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Advantage of a Co-expression System for Estimating Physiological Effects of Functional Interaction Between Cytochrome P450 3A4 and Uridine 5'-Diphospho-Glucuronosyltransferase 2B7

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Cytochrome P450 (CYP, P450) and uridine 5'-diphospho-glucuronosyltransferase (UGT) play crucial roles in drug metabolism phase I and II, respectively. Our previous studies suggest that there are functional interactions between P450 and UGT. We previously established a co-expression system featuring CYP3A4 and UGT2B7 using baculovirus-infected insect cells. Commercial microsomes are available that individually express CYP3A4/NADPH P450 reductase (CPR) or UGT2B7. It would be much easier if we could evaluate the functional interaction of CYP3A4 and UGT2B7 by simply mixing the microsomes. To address this issue, we presently compared our established co-expression system with a simple microsome mixing system. Co-expressed UGT2B7 suppressed CYP3A4 activity. On the contrary, adding UGT2B7 microsomes to CYP3A4/CPR microsomes significantly enhanced CYP3A4 activity. The enhancement was systematic and strongly dependent on UGT2B7 microsomes, and was abrogated by detergent treatment. The collective results suggested that the enhancement of CYP3A4 activity resulted from a non-physiological interaction between CYP3A4 and UGT2B7, which were both expressed on different membranes. The phenomenon was distinguishable and hardly ever reflected the physiological interaction. This pitfall can be avoided by not using simple mixing procedures. In selecting experimental materials and methods depending on the subject of the study, a co-expression system should be applied in the analysis of functional P450-UGT interaction.

Key words cytochrome P450, uridine 5'-diphospho-glucuronosyltransferase, protein-protein interaction, microsomes, endoplasmic reticulum, expression system

INTRODUCTION

Cytochrome P450 (P450, CYP) 3A4 and uridine 5'-diphospho-glucuronosyltransferase (UGT) 2B7 are major human P450 and UGT isoforms that are involved in the metabolism of endogenous substances, as well as a huge number of drugs.[1–3] Although it is important to predict the *in vivo* functions of CYP3A4 and UGT2B7 for better clinical treatment, it is still difficult to explain their large inter-individual differences by their hepatic mRNA levels alone.[4,5] To address this issue, we have been focusing on the protein-protein interaction between CYP3A4 and UGT2B7. Yamada and co-workers reported the specific binding of UGTs to a CYP1A1-immobilized affinity column,[6] and demonstrated that the interaction can modulate the functions of both.[7–9] Our previous study indicated that UGT2B7 suppresses CYP3A4 activity by inhibiting substrate-binding to P450.[10] We anticipate that the P450-UGT interaction is one aspect of the post-translational regulation of P450 and UGT activities, knowledge of which could improve our understanding of their large inter-individual differences.

Recombinant P450 and UGT formed using heterologous expression systems are commonly used for *in vitro* studies.[11] In the case of P450, there is a well-established purification protocol from an *Escherichia coli* expression system, and such a recombinant P450 can be utilized in a reconstituted system.[12,13] In contrast, UGT is not appropriate for expression in *E. coli* because UGT has several glycosylation sites,[14,15] and removing membrane components from UGT diminishes its catalytic activity.[16] Given these findings, we co-expressed CYP3A4 and UGT2B7 with a baculovirus-insect cell system in a previous study to assess the role of UGT on CYP3A4 activity.[10] Co-expression of P450 and UGT can mimic the physiological endoplasmic reticulum (ER) membrane, in which the proteins reside with different topologies. However, this approach is time consuming, requiring at least one month to construct such a co-expression system. Microsomal fractions expressing P450 or UGT are commercially available, although the enzymes are separately expressed. It was reported that cytochrome b_5 (b_5) can interact with CYP3A4, even though they are expressed in different membranes. CYP3A4 activity is increased in the presence of *E. coli* membranes in which b_5 is expressed.[17] If mixing microsomes that separately express CYP3A4/NADPH P450 reductase (CPR) and UGT2B7 can practically reflect physiological conditions, it would be much easier to comprehensively examine the functional P450-UGT interaction.

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To address this issue, we presently compared our established co-expression system with a simple mixing method using commercially available microsomes which expressed CYP3A4/CPR and UGT2B7 separately. The comparison assessed the effect of UGT on CYP3A4 activity. We describe the advantage of using a co-expression system for investigating the functional P450-UGT interaction.

MATERIALS AND METHODS

Reagents Microsomal fractions expressing drug metabolizing enzymes were purchased from BD Gentest (Franklin Lakes, NJ). The enzymes used included Human CYP3A4 + P450 Reductase Microsomes (Cat. 455107, Lot. 98); Human CYP3A4 + P450 Reductase Supersomes (Cat. 456207, Lot. 17); Human CYP3A4 + P450 Reductase + Cytochrome b_5 Supersomes (Cat. 456202, Lot. 50); Human UGT2B7 Supersomes (Cat. 456427, Lot. 28062); UGT Insect Cell Control Supersomes (Cat. 456400, Lot. 13514 and 22928); and Pooled human microsomes (HLMs) from 50 donors (Cat. 452156, Lot. 88114). Supersomes refer to microsomes prepared from insect cells. D-Luciferin potassium salt and *n*-octyl β-D-glucopyranoside (OG) were purchased from Nacalai Tesque (Kyoto, Japan). Nicotinamide adenine dinucleotide phosphate, the reduced form (NADPH), was purchased from Oriental Yeast (Tokyo, Japan).

Culture of Sf9 Cells and Expression of Recombinant Enzymes Sf9 insect cells were cultured as previously described.[18] Preparation of recombinant baculovirus encoding CYP3A4, CPR, and UGT2B7 was described previously.[10] Sf9 cells (2×10^6) were seeded in wells of 6-well plates 24 h before infection with recombinant virus. Hemin was added to the medium as a complex with bovine serum albumin (BSA) at a final concentration of 1 μg/mL 24 h after infection to obtain the CYP3A4 holoenzyme.[10] The infected cells were collected at 72 h after infection in homogenization buffer that contained 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10% glycerol, and 1× protease inhibitor cocktail (Nacalai Tesque), and sonicated with UT-51N ultrasonic cleaner (SHARP, Osaka, Japan) to prepare homogenates. Protein concentrations in the homogenates were determined by Protein Assay CBB Solution (Nacalai Tesque) with BSA as a standard.

Immunoblotting Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 2% skim milk in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 (TBS-T), the membrane was incubated with primary antibody diluted 2000-fold. The following primary antibodies and detection kit were utilized: rabbit anti-UGT1A antibody (H-300), mouse anti-CPR antibody (F-10) (both from Santa Cruz Biotechnology, Dallas, TX), rabbit anti-UGT2B7 antibody (Proteintech, Rosemont, IL), and WB-MAB-3A Human CYP3A Western Blotting Kit (BD Gentest). Each membrane was extensively washed with TBS-T prior to incubation with horseradish peroxidase (HRP)-labeled secondary antibodies (donkey anti-rabbit antibody and sheep anti-mouse antibody; GE Healthcare, Little Chalfont, UK) diluted 10000-fold. EzWestLumi plus (ATTO, Tokyo, Japan) was used as a substrate of HRP, and signals were visualized and quantified with the ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA). Human CYP3A4 + P450 Reductase Supersomes, recombinant CPR (Panvera, Madison, WI), and HLM were used as the respective standards for CYP3A4, CPR, and UGT2B7.

CYP3A4 Assay CYP3A4 activity was measured with the P450-Glo-CYP3A4-Assay-and-Screening-System (Luciferin-IPA; Promega, Madison, WI) with a slight modification. Our previous study suggested that $NADP^+$, a component of the NADPH regeneration system, works as an inhibitor of UGT.[19] Thus, we used 100 μM NADPH (final concentration) instead of the NADPH regeneration system. Chemiluminescence was detected using the Fluoroscan Asent FL device (Thermo Labsystems, Franklin, MA). In the co-expression assay system, we used 20 μg homogenates per well, and calculated the CYP3A4 activity with the P450 contents determined by immunoblotting. In the microsome mixing system, we used commercially available microsomes expressing CYP3A4 (Cat. 455107, 456207, or 456202 mentioned above) as CYP3A4 sources, and applied 0.1 pmol CYP3A4 per well. Control and/or UGT2B7 expressing microsomes (total 10 μg protein) were added to the CYP3A4 expressed microsomes. When we examined the effects of detergent, OG was added to the mixed microsomes in a detergent:protein ratio (mg detergent/mg protein) from 0.0 to 1.0. All samples were kept on ice for 30 min, and the detergent-treated microsomes were used for the CYP3A4 assay.

Kinetic and Statistical Analyses All analyses were performed using GraphPad Prism 5.04 software (GraphPad Software, La Jolla, CA). Kinetics data were curve-fitted to the following Michaelis-Menten equation:

$$V = V_{max} \times S/(K_m + S)$$

where V is the reaction rate, S is the substrate concentration, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant, which is the concentration of substrate necessary for half-maximal velocity. The kinetic analysis was repeated four times, and parameters were calculated in each analysis to elucidate the effect of mixing UGT2B7 microsomes. Statistical significance was determined by Student's t test, extra sum-of-squares F test, or ANOVA followed by Dunnett's test. The specific statistical test applied was described in each figure legend (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

RESULTS

First, we confirmed the effect of UGT2B7 on CYP3A4 activity in the co-expression system. We prepared Sf9 homogenates dually expressing CYP3A4/CPR (UGT2B7-absence) and ternary expressing CYP3A4/CPR/UGT2B7 (UGT2B7-presence) with a baculovirus expression system. Expressions of the enzymes were confirmed and quantified by immunoblotting in 12 UGT2B7-absence samples and nine UGT2B7-presence samples (Fig. 1A left and right panels, respectively). Based on the result, we calculated the P450/CPR ratio, which is critical for P450 activity, and measured CYP3A4 activity using preluciferin as a substrate. As expected, CYP3A4 activity was highly dependent on the P450/CPR ratio; a higher level of co-expressed CPR resulted in higher CYP3A4 activity (Fig. 1B). In addition, the activities showed linearity between the P450/CPR ranges (r^2=0.89 and 0.86 in the absence and presence of UGT2B7, respectively). Co-expression of UGT2B7 significantly reduced the y-intercept but had no effect on the slope, implying that UGT2B7 had no effect on CYP3A4 affinity to CPR and directly suppressed CYP3A4 itself (Fig. 1B). The result was consistent with our previous study.[10]

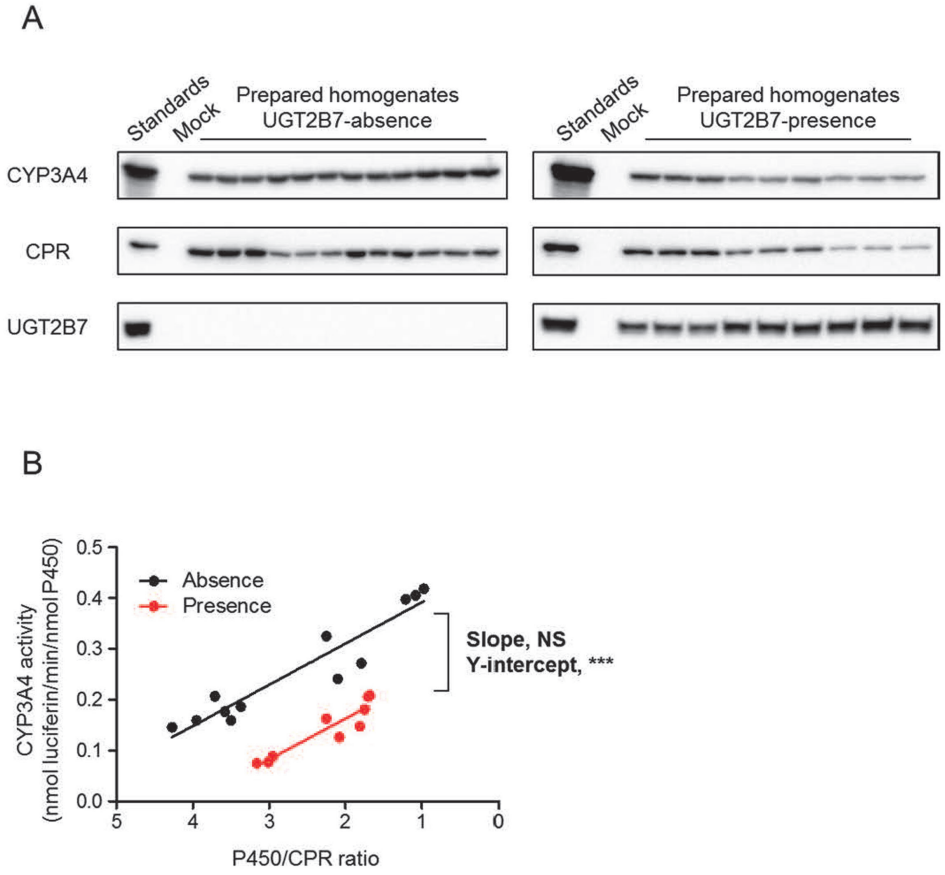

Fig. 1. Suppression of CYP3A4 Activity by Co-Expressed UGT2B7

(A) Western blots confirmed and quantified the expressions of CYP3A4, CPR, and UGT2B7 in a co-expression system. Baculovirus-infected Sf9 cells were harvested and sonicated to prepare homogenates. A portion of the homogenate (10 μg protein) was analyzed. CYP3A4 in Supersomes (5 pmol P450; BD Gentest, Cat. 456207, Lot. 17), 100 ng recombinant CPR (Panvera), and HLM (10 μg protein) were used as respective standards of CYP3A4, CPR, and UGT2B7. Mock are homogenates infected with Control virus. (B) Suppression of CYP3A4 activity by co-expressed UGT2B7. CYP3A4 activity was measured with a pre-luciferin (luciferin-IPA, Promega) and homogenate (10 μg protein) as a substrate and enzyme source, respectively, in a singlicate assay. The expressed ratio of P450/CPR was determined from the quantitative western blots shown in (A). Approximate lines were calculated in each group, and statistical differences in their slope and y-intercept were determined by extra sum-of-squares F test (NS, not significant; ***$P < 0.001$).

Next, we mixed CYP3A4/CPR-expressing microsomes prepared from lymphoblastoid cells with UGT2B7 microsomes from a baculovirus system to elucidate whether the suppressive effects are reproducible in this mixing method. Interestingly, however, CYP3A4 activity was enhanced in a manner that was dependent on UGT2B7 microsomes (Fig. 2A). This UGT2B7 microsome-dependent enhancement of CYP3A4 activity was also observed when we switched the CYP3A4 source from the lymphoblastoid sample to CYP3A4/CPR and CYP3A4/CPR/b_5 microsomes prepared from a baculovirus system (Fig. 2B). For further estimation of this enhancement of CYP3A4 activity by mixing microsomes, we performed a kinetic analysis. We added Control microsomes, Control/UGT2B7 microsomes (50:50), or UGT2B7 microsomes prepared from insect cells to CYP3A4/CPR microsomes from lymphoblastoid cells. The kinetic analysis was repeated four times; a representative result is shown in Figure 2C. Four pairs of V_{max} and K_m values are presented in Figure 2D. Adding UGT2B7 microsomes significantly increased the V_{max} value of CYP3A4 activity in a UGT2B7 microsomes-dependent manner and had no effect on K_m (Fig. 2D), which was opposite from the suppressive result observed in the co-expression system (Fig. 1B). Further, we examined the effect of the nonionic detergent, OG, on the UGT2B7 microsome-dependent enhancement of CYP3A4 activity to predict its molecular mechanism. The mixed microsomes were treated with the detergent, and utilized in the CYP3A4 assay. Enhancement was diminished in an OG-dependent fashion, and significance disappeared at a detergent:protein ratio (mg detergent/mg protein) of 1.0 (Fig. 3).

DISCUSSION

We evaluated whether a simple microsome mixing system was valid for estimation of the functional interaction between P450 and UGT. In the co-expression system, we used insect cell homogenates as the enzyme source, in which CYP3A4 and UGT2B7 were expressed in the same membrane, which closely mirror physiological conditions (Fig. 4, left image). CYP3A4 activity is highly dependent on the co-expression of CPR, so we carefully checked the P450/CPR ratio of the homogenates so as not to erroneously estimate the effect of UGT2B7 (Fig. 1A). UGT2B7 significantly suppressed CYP3A4 activity in the range of P450/CPR ratios used without affecting affinity of P450 to CPR (Fig. 1B). Our previous study revealed that UGT2B7 had no effect on CPR activity, and suppressed the entire catalytic cycle of CYP3A4, including substrate oxidation, NADPH consumption, and generation of hydrogen peroxide, by inhibiting the binding of substrate to P450.[10] Thus, the result shown in Fig. 1B is consistent with

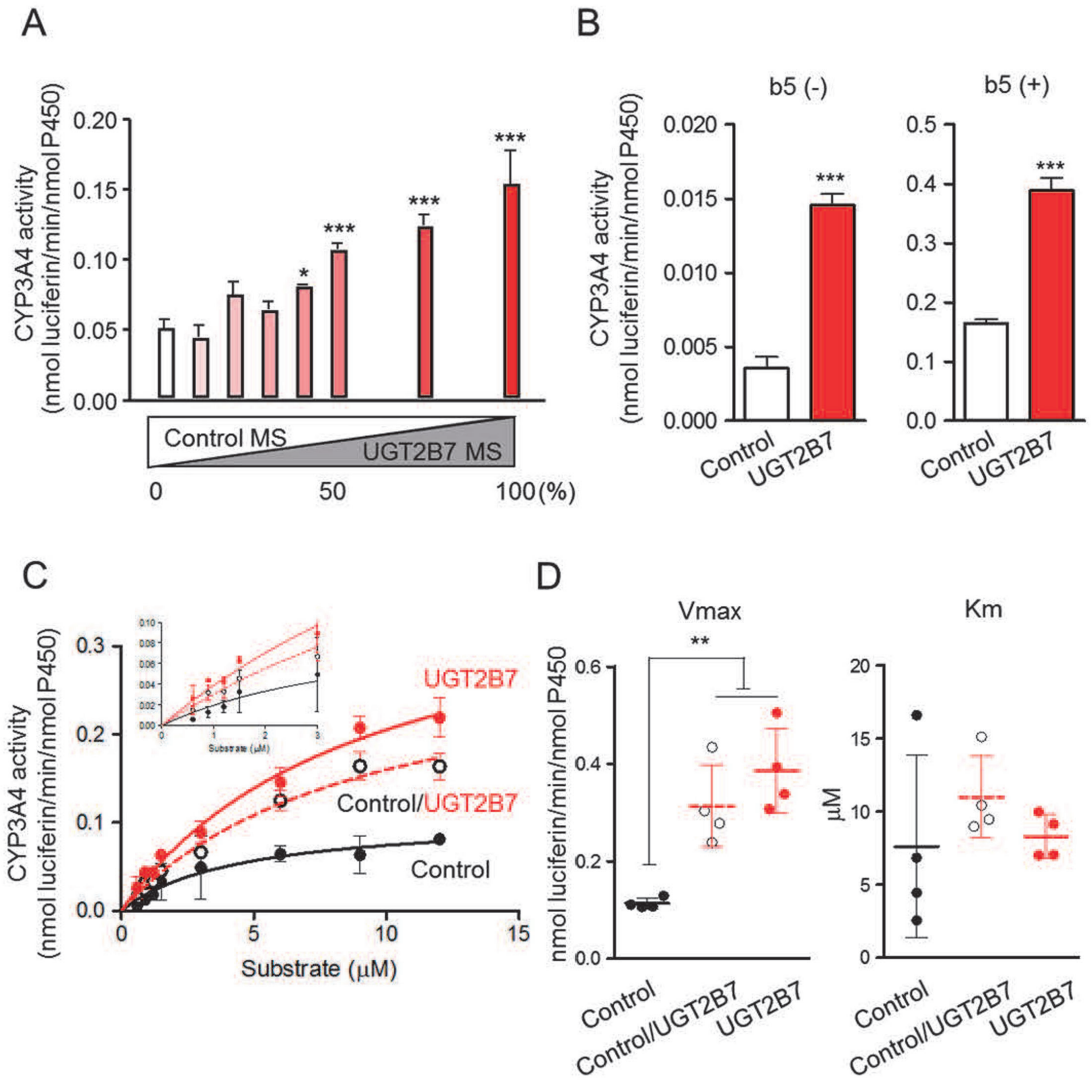

Fig. 2. Enhancement of CYP3A4 Activity by the Addition of UGT2B7-Expressing Microsomes

All the data for CYP3A4 activities are shown as the mean ± S.D. value of a triplicate assay. (A) Effect of the addition of UGT2B7-expressing microsomes on CYP3A4 activity. Microsomes prepared from lymphoblastoid cells (0.1 pmol P450) were used as CYP3A4 source, and Control and/or UGT2B7 microsomes from insect cells (total 10 μg protein) were mixed. CYP3A4 activity was measured with luciferin-IPA as a substrate. Statistical significance was determined by ANOVA followed by Dunnett's test vs adding Control (UGT2B7 0%) (*P < 0.05, ***P < 0.001). (B) CYP3A4 sources were switched from lymphoblastoid microsomes to insect microsomes, and the effect of adding UGT2B7 microsomes were analyzed. Control or UGT2B7 microsomes (10 μg protein) were added to the CYP3A4-expressed microsomes (0.1 pmol P450) with and without co-expression of cytochrome b_5 (right and left panels, respectively). Statistical significance was determined by Student's t test (***P < 0.001). (C) Kinetic analysis of mixed microsomes methods. Lymphoblastoid CYP3A4 microsomes (0.1 pmol P450) were mixed with Control and/or UGT2B7 microsomes (10 μg protein) and P450 activity was measured. Data were fit to the Michaelis-Menten equation. Analysis were performed in triplicate and was repeated four times. The mean ± S.D. values of a representative analysis are presented. Data obtained at substrate concentrations less than 3 μM are shown as a magnified graph in the inset. (D) Kinetic parameters determined by the four analyses are shown. Each plot represents the V_{max} or K_m value obtained by serial analysis, and the mean ± S.D. values are presented. Statistical significance was determined by ANOVA followed by Dunnett's test vs the addition of Control microsomes (**P < 0.01).

the previous findings.

In contrast, mixing UGT2B7-expressing microsomes with CYP3A4/CPR microsomes enhanced CYP3A4 activity in a manner that was dependent on UGT2B7-microsomes and significantly increased the V_{max} of CYP3A4 activity (Fig. 2). P450 and UGT are both localized in the ER membrane, but their membrane topologies are quite different: P450 faces the cytosol, while the main body of UGT is located at the luminal side.[20,21] UGT has a cytoplasmic domain in its C-terminus that consists of approximately 20 residues. This domain was believed to work in ER targeting, but our recent study indicated that UGT2B7 can be retained in the ER in a domain-independent fashion.[22] We also determined that the luminal membrane binding site of UGT2B7 is necessary for the interaction with CYP3A4, and that this P450-UGT interaction occurs through the ER membrane.[10] Since it is unlikely that the luminal domain of UGT2B7 associates with CYP3A4, which is in a different membrane, in the absence of detergent, only the cytoplasmic domain of UGT2B7 can associate with CYP3A4 in the simple mixing of microsomes method (Fig. 4, right image). Thus, the UGT2B7-dependent enhancement of CYP3A4 observed in the mixing-microsomes method should have resulted from a non-physiological CYP3A4-UGT2B7 interaction, which was quite different from the physiological and suppressive modulation observed in the co-expression system (Fig 4). This non-physiological interaction seemed to arise in a system-dependent manner because CYP3A4 in all the enzyme sources we tested were enhanced equally (Fig. 2A and 2B). The collective results suggest that P450 and UGT expressed on the same membrane are essential to mimic the physiological membrane and correctly analyze the functional P450-UGT interaction.

Co-expression of UGTs on the same membrane is also important in UGT-UGT interaction. We have reported that UGT2B22 can interact with and alter the regioselectivity of UGT2B21-catalyzed morphine glucuronidation in guinea pigs.[23] Co-expression of UGT2B22 increased the formation of morphine-6-glucuronoide (M6G). This effect was only observed in the co-expression system and not when microsomes expressing UGT2B22 were mixed with microsomes expressing UGT2B21. In addition, Reed *et al.* demonstrated that reconstitution of CYP1A2 and CYP2B4 in the same phospholipid vesicle is necessary for functional P450-P450 interaction.[24] The CYP1A2-CYP2B4 interaction enhanced 7-ethoxyresorfin *O*-dealkylation that was mainly catalyzed by CYP1A2 when P450 was located in the same membrane. Such an enhancement was not observed when CYP1A2/CPR vesicles and CYP2B4/CPR vesicles were mixed. These findings support our idea that proper positioning of P450 and UGT on the same membrane is necessary for their physiological interactions and for the precise assessment of effects of the interactions on P450 and UGT activities.

The effects of detergent treatment also differed between the co-expression system and the simple mixing method. In our previous studies, we detected CYP3A4-UGT2B7 and CYP3A1-UGT2B3 (rat isoforms) in solubilized microsomes co-expressing P450 and UGT.[10,25] On the other hand, enhancement of CYP3A4 activity in the simple microsomes mixing method was diminished and was abrogated by detergent treatment in the present study. Detergent was also utilized when we elucidated the role of CYP3A4 on UGT2B7-catalyzed morphine glucuronidation.[26] Purified recombinant CYP3A4 was added to COS-1 cell microsomes expressing UGT2B7, either treated or non-treated with detergent. CYP3A4 affects the function of UGT2B7 only in detergent-treated microsomes, resulting in enhanced M6G formation. Hence, it is reasonable to consider that a static CYP3A-UGT2B7/3 interaction is fairly stable in the presence of detergent, while the non-physiological interaction observed in the simple-mixing method is disrupted by detergent (Fig. 3). The molecular mechanism underlying the non-physiological and systematic CYP3A4-UGT2B7 interaction remains unclear. However, the likely interaction sites are the main body of CYP3A4 and cytoplasmic domain of UGT2B7. We predict that such a non-physiological interaction increases the hydrophobicity of the microenvironment around P450 and the concentrated substrate. Detergent could partially solubilize UGT2B7 and inhibit such an accumulation of substrate, resulting in the abrogation of the enhanced CYP3A4 activity (Fig. 3). Although we cannot rule out other possible mechanisms, the collective findings support this prediction.

In conclusion, there was a pitfall if we chose the simple-mixing method to evaluate the functional interaction of CYP3A4 and UGT2B7. We have demonstrated contradictory effects of UGT2B7 on CYP3A4 activity between the two methods. There is no doubt that commercially available microsomes are useful tools to predict metabolite and isoform specificity of P450 and UGT. However, we have to select a co-

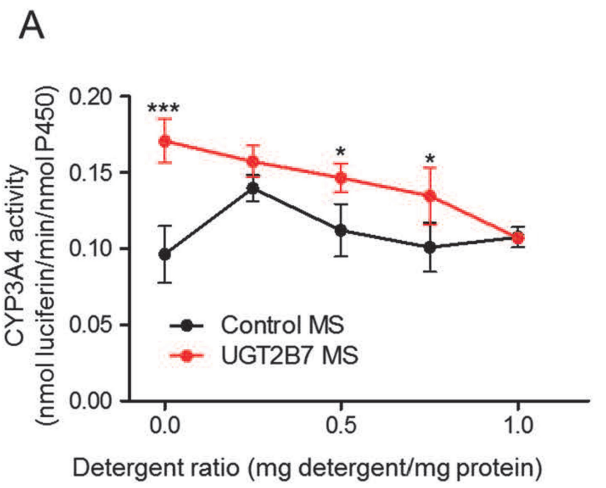

Fig. 3. Prediction of the Molecular Mechanism Underlying Non-Physiological CYP3A4-UGT2B7 Interaction

Enhanced CYP3A4 activity was diminished and disappeared upon detergent treatment. Mixed microsomes (CYP3A4/CPR + Control or UGT2B7) were solubilized with *n*-octyl β-D-glucopyranoside for 30 min, at a detergent:protein ratio (mg detergent/mg protein) ranging from 0.0 to 1.0. The solubilized microsomes were utilized for the following CYP3A4 assay. CYP3A4 activities are shown as the mean ± S.D. of a triplicate assay. Statistical significance (addition of Control vs UGT2B7 in each quantity of detergent) was determined by Student's t test (***P < 0.001; *P < 0.05).

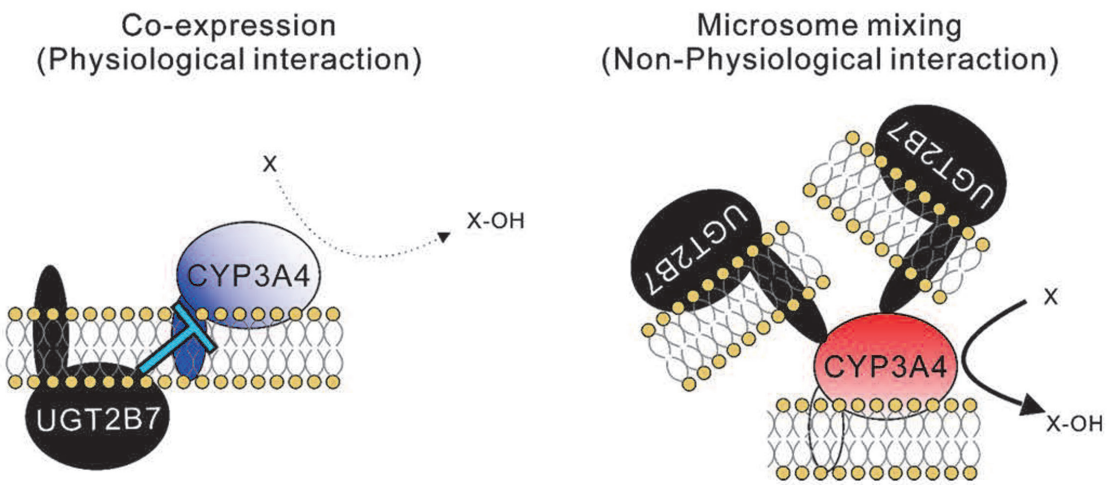

Fig. 4. Different Effects of UGT2B7 on CYP3A4 Activity between the Co-Expression System and Microsome Mixing Method

X, substrate of CYP3A4; X-OH, metabolite of CYP3A4

expression system when we analyze some complex reactions in which several drug-metabolizing enzymes are involved, such as the sequential metabolism and effect of protein-protein interactions among drug-metabolizing enzymes. Further studies are necessary for a better understanding of the P450-UGT interaction and its physiological role on the regulation of enzymes.

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Conflict of interest The authors declare no conflict of interest.

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