Different Effects of Desipramine on Bufuralol 1″-Hydroxylation by Rat and Human CYP2D Enzymes

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Inhibitory effects of desipramine (DMI) on rat and human CYP2D enzymes were studied using bufuralol (BF) 1″-hydroxylation as an index. Inhibition was examined under the following two conditions: 1) DMI was co-incubated with BF and NADPH in the reaction mixture containing rat or human liver microsomes or yeast cell microsomes expressing rat CYP2D1, CYP2D2 or human CYP2D6 (co-incubation); 2) DMI was preincubated with NADPH and the same enzyme sources prior to adding the substrate (preincubation). When either rat liver microsomes or recombinant CYP2D2 was employed, the preincubation with DMI (0.3 μM) caused a greater inhibition of BF 1″-hydroxylation than the co-incubation did, whereas BF 1″-hydroxylation by rat CYP2D1 was not markedly affected under the same conditions. The inhibitory effect of DMI on BF 1″-hydroxylation by human liver microsomal fractions or recombinant CYP2D6 was much lower than that on the hydroxylation by rat liver microsomes or CYP2D2. Kinetic studies demonstrated that the inhibition-type changed from competitive for the co-incubation to noncompetitive for the preincubation in the case of CYP2D2, whereas the inhibition-type was competitive for both the co-incubation and the preincubation in the case of CYP2D6. Furthermore, the loss of activity of rat CYP2D2 under the preincubation conditions followed pseudo-first-order kinetics. Binding experiments employing the recombinant enzymes and [^{3}H]-DMI revealed that CYP2D2 and CYP2D6 were the only prominent proteins to which considerable radioactive DMI metabolite(s) bound. These results indicate that rat CYP2D2 biotransforms DMI into reactive metabolite(s), which covalently bind to CYP2D2, resulting in inactivation of the enzyme. In contrast, human CYP2D6 may also biotransform DMI into some metabolite(s) that covalently bind to CYP2D6, but that do not inactivate the enzyme.

Key words desipramine; CYP2D2; CYP2D6; reactive metabolite; binding; inactivation

Imipramine (IMI) and desipramine (DMI) are tricyclic antidepressants that are widely used clinically. As shown in Fig. 1, IMI is oxidized by cytochrome P450 (CYP) mainly *via* two pathways: side-chain *N*-demethylation and aromatic ring 2-hydroxylation, forming DMI and 2-hydroxyimipramine (2-OH-IMI), respectively.[1] DMI and 2-OH-IMI further undergo 2-hydroxylation and *N*-demethylation, respectively, forming 2-hydroxy-DMI as the common metabolite (Fig. 1). In the human liver, CYP2D6 is mainly responsible for IMI 2-hydroxylation whereas CYP2C19 and CYP1A2 are involved in *N*-demethylation.[2,3]

It has been reported that repeated administration of IMI to rats changed hepatic CYP-dependent monooxygenase activities.[4—6] We have also found that repetitive oral administration of IMI to rats caused a decrease in hepatic microsomal CYP2D-dependent reactions such as debrisoquine 4-hydroxylation, bunitrolol 4-hydroxylation, lidocaine 3-hydroxylation and propranolol 4-, 5- and 7-hydroxylations.[7] We have proposed that binding of a reactive metabolite of IMI to rat CYP2D enzyme(s) resulted in the decreased enzyme activities.[7] As a possible mechanism, we speculated that an epoxy metabolite of IMI (1,2- or 2,3-epoxide) was involved in the inactivation of rat CYP2D enzyme(s).[7,8]

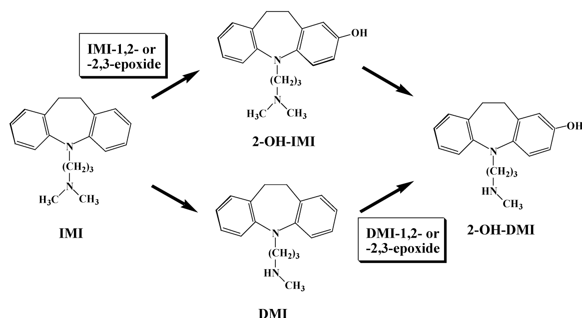

Fig. 1. Parallel Metabolic Pathways of the Conversion of IMI to 2-OH-DMI

If the epoxy metabolite(s) of IMI are responsible for the inactivation, DMI would also inactivate the rat CYP2D enzyme(s). Because several CYP2D enzymes are known to be expressed in the rat liver,[9] it is interesting to know what kind of CYP2D isoenzyme(s) are inhibited by IMI or DMI. Furthermore, there is a possibility that CYP2D6, a human functional CYP2D enzyme, is also inactivated by IMI and DMI in a similar manner. The present study was thus conducted to examine these possibilities using radiolabeled and unlabeled DMI and recombinant human and rat CYP2D enzymes.

MATERIALS AND METHODS

Materials DMI as the hydrochloride and propranolol racemate as the hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); bufuralol (BF) and

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1″-hydroxybufuralol (1″-OH-BF) (both as hydrochlorides) were from Daiichi Pure Chemical Co. (Tokyo, Japan); dithiothreitol was from Nacalai Tesque (Kyoto, Japan); [^{3}H]-labeled DMI (specific activity 2.96 TBq/mmol, radiochemical purity above 97%) was from NEN Life Science Products, Inc. (Boston, MA, U.S.A.); glucose 6-phosphate (G-6-P), G-6-P dehydrogenase and NADPH were from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and organic solvents used were of analytical grade.

Rat and Human Liver Microsomes Male Wistar rats (6 weeks old) were purchased from Clea Japan Co. (Shizuoka, Japan). Liver microsomal fractions were prepared from rats by a published method.[10] Human liver microsomal fractions (n=3, all male Caucasians from 25 to 56 years old) were supplied from Human and Animal Bridge Discussion Group (HAB) (Chiba, Japan). This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Okayama University.

Recombinant CYP2D Enzymes Recombinant rat CYP2D2[9] and human CYP2D6[11] were expressed in yeast cells (*Saccharomyces cerevisiae* AH-22 strain) according to methods previously reported. Rat CYP2D2-G45V mutant protein, which has valine instead of glycine at position 45 of CYP2D2, was expressed in yeast cells as reported elsewhere.[12] The contents of CYP2D enzymes in yeast cell microsomal fractions were 52.8, 92.0, 2.1 and 6.5 pmol/mg protein for CYP2D6, CYP2D1, CYP2D2 and CYP2D2-G45V, respectively.

Incubation of Rat or Human Liver Microsomal Fractions with IMI or DMI The inhibitory effects of DMI on rat or human liver microsomal BF 1″-hydroxylation were examined under three conditions as summarized in Chart 1a—c: rat or human liver microsomal fraction (0.5 mg protein) was added to ice-cold reaction medium (final volume 500 μl) in a brown glass conical tube (10 ml) with a glass stopper containing 5 mM G-6-P, 1 IU G-6-P dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) and preincubated for 5 min. NADPH (final concentration 0.5 mM) with or without DMI (final concentration 0.3 μM for the rat enzyme sources and 3.0 μM for the human enzyme sources) was added to the equilibrated mixture and allowed to incubate at 37 °C for 2 min, after which BF (final concentration 0.5 μM) was added and BF 1″-hydroxylation was allowed to proceed for 1 min. After the reaction was stopped by adding 1 ml of 1 M NaOH aqueous solution and vortex mixing, 1 ml of 1 M sodium carbonate buffer (pH 9.6) and propranolol racemate (100 nmol, internal standard) were added, and 1″-OH-BF was extracted into 5 ml of ethyl acetate by vigorous shaking. After centrifugation (1000×***g*** for 10 min) 4 ml of the organic layer was taken and evaporated to dryness under N_2 stream. The residue was dissolved in 100 μl of the HPLC mobile phase described below, and an aliquot (10 μl) was subjected to HPLC under the conditions described below. In the case of recombinant enzymes, yeast cell microsomal fractions containing 3 pmol of rat or human recombinant CYP were employed. In preliminary experiments, linearity of product formation as a function of time was confirmed for each case.

HPLC Conditions A Shimadzu LC-9A liquid chromatograph equipped with a Shimadzu RF-10A fluorescence detector, a Rheodyne Model 7125 injector and a Shimadzu C-R4A Chromatopac data processor were used with the following: column, Inertsil ODS (4.6 mm×250 mm, GL Science, Tokyo, Japan); mobile phase, acetonitrile/20 mM perchloric acid (pH 2.5) (35 : 65, by volume) at a flow rate of 1.5 ml/min; detection, fluorescence 252/302 nm (excitation/emission).

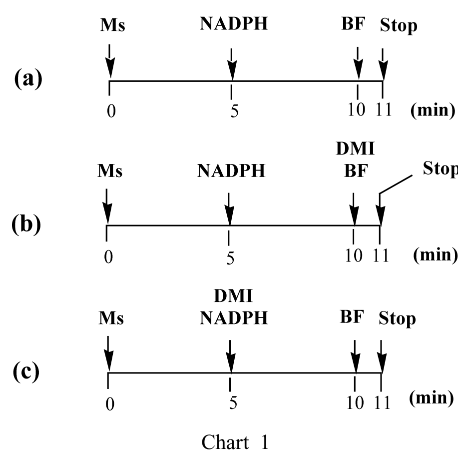

Chart 1

Kinetic Analysis Kinetic studies of BF 1″-hydroxylation by the rat liver microsomes or the recombinant CYP2D enzymes were performed using a BF concentration range from 0.2 to 20 μM and DMI concentration of 0.3 μM for the rat enzymes and 3.0 μM for the human enzyme. Apparent Michaelis constant (K_m) and maximal velocity (V_{max}) were analyzed using the nonlinear least squares regression analysis program MULTI.[13] Inhibition experiments were analyzed by fitting expressions describing competitive inhibition (Eq. 1) and noncompetitive inhibition (Eq. 2) using the same program mentioned above.

$$v = \frac{V_{max} \cdot S}{K_m + (K_m/K_i) \cdot I + S} \quad (1)$$

$$v = \frac{K_i \cdot V_{max} \cdot S}{(K_i + I)(K_m + S)} \quad (2)$$

where v is the rate of formation of metabolite, V_{max} is the maximum rate of metabolite formation, S is the substrate concentration, K_m is the Michaelis constant, I is the inhibitor concentration and K_i is the inhibition constant.

Inactivation of CYP2D2 Microsomes from yeast cells expressing CYP2D2 were preincubated with various concentrations of DMI (0, 0.3, 1.0, 2.0, 5.0 μM) at 37 °C for an appropriate time (0, 0.5, 1, 1.5, 2 min) in the presence of NADPH (0.5 mM). After the preincubation, BF 1″-hydroxylase activities of the preincubated microsomes were assayed as described above. The initial rate constant for the inactivation (K_{obs}) was obtained as slopes of initial linear phase plotting logarithm of remaining activity against the preincubation time. The maximum rate constant for inactivation (K_{inact}) and the dissociation constant for the enzyme-inactivation (K_I) were determined according to the published method.[14]

Binding Studies The yeast cell microsomal fraction expressing CYP2D2 or CYP2D6 (50 pmol) was added to equilibrated incubation mixture containing the same ingredients as described above in the enzyme assay, and preincubated at 37 °C for 5 min. DMI (final concentration 10 μM, 3600000 dpm) was then added, and the mixture was incu-

bated at 37 °C for 30 min in the presence or absence of NADPH (final concentration 0.5 mM). The entire sample mixture was transferred into a dialysis tube, and dialyzed at 4 °C for 6 h against 50 mM Tris–HCl buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol (500 ml×2).

A portion of the dialyzed sample (110 μl) was solubilized and subjected to SDS-PAGE using a 10% slab-gel. The gel was stained with Coomassie Brilliant Blue, and cut into 2-mm strips. The gel strips were then solubilized with hydrogen peroxide, and the radioactivity of the samples was measured using a liquid scintillation counter (LSC-3100, Aloka Co., Tokyo, Japan). The scintillation medium consisted of one volume of Triton X-100 and two volumes of toluene phosphor including 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene per liter of toluene.

A small portion (10 μl) of the dialyzed sample was subjected to SDS-PAGE using a 10% slab-gel. After electrophoresis, proteins in the gel were transblotted to a PVDF membrane and the CYP2D protein was probed by Western blot analysis using polyclonal antibodies (rabbit IgG) raised against CYP2D1 according to a published method.[15] Various amounts of the recombinant CYP2D enzyme were electrophoresed and transferred to the same membrane, and calibration curves were made by scanning the protein bands corresponding to the CYP2D enzyme using NIH Image (version 1.2) installed in a Power Macintosh G4 equipped with an Epson CC-550L scanner. The localization of the radiolabeled CYP2D enzymes on the PVDF membrane and their radioactivity were measured by imaging analysis with the BAS2000 system (Fuji Film, Co., Tokyo, Japan). The PVDF membrane on which radioactive DMI metabolite(s)-bound proteins were transblotted and another PVDF membrane on which various amounts of [^{3}H]-DMI were spotted were attached to an imaging plate (Fuji Film, Co) and exposed for 2 weeks. Then the imaging plate was scanned using the BAS2000 system, and the radioactivity of the CYP2D2 or CYP2D6 protein band on the membrane was calculated on the basis of the calibration curves.

Others Protein concentrations were determined by the method of Lowry *et al.*[16] Total holo-CYP content was spectrophotometrically measured from reduced carbon monoxide (CO) spectra according to the method of Omura and Sato[10] using 91 mM^{-1} cm^{-1} as an absorption coefficient. Statistical significance was calculated with Student's *t*-test using Prism version 3.0 (Graph Pad Software, San Diego, CA, U.S.A.), and differences were considered to be statistically significant when p was <0.05.

RESULTS

Comparison of Inhibitory Effects of DMI on BF 1″-Hydroxylation by Rat and Human Liver Microsomes and Recombinant CYP Enzymes In our previous report, we proposed that rat liver microsomal CYP2D enzyme(s) might be inactivated by reactive intermediate(s) formed during IMI metabolism.[8] We thought similarly that CYP2D2 might be a target enzyme that is inactivated during the metabolism of DMI in rat liver microsomes. As the first stage of the present study to test this possibility, we thus compared the inhibitory effects of DMI on BF 1″-hydroxylation between rat liver microsomes (Fig. 2A) and yeast cell microsomes expressing CYP2D1 (Fig. 2B) or CYP2D2 (Fig. 2C) using a DMI concentration of 0.3 μM and a BF concentration of 0.5 μM.

These concentrations of the inhibitor and substrate were chosen to distinguish the inhibition-types of DMI for BF 1″-hydroxylation as clearly as possible, because preliminary

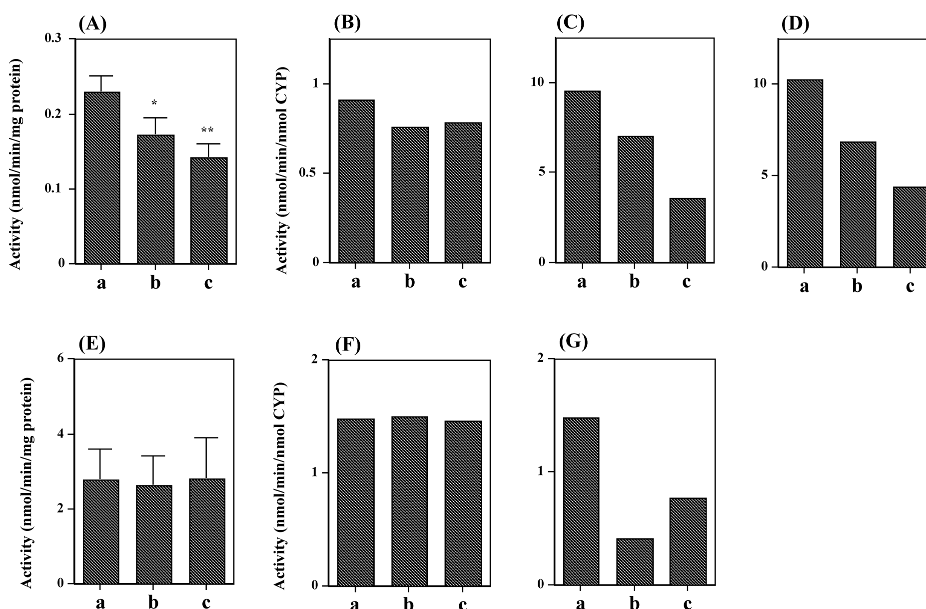

Fig. 2. Comparison of the Inhibitory Effects of DMI on BF 1″-Hydroxylation by Rat or Human Liver Microsomes or Yeast Cell Microsomes Expressing Recombinant CYP Enzymes

The reaction mixtures (final 500 μl) containing various enzyme sources were incubated with BF (0.5 μM) in the presence or absence of DMI (0.3 μM) under the conditions given in Charts 1a, b and c. The enzyme sources were; (A) rat liver microsomes, (B) yeast cell microsomes expressing rat CYP2D2, (C) yeast cell microsomes expressing rat CYP2D2-G45V, (D) human liver microsomes, (E) and (F) yeast cell microsomes expressing human CYP2D6. The concentration of DMI was 0.3 μM except in (F) (3.0 μM). Each value in (A) and (E) is the mean±S.D. ($n=3$). Each value in (B), (C), (D), (F) and (G) is the mean value of two independent determinations. Significantly different from the control (a): * $p<0.05$, ** $p<0.01$.

studies indicated that most of 0.3 μM DMI disappeared from the reaction mixture during a 2 min incubation and at least 50% of 0.5 μM BF remained after a 1 min incubation under the conditions employed. The co-incubation of BF with DMI significantly inhibited rat hepatic microsomal BF 1″-hydroxylation (Fig. 2A-b) and the preincubation of microsomes with DMI in the presence of NADPH caused a greater inhibition (Fig. 2A-c).

When the recombinant CYP2D1 was used as enzyme source, DMI did not exhibit any clear inhibitory effect on BF 1″-hydroxylase activity (Fig. 2B). On the other hand, DMI produced a greater inhibition of BF 1″-hydroxylation by recombinant CYP2D2 (Fig. 2C). The preincubation of DMI with yeast cell microsomes expressing CYP2D2 in the presence of the cofactor suppressed the activity to 37% of the control level (Fig. 2C-c).

It has been reported that three CYP2D enzymes (CYP2D1, CYP2D2 and CYP2D3) are expressed in the rat liver, and that the microsomal contents of functional CYP2D2 are much lower than those of CYP2D1 and CYP2D3.[17] We recently found that the difference of amino acid residues at positions 43 (tryptophan for CYP2D1 and leucine for CYP2D2) and 45 (valine for CYP2D1 and glycine for CYP2D2) within or near the proline-rich region of the N-terminal region causes the difference in the microsomal functional P450 contents between CYP2D1 and CYP2D2.[12] In that study, we prepared recombinant CYP2D2-G45V having valine instead of glycine-45 and found that the yeast cell microsomal content of the functional holoprotein, P450, was 2- to 3-fold that of wild-type CYP2D2.[12] In the present study, DMI showed a similar inhibition profile for BF 1″-hydroxylation by the wild-type CYP2D2 (Fig. 2C) and by the mutant CYP2D2 (Fig. 2D). Therefore, the inhibitory properties of DMI for CYP2D2 and CYP2D2-G45V are thought to be essentially the same. On the basis of these results, CYP2D2-G45V was used as CYP2D2 in further experiments.

The inhibitory properties of DMI were also examined for human liver microsomes (Fig. 2E) and for yeast cell microsomes expressing recombinant human CYP2D6 (Figs. 2F, G). Interestingly, DMI (0.3 μM) did not cause any inhibitory effect on BF 1″-hydroxylation by either the human liver microsomes (Fig. 2E) or the recombinant CYP2D6 (Fig. 2F). When a 10-times higher concentration of DMI (3 μM) was employed, a considerable inhibition was observed (Fig. 2G). These results indicate that the inhibitory effect of DMI on BF 1″-hydroxylation is much stronger with CYP2D2 than with CYP2D1 and CYP2D6.

Kinetic Studies of the Inhibition by DMI of BF 1″-Hydroxylation by Rat and Human Liver Microsomes and by the Recombinant CYP2D Enzymes Figure 3 shows typical Lineweaver-Burk plots for the inhibition of BF 1″-hydroxylation by rat liver microsomes (A), CYP2D2 (B) and CYP2D6 (C). In plots in (A) and (B), the co-incubation of DMI and BF yielded a competitive-type inhibition, whereas the preincubation of DMI with rat liver microsomes and CYP2D2 in the presence of NADPH changed the inhibition-type from competitive to noncompetitive. The calculated kinetic parameters are summarized in Table 1.

These results demonstrate that BF 1″-hydroxylation was inhibited competitively when rat CYP2D2 was co-incubated with DMI and BF in the presence of NADPH (Chart 1b) and noncompetitively when the enzyme was preincubated with DMI and NADPH before the incubation with BF (Chart 1c). The K_i value for the recombinant CYP2D2 was 0.3 μM, which was lower than that (0.6 μM) for the rat liver microsomes. It is possible that the rat liver microsomes contain not

Table 1. Kinetic Parameters of BF 1″-Hydroxylation by Microsomal Fractions from Rat Livers and Yeast Cells Expressing Rat CYP2D2 or Human CYP2D6

	K_m (μM)	V_{max} [a]	K_i (μM)
Rat liver microsomes			
(A) Control	1.6	2.73	—
(B) Co-incubation[b]	2.3	2.66	0.62
(C) Preincubation[c]	1.7	1.81	0.58
Recombinant CYP2D2			
(A) Control	0.9	94.3	—
(B) Co-incubation[b]	1.7	95.8	0.33
(C) Preincubation[c]	0.9	42.5	0.25
Recombinant CYP2D6			
(A) Control	3.6	16.7	—
(B) Co-incubation[b]	10.3	14.9	1.61
(C) Preincubation[c]	9.8	15.9	1.74

a) nmol/min/mg protein for rat liver microsomes; nmol/min/nmol CYP for recombinant enzymes. *b*) DMI (0.3 μM for the rat-derived enzymes; 3 μM for CYP2D6) together with BF (0.2 to 20 μM) was added to the equilibrated mixture and the mixture was incubated for 1 min. *c*) DMI (0.3 μM for the rat-derived enzymes; 3 μM for CYP2D6) was preincubated with the microsomal fraction in the presence of NADPH (0.5 M) at 37 °C for 2 min, followed by incubation with BF (0.2 to 20 μM) for 1 min. Each value is the mean of two independent determinations.

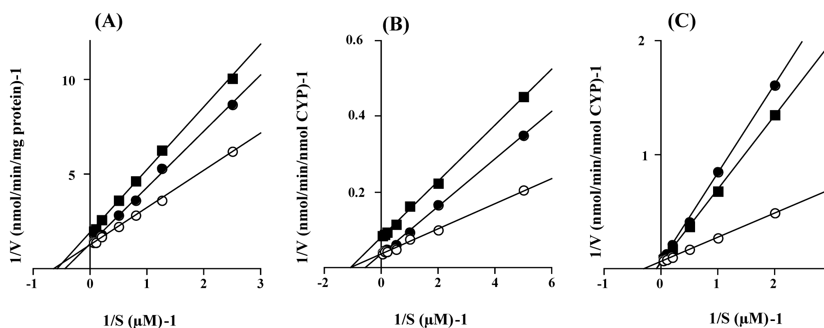

Fig. 3. Lineweaver-Burk Plots Showing the Inhibitory Effects of DMI on BF 1″-Hydroxyation by Rat Liver Microsomes and Yeast Cell Microsomes Expressing Rat CYP2D2 or Human CYP2D6

In the cases of rat liver microsomes (A) and CYP2D2 (B), DMI (0.3 μM) was used. In the case of human CYP2D6 (C), DMI (3.0 μM) was employed. Open circles, control; closed circles, co-incubation; closed squares, preincubation. The protocols for the co-incubation and preincubation are described in Chart 1. Each value represents the mean of duplicate determinations.

only CYP2D2 but also other enzyme(s) that function as BF 1″-hydroxylase and that are relatively resistant to the inactivation by DMI.

In the plots in Fig. 3C using human CYP2D6, on the other hand, the inhibition type was the same, *i.e.*, competitive inhibition, for both co-incubation and preincubation. Furthermore, the K_i value calculated for the human recombinant enzyme was 1.7 μM, which was 5 to 6 times higher than that for the rat recombinant enzyme. This result supports the results in Fig. 2 showing that the inhibitory effect of DMI was much higher for BF 1″-hydroxylation by CYP2D2 than for that by CYP2D6.

Inactivation of CYP2D2 by DMI In order to further characterize the inactivation of CYP2D2 by DMI, the recombinant enzyme was preincubated with various concentrations of DMI in the presence of NADPH, and remaining enzyme activities were assayed. As shown in Fig. 4A, pseudo-first order kinetics were observed for the initial phase of the inactivation. Double reciprocal plots of the rate of inactivation of BF 1″-hydroxylase activity as a function of DMI concetration yielded K_{inact} and K_I values to be 0.19 min^{-1} and 0.76 μM, respectively (Fig. 4B).

Binding of DMI Metabolite(s) to CYP2D Enzymes [^{3}H]-DMI (10 μM, 3600000 dpm) was incubated with yeast cell microsomal fractions expressing CYP2D2 or CYP2D6 (50 pmol) in the presence of an NADPH-generating system. After the incubation, a portion of the reaction mixture was subjected to SDS-PAGE. Proteins in the gels were transblotted to a PVDF membrane and were analyzed by Western blotting using polyclonal antibodies (rabbit IgG) raised against CYP2D1. In Fig. 5, the upper panels A and B are for CYP2D2 and the lower panels C and D for CYP2D6. In the Western blot analysis of CYP2D2 incubated with radioactive DMI (Fig. 5 panel A-b), there was only one protein band whose molecular weight was 51 kDa on the PVDF membrane. Imaging analysis of the membrane using the BAS2000 system showed that the 51 kDa protein was the only protein band with considerable radioactivity (Fig. 5, panel A-c).

The localization of radioactivity was also confirmed by cutting the slab gel after SDS-PAGE and measuring the radioactivity in the gel strips by liquid scintillation counting. As depicted in Fig. 5 panel B, there was a prominent radioactive peak whose location coincided with that of CYP2D2. On

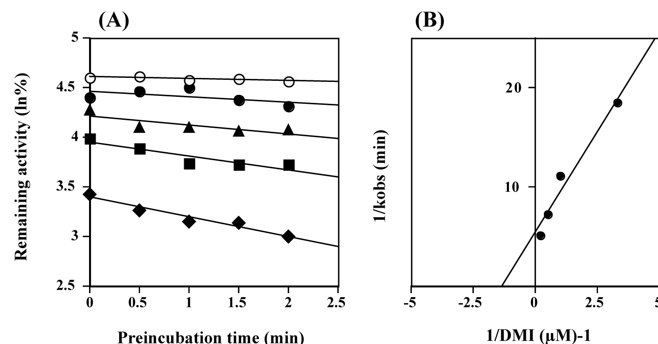

Fig. 4. Time- and Concentration-Dependent Loss of BF 1″-Hydroxylase Activity of CYP2D2 by Preincubation with DMI (A)

Incubation conditions were described under Materials and Methods. The concentrations of DMI were: open circles 0; closed circles 0.3 μM; closed triangles 1 μM; closed squares 2 μM; closed diamonds 5 μM. The reciprocal of first-order inactivation constants obtained from (A) and that of DMI concentrations were plotted (B), yielding the maximal rate of the inactivation (K_{inact}, 0.19 min^{-1}) and the inhibitor concentration required for the half-maximal rate of inactivation (K_I, 0.76 μM).

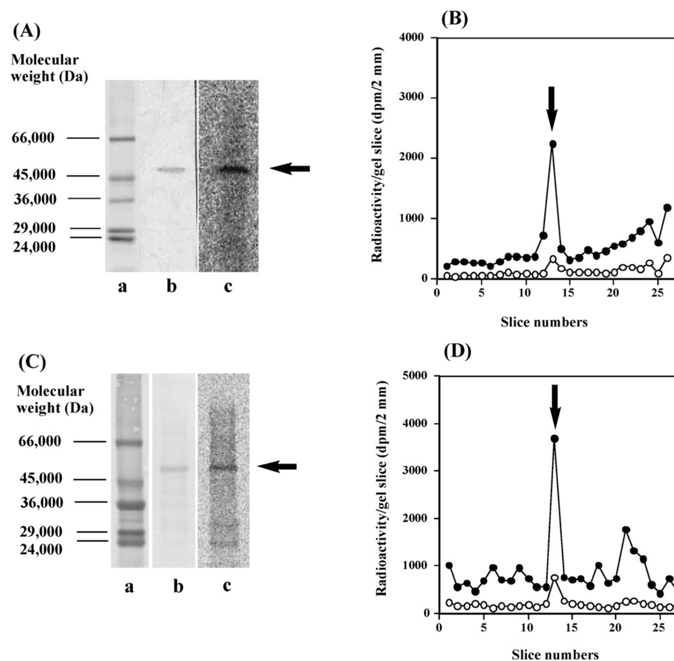

Fig. 5. Detection of Radioactive DMI Metabolite(s)-Bound Proteins by Western Blotting, BAS2000 Imaging Analysis and SDS-PAGE

The yeast cell microsomal fraction expressing rat CYP2D2 or human CYP2D6 (50 pmol each) was incubated with DMI under the conditions described in Materials and Methods. A part of the reaction medium was subjected to SDS-PAGE, and proteins in the gel were transblotted to a PVDF membrane. Proteins and their radioactivities were analyzed by Western blotting and using a BAS2000 image analyzer, respectively (left panels). Another part of the reaction medium was subjected to SDS-PAGE using a 10% slab-gel. The gel was cut into slices of 2 mm each, and their radioactivity was measured by liquid scintillation counting (right panels). Upper panels, rat CYP2D2; lower panels, human CYP2D6. The left panels: a, molecular weight markers; b, a PVDF membrane on which radiolabeled proteins were transblotted was analyzed by Western blotting; c, a scanning image of the PVDF membrane obtained using the BAS2000 imaging analyzer. The arrow shows the CYP2D proteins. The right panels: the sample was incubated in the presence (closed circles) or absence (open circles) of NADPH. Molecular weight markers; bovine albumin 66000; egg albumin, 45000; rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 36000; bovine carbonic anhydrase, 29000; bovine pancreas trypsinogen, 24000.

the basis of the radioactivity in the gel strip where CYP2D2 was localized, 33 pmol of radioactive metabolite(s) derived from DMI were calculated to bind to 50 pmol of CYP2D2. This means that DMI metabolite(s) were bound to about 70% of the enzyme during the incubation assuming that the binding mol ratio of the enzyme to the metabolite is 1. Results from the imaging analysis using the BAS2000 system also supported these conclusions (data not shown).

The results of binding experiments using human CYP2D6 were similar to those using rat CYP2D2 described above. In this case, 46 pmol of radioactive metabolite(s) derived from DMI were calculated to bind to 50 pmol of CYP2D6. However, in addition to the major peak of CYP2D6 protein, some radioactive peaks with molecular weights smaller than that of CYP2D6 were observed in Fig. 5 panel D, although they were not very prominent. BAS2000 imaging analysis also gave similar results (Fig. 5 panel C-c).

DISCCUSSION

Our previous studies suggested that rat hepatic microsomal CYP2D enzyme(s) were inactivated by IMI during its oxidative metabolism.[8] In the present study, we confirmed that DMI also inhibited BF 1″-hydroxylation by recombinant CYP2D2 as well as by rat liver microsomes. However, CYP2D1, another major CYP2D enzyme in the rat liver, was not markedly affected by DMI under the conditions used.

Interestingly, BF 1″-hydroxylation by human liver microsomes was not affected by DMI at 0.3 μM, which efficiently inhibited the same reaction by rat liver microsomes and recombinant CYP2D2. The human liver microsmal preparations ($n=3$) used in this study were examined for their CYP2D6 contents (17.6 ± 6.4 pmol/mg protein) and debrisoquine 4-hydroxylase activities (39.6 ± 14.6 nmol/min/mg protein) prior to starting the present study. These results indicate that the functions of CYP2D6 in the human liver microsomal fractions used in the present study were within normal levels. Our finding that BF 1″-hydroxylation by recombinant CYP2D6 was not inhibited by DMI at 0.3 μM but was inhibited at 3.0 μM also suggests that the sensitivity of human CYP2D6 to DMI is lower than that of rat CYP2D2. The difference in the inhibition constants (0.3 μM for CYP2D2 and 1.7 μM for CYP2D6) well supports this notion.

In BF 1″-hydroxylation by the rat enzyme, the inhibition-type of DMI was found to change from competitive under the co-incubation conditions to noncompetitive under the preincubation conditions. To further characterize the inhibition properties, CYP2D2 was preincubated for 0.5 to 2 min with various concentrations of DMI in the presence of NADPH, followed by the assay of BF 1″-hydroxylase activity. The loss of the activity was found to be kinetically pseudo-first-order and saturable, indicating that DMI is a mechanism-based inhibitor for BF 1″-hydroxylation by CYP2D2.

From the profile of the change in the enzyme activity following co-incubation (Fig. 2 b) and preincubation (Fig. 2 c), the activity of the preincubated microsomes from yeast cell expressing CYP2D2 tends to be lower than that of the co-incubated microsomes (Figs. 2B, C). It is reasonable to think that competitive inhibition is responsible for the decreased activity in the co-incubation, whereas mechanism-based inactivation is mainly responsible for the decreased activity in the preincubation. In contrast, the activity of preincubated microsomes expressing CYP2D6 is higher than that of co-incubated microsomes. This result suggests that in this case, mechanism-based inactivation does not occur or if occurs, to much lesser extent as compared to the case of CYP2D2, and competitive inhibition is mainly responsible for the decreased activity of co-incubated and preincubated microsomes. The phenomenon that the activity of the preincubated microsomes was higher than that of the co-incubated microsomes may be due to the consumption to some extent of DMI during the preincubation with microsomes and the cofactor.

In another experiment using human CYP2D6, preincubation time was prolonged from 2 min to 4 or 6 min, yielding similar results in which the activities in the case of preincubation were higher than those in the case of co-incubation (data not shown). Therefore, it is feasible that, compared to rat CYP2D2, human CYP2D6 is resistant to the inactivation by DMI.

The binding study demonstrated that radioactivity derived from [^{3}H]-DMI bound to CYP2D2. Taking the results of the binding study into account, it is reasonable to think that some reactive metabolite(s) might be formed by CYP2D2 during the preincubation and bind to the enzyme, probably to an amino aid residue which is located in the active site of the enzyme, resulting in the inactivation of the enzyme.

It is noteworthy that in contrast to the results obtained with the rat enzyme, the human enzyme was not inactivated by preincubation with DMI and NADPH, though considerable radioactivity derived from [^{3}H]-DMI was shown to bind to the human enzyme. It is possible that, similarly to the case of the rat CYP2D2, some reactive metabolite(s) are formed from DMI by human CYP2D6 and bind to the enzyme. In this case, however, the binding of the metabolite(s) may not affect the function of the enzyme. It is unclear at present what species of reactive metabolite(s) of DMI and what amino acid residues of the enzymes are involved in the binding described above. Further studies proceeding in this laboratory will clarify these points in the near future.

In summary, the inhibitory effects of DMI on rat and human CYP2D enzymes were studied using BF 1″-hydroxylation as an index. BF 1″-hydroxylation was inhibited competitively when rat CYP2D2 was co-incubated with DMI and BF in the presence of NADPH and noncompetitively when the enzyme was preincubated with DMI and NADPH before the incubation with BF, whereas BF 1″-hydroxylation by rat CYP2D1 was not markedly affected under the same conditions. In contrast, BF 1″-hydroxylation by human liver microsomes and recombinant CYP2D6 was competitively inhibited by DMI in both the co-incubation and the preincubation. The loss of activity of rat CYP2D2 under the preincubation conditions followed pseudo-first-order kinetics. Binding experiments using the recombinant CYP2D enzymes revealed that CYP2D2 and CYP2D6 were the only prominent proteins to which considerable radioactive DMI metabolite(s) bound. These results indicate that DMI was metabolized by the CYP2D enzymes to reactive metabolite(s), which bind to CYP2D proteins, resulting in the inactivation of rat CYP2D2 but not human CYP2D6.

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