min and a 100 μl portion of the supernatant was subjected to HPLC. The HPLC equipment was the same as described above. The analytical column was a Capcell pak C_{18} UG120 (4.6 x 250 mm, 4 μm, Shiseido). The eluent was monitored at 220 nm. The mobile phase was methanol/acetonitrile/10 mM potassium phosphate buffer (pH 7.4), 27:18:55 (v/v/v). The flow rate was 1.0 ml/min and the column temperature was 35°C.

Kinetic Analysis

In determining the kinetic parameters, the concentrations of substrates were as follows: azelastine, 1 – 200 μM; ethoxyresorufin, 0.05 – 1 μM; phenacetin, 5 – 100 μM; bufuralol, 0.5 – 20 μM, debrisoquin, 1 – 200 μM; testosterone, 5 – 200 μM, midazolam, 0.5 – 10 μM. Kinetic parameters were estimated from the fitted curves using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis. The intrinsic clearance(CL)s were calculated as V_{max}/ K_m except for azelastine *N*-demethylation in human liver microsomes. The *N*-demethylation clearances of azelastine in human liver microsomes were estimated as the rate of metabolism under nonsaturating conditions,[7] because such a condition is considered to be comparable to that in *in vivo*.

Contributions of CYP1A2, CYP2D6 and CYP3A4 to Azelastine *N*-Demethylase Activity in Human Liver Microsomes

The percent contributions of each CYP to the azelastine *N*-demethylase activity in human liver microsomes were estimated as described previously[7] by applying RAF as the ratio of intrinsic clearance values (RAF_{CL}). The RAF_{CL} values for CYP1A2 ($RAF_{CL, CYP1A2}$) were determined as the ratios of the intrinsic clearance of EROD or POD in human liver

microsomes to the values for recombinant CYP1A2. The RAF_{CL} values for CYP2D6 ($RAF_{CL, CYP2D6}$) were determined as the ratios of the intrinsic clearance of BFOH or DEBOH in human liver microsomes to the values for recombinant CYP2D6. The RAF_{CL} values for CYP3A4 ($RAF_{CL, CYP3A4}$) were determined as the ratios of the intrinsic clearance of TESOH or MDZOH in human liver microsomes to the values for recombinant CYP3A4. Using RAF, the *N*-demethylation clearances of azelastine by CYP1A2, CYP2D6 and CYP3A4 in human liver microsomes (CL_{CYP1A2}, CL_{CYP2D6} and CL_{CYP3A4}, respectively) were expressed as follows:

$$CL_{CYP1A2} = CL_{rec\text{-}CYP1A2} \cdot RAF_{CL, CYP1A2} \quad (1)$$

$$CL_{CYP2D6} = CL_{rec\text{-}CYP2D6} \cdot RAF_{CL, CYP2D6} \quad (2)$$

$$CL_{CYP3A4} = CL_{rec\text{-}CYP3A4} \cdot RAF_{CL, CYP3A4} \quad (3)$$

where $CL_{rec\text{-}CYP1A2}$, $CL_{rec\text{-}CYP2D6}$ and $CL_{rec\text{-}CYP3A4}$ are the intrinsic clearances of azelastine *N*-demethylation for recombinant CYP1A2, CYP2D6 and CYP3A4, respectively. The contributions of CYP1A2, CYP2D6 and CYP3A4 to the clearance of azelastine *N*-demethylation by human liver microsomes were calculated using the following equations:

$$\text{Contribution of CYP1A2 (\%)} = (CL_{CYP1A2} / CL_{HL}) \times 100 \quad (4)$$

$$\text{Contribution of CYP2D6 (\%)} = (CL_{CYP2D6} / CL_{HL}) \times 100 \quad (5)$$

$$\text{Contribution of CYP3A4 (\%)} = (CL_{CYP3A4} / CL_{HL}) \times 100 \quad (6)$$

where the CL_{HL} values are the *N*-demethylation clearances of azelastine in human liver microsomes.

Statistical Analysis

Correlations between the $RAF_{CL, CYP1A2}$ using EROD and $RAF_{CL, CYP1A2}$ using POD, between the $RAF_{CL, CYP2D6}$ using BFOH and $RAF_{CL, CYP2D6}$ using DEBOH, and between the $RAF_{CL, CYP3A4}$ using TESOH and $RAF_{CL, CYP3A4}$ using MDZOH were determined by linear regression

analyses. The contributions of each CYP using two different marker activities were compared by paired t-test. Differences were identified as statistically significant when $p < 0.05$. Results are expressed as means ± SD.

RESULTS

Azelastine *N*-Demethylation in Human Liver Microsomes and Recombinant CYPs

The clearances of azelastine *N*-demethylation in six human liver microsomes were determined previously[7] as follows: HLG1, 72.1 μl/min/nmol CYP; HLG4, 66.9 μl/min/nmol CYP; HLG6, 47.2 μl/min/nmol CYP; HLG7, 42.5 μl/min/nmol CYP; HLG10, 91.2 μl/min/nmol CYP; HLG11, 38.0 μl/min/nmol CYP. Kinetic parameters of azelastine *N*-demethylation in microsomes from baculovirus-infected insect cells expression CYP1A2, CYP2D6, and CYP3A4 are shown in Table 2. Using three lots of microsomes, the K_m values of CYP1A2 were 125.4 – 273.3 μM and the V_{max} values of CYP1A2 were 14.33 – 20.42 pmol/min/pmol CYP, resulting in a $CL_{rec\text{-}CYP1A2}$ of 74.7 – 131.2 μl/min/nmol CYP. The K_m values of CYP2D6 were 1.8 – 2.4 μM and the V_{max} values of CYP2D6 were 1.73 – 2.06 pmol/min/pmol CYP, resulting in a $CL_{rec\text{-}CYP2D6}$ of 781.7 – 945.6 μl/min/nmol CYP. The K_m values of CYP3A4 were 51.1 – 69.4 μM and the V_{max} values of CYP3A4 were 4.45 – 10.10 pmol/min/pmol CYP, resulting in a $CL_{rec\text{-}CYP3A4}$ of 87.1 – 145.9 μl/min/nmol CYP. Thus, the differences in the kinetic parameters between the three lots were at most ~2 fold.

RAF calculated with the three lots of recombinant CYPs and two kinds of marker activities

The CL values of EROD in the six human liver microsomes ranged from 0.02 to 1.33 μl/min/pmol CYP. Using the CL of lot 9 recombinant CYP1A2 (16.17 μl/min/pmol), the

$RAF_{CL, CYP1A2}$ values were calculated as 0.001 to 0.082 (Table 3). However, using the CL of lot 12 (19.83 μl/min/pmol) and lot 13 (14.94 μl/min/pmol), the $RAF_{CL, CYP1A2}$ values were calculated as 0.001 to 0.067 and 0.001 to 0.089, respectively. The CL values of POD in six human liver microsomes ranged from 0.01 to 0.12 μl/min/pmol CYP. Using the CL of lot 9 recombinant CYP1A2 (0.88 μl/min/pmol), the $RAF_{CL, CYP1A2}$ values were calculated as 0.006 to 0.132 (Table 3). Using the CL of lot 12 (2.94 μl/min/pmol) and lot 13 (1.53 μl/min/pmol), the $RAF_{CL, CYP1A2}$ values were calculated as 0.002 to 0.039 and 0.003 to 0.075, respectively. With all three lots of recombinant CYP1A2, the $RAF_{CL, CYP1A2}$ values using EROD and the $RAF_{CL, CYP1A2}$ values using POD were significantly correlated ($r = 0.940$, $p < 0.01$).

The K_m value of BFOH in HLG4 (30.13 μM) was prominently higher than those in the other human liver microsomes (2.81 to 8.86 μM). The CL values of BFOH in the six human liver microsomes ranged from 0.01 to 0.015 μl/min/pmol CYP. Using the CL of lot 8 recombinant CYP2D6 (4.35 μl/min/pmol CYP), the $RAF_{CL, CYP2D6}$ values were calculated as 0.001 to 0.035 (Table 3). However, using the CL of lot 11 (4.35 μl/min/pmol) and lot 14 (6.59 μl/min/pmol), the $RAF_{CL, CYP2D6}$ values were calculated as 0.001 to 0.026 and 0.001 to 0.023, respectively. DEBOH activity in HLG 4 was not detected at any substrate concentrations used. The CL values of DEBOH in five human liver microsomes ranged from 0.25 to 1.62 μl/min/pmol CYP. Using the CL of lot 8 recombinant CYP2D6 (37.93 μl/min/pmol CYP), the $RAF_{CL, CYP2D6}$ values were calculated as 0.000 to 0.044 in the six human liver microsomes (Table 3). Using the CL of lot 11 (52.80 μl/min/pmol) and lot 14 (86.76 μl/min/pmol), the $RAF_{CL, CYP2D6}$ values were calculated as 0.000 to 0.031 and 0.000 to 0.019, respectively. In lot 8 ($r = 0.962$, $p < 0.01$), lot 11 ($r = 0.970$, $p < 0.05$), and lot 14 ($r = 0.974$, $p < 0.01$), the $RAF_{CL, CYP2D6}$ values using BFOH and the $RAF_{CL, CYP2D6}$ values using DEBOH were significantly correlated.

The CL values of TESOH in the six human liver microsomes ranged from 0.23 to 0.93 μl/min/pmol CYP. Using the CL of lot 1 recombinant CYP3A4 (0.94 μl/min/pmol), the $RAF_{CL, CYP3A4}$ values were calculated as 0.238 to 0.983 (Table 3). Using the CL of lot 19 (1.48 μl/min/pmol) and lot 23 (2.19 μl/min/pmol), the $RAF_{CL, CYP3A4}$ values were calculated as 0.152 to 0.628 and 0.103 to 0.424, respectively. The CL values of MDZOH in the six human liver microsomes ranged from 1.47 to 5.71 μl/min/pmol CYP. Using the CL of lot 1 recombinant CYP3A4 (8.56 μl/min/pmol), the $RAF_{CL, CYP3A4}$ values were calculated as 0.172 to 0.667 (Table 3). Using the CL of lot 19 (12.60 μl/min/pmol) and lot 23 (9.90 μl/min/pmol), the $RAF_{CL, CYP3A4}$ values were calculated as 0.117 to 0.453 and 0.149 to 0.577, respectively. In lot 1 ($r = 0.892$, $p < 0.05$), lot 19 ($r = 0.893$, $p < 0.05$), and lot 23 ($r = 0.892$, $p < 0.05$), the $RAF_{CL, CYP3A4}$ values using TESOH and the $RAF_{CL, CYP3A4}$ values using MDZOH were significantly correlated.

Contributions of CYP1A2, CYP2D6 and CYP3A4 to Azelastine *N*-Demethylase Activity in Human Liver Microsomes

The contributions of CYP1A2, CYP2D6 and CYP3A4 to the azelastine *N*-demethylation clearance in microsomes from the six human livers (HLG1, 4, 6, 7, 10 and 11) were estimated using the equations (1)-(6). Using EROD as a marker activity, the contributions of CYP1A2 were calculated as 0.2 – 25.1%, 0.1 – 11.7%, and 0.2 – 16.4% with lot 9, lot 12, and lot 13 recombinant CYP1A2, respectively (Table 4). The differences in the contributions between the three lots were at most 2.1 fold. Using POD as a marker activity, the contributions of CYP1A2 were calculated as 1.0 – 28.2%, 0.2 – 4.8%, and 0.3 – 9.7% with lot 9, lot 12, and lot 13 recombinant CYP1A2, respectively. The differences in the contributions between the three lots were 5.9 fold. There were no significant differences ($p = 0.513$) in the contributions

of CYP1A2 using EROD and POD as marker activities in six human liver microsomes (Figure 1).

Using BFOH as a marker activity, the contributions of CYP2D6 were calculated as 1.4 – 46.3%, 1.2 – 38.1%, and 1.3 – 41.8% with lot 8, lot 11, and lot 14 recombinant CYP2D6, respectively (Table 4). The differences in the contributions between the three lots were only 1.2 fold. Using DEBOH as a marker activity, the contributions of CYP2D6 were calculated as 0.0 – 47.4%, 0.0 – 37.6%, and 0.0 – 24.9% with lot 8, lot 11, and lot 14 recombinant CYP2D6, respectively. The differences in the contributions between the three lots were 1.9 fold. There was no significant difference ($p = 0.904$) in the contributions of CYP2D6 using BFOH and DEBOH as marker activities in six human liver microsomes (Figure 1).

Using TESOH as a marker activity, the contributions of CYP3A4 were calculated as 48.8 – 101.7%, 41.6 – 86.7%, and 35.3 – 73.5% with lot 1, lot 19, and lot 23 recombinant CYP3A4, respectively (Table 4). The differences in the contributions between the three lots were only 1.4 fold. Using MDZOH as a marker activity, the contributions of CYP3A4 were calculated as 35.2 – 54.0%, 31.9 – 49.0%, and 51.0 – 78.3% with lot 1, lot 19, and lot 23 recombinant CYP3A4, respectively. The differences in the contributions between the three lots were 1.6 fold. There were no significant differences ($p = 0.070$) in the contributions of CYP3A4 using TESOH and MDZOH as marker activities in six human liver microsomes. Collectively, the contributions of CYP1A2, CYP2D6 and CYP3A4 to azelastine *N*-demethylation clearance in human liver microsomes were coincident with the data that we previously demonstrated.[7] The sum of the contributions of CYP1A2, CYP2D6 and CYP3A4 to the *N*-demethylation clearance was almost 100% for each of the six human liver microsomes.

DISCUSSION

Recently, extrapolations from *in vitro* data to intrinsic clearance of drugs in humans have received increasing attention, since it is important for predicting drug-drug interactions *in vivo*. cDNA-expressed human recombinant CYP has become increasingly important for *in vitro* drug metabolism studies. This recombinant CYP is useful for rapid screening of the metabolic capacity. However, the activity obtained with the recombinant CYP can not be applied directly to the activity in human liver microsomes. For scaling drug biotransformation data from recombinant CYP to human liver microsomes, there are two primary methods: one approach is based on the capability of recombinant CYP to metabolize a drug and the abundance of the CYP protein in human liver microsomes, and the other approach is RAF as proposed by Crespi.[4] In our previous study,[7] we evaluated several prediction methods using azelastine *N*-demethylation as a model. We demonstrated that the estimation with RAF was preferable to the method using the abundance of CYP proteins, which was supported by a recent study by Störmer et al.[13] We also reported that RAF as the ratio of intrinsic clearance was the most appropriate approach for estimating the contributions of CYPs, comparing with RAF as the ratio of the activity at a substrate concentration or RAF as the ratio of the Vmax value. Furthermore, it was suggested that the use of recombinant CYPs from baculovirus-infected insect cells is more suitable for the estimation than recombinant CYPs from human B-lymphoblastoid cells.[7] However, even with this prediction method, several problems remain to be solved: (1) Do the differences in the expression levels of OR or b_5 in expression system affect the prediction? (2) Is the RAF of each CYP isoform independent of the kind of marker activity? To address these problems, we performed the present study.

For CYP, the interaction with OR is obviously essential[14] and the association with b_5 is required for certain reactions.[15] It is known that the turnover number of CYP is affected by

OR and b_5.[16] In human liver microsomes, the OR/CYP ratios for CYP1A2, CYP2D6 and CYP3A4 have been reported to be 0.9 – 19.6, 2.4 – 14.6, and 0.12 – 16.3, respectively.[17] In microsomes from the baculovirus-infected insect cells used in the present study, the OR/CYP ratios were estimated to be 3.9 – 4.8 for CYP1A2, 5.1 – 8.7 for CYP2D6, and 8.0 – 11.3 for CYP3A4. It has been reported that the RAF values reach to a plateau when increasing the OR/CYP1A2 ratio (~4).[17] Our results suggested that the difference in the OR/CYP ratio in the co-expression system did not affect the calculation of RAF. It has been established that b_5 does not stimulate CYP1A2 and CYP2D6 activities.[18,19] In contrast, CYP3A4 has been reported to be stimulated by b_5, although the effects were dependent on the substrates.[9,20] Our previous study confirmed that b_5 co-expressed in baculovirus-infected insect cells enhanced the azelastine *N*-demethylase activity.[7] It has been reported that the b_5/CYP ratio of 1 – 2 resulted in the maximal stimulation of CYP3A4 in the expression system.[9,19] In the present study, it was demonstrated that the differences in b_5/CYP ratio of 2.1 – 18.7 in the expression system did not affect the calculation of RAF. Accordingly, it was suggested that co-expression of OR (and b_5 if required) in the expression system is important, and the several-folds differences in the expression levels would not be a critical factor for the quantitative prediction. In other words, if the expression level of OR (and b_5) in the expression system is sufficient for CYP activity, the over-expression might not affect the prediction using RAF. In contrast, Roy et al.[21] reported successful prediction using RSF (relative substrate-activity factor, i.e., modified RAF) with recombinant CYP2B6 expressed in human B-lymphoblastoid cells without b_5. As they suggested, if the stimulation effects of b_5 on marker activity and test activity were different from each other, one must pay attention to the prediction using both recombinant CYPs with and without co-expressing b_5. In such a scenario, the validity of the RSF-based prediction must be tested using other means such as using CYP isoform-specific inhibitors.

Whereas EROD is specific for CYP1A2 in human livers,[22] POD is catalyzed by CYP2E1[23] and CYP3A4[10] in addition to CYP1A2 at high substrate concentrations. Since the K_m values of CYP2E1 and CYP3A4 were 785 ± 125 μM and > 1 mM, respectively,[23] we determined the kinetic parameter of POD at 5 – 100 μM to reflect only the CYP1A2 activity. According to the results that the RAFs as the ratios of intrinsic clearance of EROD and POD were not significantly different from each other, it was revealed that both activities were suitable for the RAF of CYP1A2 as the marker activity under the present condition. When POD was used for marker activity of CYP1A2, the differences in the contribution between the three lots were large as 5.9 fold. Since the contribution of CYP1A2 to azelastine *N*-demethylation is minor, the difference would not be clinically meaningful. However, we should be in mind that if the contribution of CYP1A2 to a certain drug metabolism would be large, such difference between lots might yield different conclusion in the prediction.

Whereas DEBOH is specific for CYP2D6 in human livers,[24] BFOH is catalyzed by CYP1A2 in addition to CYP2D6 at high substrate concentrations.[25] Faint BFOH was detected in HLG4 microsomes, although it was from a poor metabolizer of CYP2D6. Since the K_m value of BFOH in HLG4 was close to the K_m value of recombinant CYP1A2 (38.1 μM),[7] it was implicated that the RAF using BFOH might have resulted in an overestimation of the contribution of CYP2D6 by the inclusion of CYP1A2. However, the RAFs as the ratios of intrinsic clearances using BFOH and DEBOH were not significantly different from each other, indicating that the extent of the overestimation by RAF using BFOH might be negligible. TESOH is catalyzed by CYP3A4.[26] According to an immunoinhibition experiment with anti-peptide antibodies, CYP3A4 accounts for nearly all of the TESOH in human liver microsomes, whereas hepatic CYP3A5 makes little or no contribution to this activity.[27] MDZOH is catalyzed by CYP3A4 and CYP3A5.[28] In a reconstituted system, it was reported that the clearance value of MDZOH by CYP3A5 was higher than that by CYP3A4.[28] However, Wang

and Lu[27] also reported that CYP3A4 is the predominant enzyme (more than 90%) responsible for MDZOH in human livers. In the six human liver microsomes used in the present study, the CYP3A5 content was provided as only 0.6 – 3.1% of the CYP3A content by the manufacturer (data not shown). Therefore, it was suggested that the relevance of CYP3A5 for the calculation of RAF using MDZOH would be negligible. The reason why the contribution of CYP3A4 calculated with RAF using TESOH was significantly higher than that with RAF using MDZOH in only one microsomal specimen (HLG4) is unknown. Although we re-examined TESOH and MDZOH in HLG4 several times to confirm the difference, the results were reproducible.

The present study revealed that there are lot-to-lot differences in activity and/or kinetic parameters in the expression systems. When investigators determine the prediction using RAF, same lot of recombinant CYP should be used for marker substrates and test substrate. Furthermore, even if the prediction using the abundance of CYP proteins would be performed, the CYP contents could not serve as a universally applicable scaling factor, because it was indicated in the present study that the scaling factors are lot-dependent. Thus, the prediction method using RAF with same lot for marker activity and test activity in the simultaneous experiment is superior to the approach using the abundance of CYP proteins.

In conclusion, the data from the present study show evidence that the difference in the expression levels of OR and b_5 in microsomes from baculovirus-infected insect cells did not significantly affect the calculation of RAF. Differences in the kinds of marker activities also did not remarkably affect the calculation of RAF. It was suggested that the RAF approach using recombinant CYPs from baculovirus-infected insect cells co-expressing OR (and b_5 if required) could be valuable for the prediction of the contribution of each CYP.

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Table 1. Expression levels of CYP, OR and b_5, and specific activities of CYPs in microsomes from six human livers and from baculovirus-infected insect cells expressing human CYP

Microsomes		Total CYP	OR	OR/CYP	b_5	b_5/CYP	POD	BFOH	TESOH
	No.	pmol/mg	pmol/mg		pmol/mg			pmol/min/mg	
HLG	1	353	111		562		112	124	5854
	4	571	124		623		2102	ND	9664
	6	236	80		518		356	106	3408
	7	559	88		596		2009	140	5028
	10	428	92		500		1197	5	11732
	11	321	94		551		501	63	2090
	Lot No.							pmol/min/pmol CYP	
rec-CYP1A2	9	200	940	4.7	–		29	–	–
	12	211	1013	4.8	–		37	–	–
	13	154	597	3.9	–		39	–	–
rec-CYP2D6	8	164	1423	8.7	–		–	31	–
	11	125	1067	8.5	–		–	32	–
	14	131	667	5.1	–		–	30	–
rec-CYP3A4+b_5	1	250	–	–	530	2.1	–	–	131
	19	71	800	11.3	1330	18.7	–	–	140
	23	113	903	8.0	830	7.3	–	–	110

These data were provided by manufacturer. HLM 4 was from a poor metabolizer of CYP2D6.
Recombinant CYP1A2 and CYP2D6 were not co-expressed with b_5.
The concentrations of phenacetin and testosterone were 200 μM.
The concentrations of bufuralol were 25 μM and 100 μM for human liver microsomes and rec-CYP2D6, respectively.
ND: Not detected.
–: Not available.

Table 2. Kinetic parameters of azelastine *N*-demethylase activity in microsomes from baculovirus-infected insect cells expressing human CYP

Isoform		K_m	V_{max}	CL
	Lot No.	μM	pmol/min/pmol CYP	μl/min/nmol CYP
rec-CYP1A2	9	125.4	16.45	131.2
	12	273.3	20.42	74.7
	13	181.1	14.33	79.1
rec-CYP2D6	8	2.3	1.76	781.7
	11	2.4	2.06	861.9
	14	1.8	1.73	945.6
rec-CYP3A4+b_5	1	51.1	4.45	87.1
	19	69.4	8.07	116.3
	23	69.2	10.10	145.9

Table 3. RAF values calculated from clearances of marker activity in six human liver microsomes and recombinant CYPs

Microsomes		K_m	V_{max}	CL	RAF		
		EROD			$RAF_{CL, CYP1A2}$		
	No.	μM	pmol/min/pmol CYP	μl/min/pmol CYP	Lot 9	Lot 12	Lot 13
HLG	1	0.29	0.01	0.02	0.001	0.001	0.001
	4	0.13	0.18	1.33	0.082	0.067	0.089
	6	0.12	0.05	0.40	0.025	0.020	0.027
	7	0.13	0.17	1.32	0.081	0.066	0.088
	10	0.35	0.34	0.96	0.059	0.048	0.064
	11	0.10	0.06	0.67	0.041	0.034	0.045
	Lot No.						
rec-CYP1A2	9	0.09	1.47	16.17			
	12	0.08	1.67	19.83			
	13	0.09	1.28	14.94			
		POD					
	No.	μM	pmol/min/pmol CYP	μl/min/pmol CYP	Lot 9	Lot 12	Lot 13
HLG	1	48.23	0.23	0.01	0.006	0.002	0.003
	4	19.92	2.30	0.12	0.132	0.039	0.075
	6	44.91	1.24	0.03	0.032	0.009	0.018
	7	17.14	1.37	0.08	0.091	0.027	0.052
	10	19.70	1.70	0.09	0.099	0.029	0.056
	11	28.51	1.01	0.04	0.041	0.012	0.023
	Lot No.						
rec-CYP1A2	9	11.03	9.66	0.88			
	12	7.13	20.96	2.94			
	13	9.42	14.42	1.53			
Correlation					$r = 0.940$ $p < 0.01$	$r = 0.940$ $p < 0.01$	$r = 0.940$ $p < 0.01$
		BFOH			$RAF_{CL, CYP2D6}$		
	No.	μM	pmol/min/pmol CYP	μl/min/pmol CYP	Lot 8	Lot 11	Lot 14
HLG	1	3.14	0.47	0.15	0.035	0.026	0.023
	4	30.13	0.16	0.01	0.001	0.001	0.001
	6	8.34	0.38	0.05	0.011	0.008	0.007
	7	2.81	0.31	0.11	0.025	0.019	0.017
	10	6.66	0.12	0.02	0.004	0.003	0.003
	11	8.86	0.53	0.06	0.014	0.010	0.009
	Lot No.						
rec-CYP2D6	8	1.47	6.38	4.35			
	11	1.99	11.58	5.83			
	14	1.96	12.91	6.59			
		DEBOH					
	No.	μM	pmol/min/pmol CYP	nl/min/pmol CYP	Lot 8	Lot 11	Lot 14
HLG	1	67.86	0.11	1.62	0.044	0.031	0.019
	4		ND		0.000	0.000	0.000
	6	106.78	0.04	0.37	0.010	0.007	0.004
	7	61.99	0.06	0.97	0.025	0.018	0.011
	10	79.39	0.02	0.25	0.006	0.004	0.003
	11	72.47	0.06	0.83	0.022	0.016	0.009
	Lot No.						
rec-CYP2D6	8	35.86	1.36	37.93			
	11	19.13	1.01	52.80			
	14	11.18	0.97	86.76			
Correlation					$r = 0.962$ $p < 0.01$	$r = 0.970$ $p < 0.05$	$r = 0.974$ $p < 0.01$
		TESOH			$RAF_{CL, CYP3A4}$		
	No.	μM	pmol/min/pmol CYP	μl/min/pmol CYP	Lot 1	Lot 19	Lot 23
HLG	1	39.78	20.39	0.51	0.543	0.347	0.234
	4	29.95	22.05	0.74	0.781	0.499	0.337
	6	57.30	22.25	0.39	0.412	0.263	0.178
	7	48.62	10.92	0.23	0.238	0.152	0.103
	10	40.25	37.33	0.93	0.983	0.628	0.424
	11	27.80	8.39	0.30	0.320	0.204	0.138
	Lot No.						
rec-CYP3A4+b_5	1	37.00	34.90	0.94			
	19	45.30	66.90	1.48			
	23	51.83	113.34	2.19			
		MDZOH					
	No.	μM	pmol/min/pmol CYP	μl/min/pmol CYP	Lot 1	Lot 19	Lot 23
HLG	1	1.52	5.34	3.51	0.410	0.278	0.354
	4	1.40	3.94	2.81	0.328	0.223	0.284
	6	1.59	3.97	2.49	0.291	0.197	0.251
	7	1.01	1.48	1.47	0.172	0.117	0.149
	10	1.28	7.32	5.71	0.667	0.453	0.577
	11	1.47	2.41	1.64	0.192	0.130	0.166
	Lot No.						
rec-CYP3A4+b_5	1	0.96	8.21	8.56			
	19	1.32	16.68	12.60			
	23	2.62	25.96	9.90			
Correlation					$r = 0.892$ $p < 0.05$	$r = 0.893$ $p < 0.05$	$r = 0.892$ $p < 0.05$

ND: Not detected.

Table 4. Percentage contribution of CYP1A2, CYP2D6 and CYP3A4 to azelastine *N*-demethylation in human liver microsomes

HLG	CYP1A2			CYP2D6			CYP3A4		
		%			%			%	
No.	Lot. 9	Lot. 12	Lot. 13	Lot. 8	Lot. 11	Lot. 14	Lot. 1	Lot. 19	Lot. 23
	EROD			**BFOH**			**TESOH**		
1	0.2	0.1	0.2	37.4	30.8	33.8	65.7	56.0	47.5
4	16.1	7.5	10.5	1.4	1.2	1.3	101.7	86.7	73.5
6	6.8	3.2	4.5	17.4	14.3	15.7	76.0	64.8	54.9
7	25.1	11.7	16.4	46.3	38.1	41.8	48.8	41.6	35.3
10	8.5	4.0	5.6	3.4	2.8	3.1	93.9	80.1	67.9
11	14.3	6.6	9.3	28.3	23.3	25.6	73.3	62.5	53.0
	POD			**DEBOH**			**MDZOH**		
1	1.0	0.2	0.3	47.4	37.6	24.9	49.5	44.9	71.8
4	25.9	4.4	8.9	0.0	0.0	0.0	42.7	38.7	61.9
6	8.8	1.5	3.0	16.8	13.3	8.8	53.6	48.6	77.7
7	28.2	4.8	9.7	46.1	36.5	24.2	35.2	31.9	51.0
10	14.2	2.4	4.9	5.3	4.2	2.8	54.0	49.0	78.3
11	14.0	2.4	4.8	44.4	35.2	23.3	44.0	39.9	63.7

Figure legends

Figure 1. Percentage contributions of CYP1A2, CYP2D6 and CYP3A4 to azelastine *N*-demethylation in human liver microsomes. Extrapolation was determined by RAF using microsomes from six human livers (HLG1, 4, 6, 7, 10 and 11) and recombinant CYP1A2, CYP2D6 and CYP3A4+b_5 from baculovirus-infected insect cells. The percentage contributions of CYP1A2, CYP2D6 and CYP3A4 were calculated with RAFs as the clearance of EROD and POD, BFOH and DEBOH, and TESOH and MDZOH as marker activities, respectively, as described in Materials and Methods. Data are expressed as mean ± SD of three different lots of recombinant CYPs.

Fig. 1. Nakajima et al.