The cytochromes P450 (P450s) represent a superfamily of the enzymes, and the families CYP1, -2, and -3 seem to have evolved to metabolize xenobiotics such as drugs, carcinogens, and environmental chemicals (Gonzalez, 1988; Guengerich, 1992; Wrighton and Stevens, 1992; Nelson et al., 1996). The large multiplicity, different overlapping substrate, product specificity and heterogeneous distribution of human P450 forms require methods for determining their tissue content and contribution to the metabolism of specific drugs and other xenobiotics. The interaction and contribution of individual P450s to the metabolism of clinically used drugs and drug candidates can be crucial factors in predicting drug efficacy, drug interaction, and drug toxicity. The quantitative contribution of a single P450 isoform to the metabolism of a given drug in human tissues can be used to predict the fraction of the substrate that is metabolized by a particular P450 (f_m). The value is of considerable importance in the prediction of drug metabolism, pharmacokinetics in vivo (Rodrigues et al., 2001). Inhibitory monoclonal antibodies (MAbs) to individual P450s have been widely used in recent years as a unique tool for investigating the properties and molecular diversity of P450 enzymes and for qualitative and quantitative identification of P450s involved in drug metabolism (Park et al., 1986; Gelboin, 1993; Halpert et al., 1994; Gelboin et al., 1995, 1997, 1999; Lin and Lu, 1997; Wang and Lu, 1997; Yang et al., 1999; Mei et al., 1999, 2000; Sai et al., 2000; Shou et al., 2000). Although the MAbs inhibition approach for P450 enzymes is widely accepted, little is known about the kinetic parameters and the applications of the MAbs in qualitative and quantitative examination of P450s involved in drug metabolism.

The value is of considerable importance in the prediction of drug metabolism, pharmacokinetics in vivo (Rodrigues et al., 2001). Inhibitory monoclonal antibodies (MAbs) to individual P450s have been widely used in recent years as a unique tool for investigating the properties and molecular diversity of P450 enzymes and for qualitative and quantitative identification of P450s involved in drug metabolism (Park et al., 1986; Gelboin, 1993; Halpert et al., 1994; Gelboin et al., 1995, 1997, 1999; Lin and Lu, 1997; Wang and Lu, 1997; Yang et al., 1999; Mei et al., 1999, 2000; Sai et al., 2000; Shou et al., 2000). Although the MAbs inhibition approach for P450 enzymes is widely accepted, little is known about the kinetic parameters and the applications of the MAbs in qualitative and quantitative examination of P450s involved in drug metabolism.

INHIBITION KINETICS OF MONOCLONAL ANTIBODIES AGAINST CYTOCHROMES P450

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ABSTRACT:

Monoclonal antibodies (MAbs) inhibitory to individual cytochromes P450 (P450s) are of tremendous utility in identification of P450s responsible for the metabolism of a given drug or drug candidate in pharmaceuticals. In the present study, two inhibitory MAbs against CYP2D6 (MAb2D6-50, IgG2b, and MAb2D6-184, IgG2a) were developed by hybridoma technology to exhibit their high specificity and potency. The MAbs were further employed to assess the quantitative role (47–93%) of CYP2D6 to the metabolism of bufuralol in human liver microsomes from seven donors. Together with the MAb inhibitory to CYP3A4 as previously reported (Mei et al., 1999), the MAbs were used to study the inhibition kinetics of dextromethorphan O-demethylation (CYP2D6), testosterone 6β-hydroxylation (CYP3A4) and aflatoxin B1 3-hydroxylation (CYP3A4), respectively, with an adequate size of sample measurement. A kinetic model was proposed to fit the experimental observations with three-dimensional nonlinear regression, thereby resulting in a solution of kinetic parameters, i.e., K_I, V_max, a, and b (changes in K_I or K_S and V_max in the presence of the MAb). As a result, dissociation constants (K_I) of the MAbs for the enzymes and the maximal inhibition (b) values for the P450-catalyzed reactions were predicted to have 0.04 to 0.25 μM and ≈94%, respectively. The results have demonstrated that the model can accurately predict the kinetic parameters and provide some insights into the understanding of the mechanism of MAb interaction with P450 enzyme in nature and the applications of the MAbs in qualitative and quantitative identification of P450s involved in drug metabolism.

1 Abbreviations used are: P450, cytochrome P450; OR, cytochrome P450 oxidoreductase; MAb, monoclonal antibody; KPI, potassium phosphate buffer; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; RSS, residual sum of square.

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Chemicals. The following chemicals were obtained from commercial sources: (±)-4-hydroxymphenytoin, (S)-mephenytoin, dextromethorphan,
Preparation of Human P450s. Plasmids containing the full-length cDNAs for P450s 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 and P450 OR were provided by Dr. Frank J. Gonzalez, National Cancer Institute. The entire coding region of each cDNA was excised from the vectors by digestion with respective endonucleases and inserted into baculovirus shuttle vector, pBlueBac 4.5 (Invitrogen), downstream of the polyhedron promoter. Recombinant virus was constructed according to the manufacturer’s procedure and was isolated using Blue-Gal color selection. After plaque purification, the recombinant baculoviruses were propagated in Spodoptera frugiperda (Sf21) cells to generate high-titer virus stocks for protein expression. Sf21 insect cells (Invitrogen) were grown at 27°C in complete SF90-SFM II (Invitrogen) to a density of 1 to 2 x 10^6 cells/ml in 1-liter spinner flasks (Bellico Glass, Inc., Vineland, NJ) or 2- or 5-liter Bench-Top Fermentor (B. Braun Biotech International, Allentown, PA) with enlarged blades at 90 rpm. Cells were infected at approximately 1.0 multiplicity of infection of virus encoding individual P450s and 0.1 to 1 multiplicity of infection of virus encoding OR (Shou et al., 1999). One microgram of hemin/ml of medium in the form of a hemin-albumin complex was added. After 72 h, cells were harvested by centrifugation, resuspended in 20% glycerol in 0.1 M KPi (potassium phosphate buffer, pH 7.4), and stored at -70°C until microsomal preparation. The total P450 content was measured by the CO-difference spectrum at 450 nm. Microsomes were prepared as described below, and the resulting protein concentration was determined by bicinchoninic acid assay according to the manufacturer’s directions (Pierce Chemical Co., Rockford, IL). The activities of individual P450s coexpressed with OR were determined by the assays as described elsewhere (Mei et al., 1999).

Microsomal Preparations. Normal liver specimens were provided by the National Cancer Institute Cooperative Human Tissue Network (Philadelphia, PA). Microsomes from Sf21 cells and from human livers were prepared by two centrifugation steps (9,000g and 105,000g) and were reconstituted in a buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, 1.15% KCl, and 0.1M KPi and stored at -70°C until used. Sf21 cell microsomes containing individual P450s with or without OR were used as a source of enzyme for metabolism studies and as immunogens for MAb production, respectively.

Procedure for MAb Development. The experimental procedures were described as previously reported (Mei et al., 1999). Three female BALB/c mice were immunized intraperitoneally with 50 µg of Sf21 cell microsomes containing baculovirus-expressed CYP2D6 protein emulsified in 0.2 ml of complete Freund’s adjuvant (first immunization), followed by two booster injections with incomplete Freund’s adjuvant on the 10th and 20th days. Three days after the third injection, splenocytes of the mouse were obtained for fusion with mouse myeloma cells P3/NS1/Ag4-1 (NS-1). Fusion of the spleen cells with the NS-1 cells was performed in the presence of polyethylene glycol 5000, and the fused cells were plated in 96-well plates at a density of 1 x 10^5 cells per well and grown in RPMI 1640 medium supplemented with 1% hypoxanthine/aminopterin/thymidine and 20% fetal bovine serum. The plates were examined daily for hybridoma growth. Two weeks later, when hybridomas approached confluence, media were tested with ELISA using baculovirus-expressed CYP2D6 as antigen (0.1 µg/ml). Positive clones were selected by comparison with microsomes of the cells infected with wild-type baculoviruses. Selected hybridomas were transferred to 24-well plates for further growth until confluence, after which media were further tested by ELISA, and inhibitory activity in CYP2D6-catalyzed bufuralol 1'-hydroxylation was examined by HPLC or LC-MS for assurance of the desired MAbs excreted from the selected hybridomas.

Production of Mouse Ascites Containing the MAbs. The MAb-forming hybridomas (1 x 10^5 cells/mouse) were injected intraperitoneally into pristine-primed female BALB/c mice for production of concentrated MAbs. The ascites fluid was built up and withdrawn 2 to 3 weeks after inoculation and stored at -70°C. Concentration of IgG and total protein of the pooled ascites was measured by Ouchterlony immunodiffusion and bicinchoninic acid assay methods, respectively, according to manufacturer’s instruction.

Isotyping of Mouse Ig. Isotyping of MAbs was conducted by Ouchterlony immunodiffusion using the mouse monoclonal antibody typing kits containing anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgM from Binding Site, Inc. (Birmingham, AL).

MAb Inhibition of P450-Catalyzed Metabolism. To titrate the inhibitory activity of the MAbs, a typical 1-ml incubation containing MAbs (MAB2D6, 50) and MAB2D6, 60 (MAB2D6, 60) at a concentration range of the IgG (0–1.92 µM) and 15 or 30 pmol of recombinant CYP2D6 in 0.1 M KPi was carried out. The mixture was preincubated at room temperature for 5 min, and the reaction was initiated by the addition of 1 mM NADPH and substrate (bufuralol or dextromethorphan) in a final volume of 1 ml and incubated at 37°C for 10 to 30 min. To determine cross-inhibition of the MAbs with other P450s or to assess the contribution of CYP2D6 to the metabolism of a substrate in human liver microsomes, 5 µl of ascites (or diluted ascites containing inhibitory MAbs) was added to 950 µl of 0.1 M KPi containing 10 to 50 pmol each recombinant P450 or 50 to 100 pmol of total P450 present in human liver microsomes. The reaction was initiated by the addition of respective substrate (Table 1) and 1 mM NADPH in a total volume of 1 ml and incubated at 37°C for 15 to 30 min, depending on substrates used. Incubations were terminated by the addition of 6 volumes of dichloromethane and corresponding internal standards (Table 1). The remaining substrate and metabolites formed were extracted and centrifuged for 10 min (500g). The organic phase was evaporated to dryness under a stream of nitrogen. The residues were analyzed immediately by reverse-phase HPLC (HP1100) or LC-MS (API-150; PerkinElmer Life Sciences, Boston, MA). Assays for all individual P450-catalyzed reactions were described in the previous report (Mei et al., 1999; Sai et al., 2000).

LC-MS Analysis. To screen hybridoma clones in 96-well plates that produce MAbs inhibitory to CYP2D6 activity, bufuralol 1'-hydroxylation was analyzed by LC-MS. Bufuralol and its metabolite (1'-OH bufuralol) were analyzed using a PerkinElmer HPLC system and separated on a SB-C18 column (3.5 µm particle; 4.6 x 50 mm; MAC-MOD Analytical, Chadds Ford, PA) eluted with a mobile phase consisting of solvent A (90% water:10% MeOH: 0.1% trifluoroacetic acid) and solvent B (10% water:90% MeOH:0.1% trifluoroacetic acid). Samples were eluted with a 1.9-min linear gradient from 100% solvent A to 50% solvent B for bufuralol and its metabolite (flow rate at 1.5 ml/min). Metabolite and internal standard were identified using an API 150MCA mass spectrometer in the positive ion mode (m/z of 278.1, 1'-OH-bufuralol, and 260.2, trans-propanol, an internal standard). Percentage of control was obtained by a comparison between the presence and absence of the MAbs.

TABLE 1

<table>
<thead>
<tr>
<th>P450</th>
<th>Substrate</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>6β-OH-Testosterone</td>
</tr>
<tr>
<td>3A5</td>
<td>Testosterone</td>
<td>6β-OH-Testosterone</td>
</tr>
<tr>
<td>3A7</td>
<td>Testosterone</td>
<td>6β-OH-Testosterone</td>
</tr>
<tr>
<td>1A1</td>
<td>Phenanthrene</td>
<td>9,10-Dihydrodiol</td>
</tr>
<tr>
<td>1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin</td>
<td>7-OH-Coumarin</td>
</tr>
<tr>
<td>2B6</td>
<td>Diazepam</td>
<td>Nor Diazepam</td>
</tr>
<tr>
<td>2C8</td>
<td>Flurbiprofen</td>
<td>4'-OH-Flurbiprofen</td>
</tr>
<tr>
<td>2C9</td>
<td>Flurbiprofen</td>
<td>4'-OH-Flurbiprofen</td>
</tr>
<tr>
<td>2C19</td>
<td>(S)-Mephénytoin</td>
<td>4'-OH-Mephénytoin</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan</td>
<td>Dextromethorphan</td>
</tr>
<tr>
<td>2E1</td>
<td>Bufuralol</td>
<td>1'-OH-Bufuralol</td>
</tr>
<tr>
<td>3A4</td>
<td>Chlorozoxane</td>
<td>6-OH-Chlorozoxane</td>
</tr>
</tbody>
</table>

*Note: Table 1 provides specific assays for 12 individual P450s.*
Inhibition Kinetic Model. Each IgG molecule has two identical sites, capable of recognizing and binding to the antigen (P450). The antigen-combining site of an IgG is made up of elements from the V regions of the H and L chains (V_H and V_L). Therefore, one molecule of IgG binds to two P450 molecules with identical affinity. Scheme 1 comprises all possible combinations among MAb, enzyme, and substrate. ES is a product-forming complex that can break down to form product by a rate constant (k_p). The model postulates that MAb, even at saturating concentrations, may not completely inhibit enzyme activity (especially for some partially inhibitory MAbs), and, therefore, AES, EAES, and SEAES complexes are less productive than ES, in which the k_p may be changed by a factor, β. Similarly, the presence of the MAb or the substrate may change binding affinity of the enzyme for the substrate (K_S) or MAb (K_P) by a factor, α. Thus, a general model for the IgG-induced P450 inhibition kinetics is expressed in Scheme 1. Because one molecule of MAb (IgG) can bind to two enzymes with identical binding affinity, EAE, EAES, or SEAES is considered as 2 units of AE or AES. Therefore, velocity equation for product formation can be derived and simplified as shown below. The kinetic parameters can be solved to interpret the nature of the MAb-mediated P450 inhibition.

\[
v = \frac{v_0}{1 + \left(\frac{[Ab]}{IC_{50}}\right)^n}
\]

where [Ab] is MAb concentration; v and \(v_0\) are velocities in the presence and absence of the MAb; and n is the slope factor.

Results

Preparation of MAbs Specific to Human CYP2D6

Hybridoma cells were obtained by immunization of mice with the microsomal fraction recovered from Sf21 cells expressing human CYP2D6 followed by the fusion of mouse spleen cells with myeloma cells. Positive hybridoma clones were selected by ELISA for specific binding to CYP2D6 antigen in 96-well plates and were further screened for their inhibitory activities toward CYP2D6-catalyzed bufuralol metabolism, which were analyzed by LC-MS. Two hybridoma clones were found to produce MAbs (MAb 2D6_M and MAb 2D6_A) that are inhibitory to CYP2D6-catalyzed 1'-hydroxylation of bufuralol. The hybridoma cells were subsequently injected into mice for the preparation of ascites fluid. The murine immunoglobulin isotype of the MAb 2D6_M and MAb 2D6_A was identified as IgG2a and IgG2b, respectively, by the Ouchterlony immunodiffusion technique. The IgG concentrations of MAb 2D6_M and MAb 2D6_A in mouse ascites were measured to be 60 mg/ml (0.41 mM) and 71 mg/ml (0.48 mM), respectively, which represented majority of the protein in ascites fluid (> 90%).

Inhibition of CYP2D6 Activity

Mouse ascites containing MAb 2D6_M and MAb 2D6_A, respectively, was examined for inhibition of CYP2D6 activity in bufuralol 1'-hydroxylation (Fig. 1). Addition of ascites (0.41–0.48 μM) to a
1-ml incubation containing 10 to 15 pmol of CYP2D6, inhibited almost completely the metabolism of bufuralol (98%). The IC_{50} values for MAb 2D6–50 and MAb 2D6–184 were 0.076 \mu M (IgG) and 0.049 \mu M, respectively, for bufuralol 1'-hydroxylation (Fig. 1), and 0.040 and 0.021 \mu M, respectively, for dextromethorphan O-demethylation (Fig. 2). In addition, the MAbs also inhibited, to a lesser extent, the activity of CYP2D6*10 variant by 90% (Fig. 3).

Cross-Inhibition with Other P450s

To determine the specificity of the MAbs, marker assays for 11 other human cDNA-expressed P450s were examined (Table 1). No significant cross-inhibition toward any of the other 11 human cDNA-expressed human CYP1A1 (phenanthrene 9-hydroxylation), CYP1A2 (phenacetin O-demethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (diazepam N-demethylation), CYP2C8 (paclitaxel 6-\alpha-hydroxylation), CYP2C9 (flurbiprofen 4'-hydroxylation), CYP2C19 (S)-mephenytoin 4'-hydroxylation), CYP2E1 (chlorozoxazone 6-hydroxylation), CYP3A4 (testosterone 6\beta-hydroxylation), CYP3A5 (testosterone 6\beta-hydroxylation), and CYP3A7 (testosterone 6\beta-hydroxylation) was observed in the presence of 5 \mu l (2–2.5 \mu g) of mouse ascites (Fig. 3). Thus, the two MAbs were shown to be highly specific to CYP2D6.

Cross-Inhibition with P450s in Different Species

MAb 2D6–50 and MAb 2D6–184 were employed to examine cross-inhibition with bufuralol 1'-hydroxylase in liver microsomes from different species. The presence of each MAb partially inhibited bufuralol 1'-hydroxylase activity by approximately 91% (human), 65 to 75% (mouse and rat), 50% (rhesus monkey), and 15 to 50% (dog), respectively (Fig. 4).

Contribution of CYP2D6 to the Bufuralol 1'-Hydroxylation in Human Liver Microsomes

To assess the quantitative contribution of CYP2D6 to drug metabolism in human liver microsomes, inhibitory MAb 2D6–50 and MAb 2D6–184 were used to determine the metabolism of bufuralol in human liver microsomes from seven donors. Basal CYP2D6 activity in the human liver microsomes varied from 0.9 to 5.9 nmol/min/nmol. With the addition of the MAbs, bufuralol 1’-hydroxylation was inhibited by a range between 47 and 93%, depending on the liver donors (Fig. 5). These results suggest that other P450s, in addition to CYP2D6, also play a role in the metabolism of bufuralol. The inhibitory activity of the two antibodies in each sample was fairly consistent. Interestingly, one of the seven human liver microsomes (HL16)
exhibited the lowest CYP2D6 activity (0.9 nmol/min/nmol), and the MAbs only inhibited bufuralol $1'$/hydroxylation by 47%. This suggests that HL16 is probably a poor metabolizer, exhibiting a low level of CYP2D6 expression and activity.

**MAb Inhibition Kinetics**

**Dextromethorphan O-Demethylation.** MAb$_{2D6-184}$ (0.0–0.6 μM) and dextromethorphan (3.12–100 μM) were used to evaluate product formation (dextrorphan), providing 42 incubation data points corresponding to various combinations of concentrations. In the absence of the MAb$_{2D6-184}$, apparent $K_m$ and $V_{max}$ values for dextromethorphan were 7.5 μM and 17.3 nmol/min/nmol, respectively (Fig. 6). The addition of the MAb resulted in a negligible effect on the apparent $K_m$ and a large decrease in $V_{max}$ for dextromethorphan (Fig. 6), suggesting that the MAb-induced inhibition seems to be noncompetitive, although the MAb contains two-combining sites for the enzyme. According to the kinetic model (Scheme 1), experimental data were accepted to fit eq. 1 (Fig. 7). The scatter plot indicates actual measurements of the reaction rate ($z$-axis, vertical) of dextromethorphan ($x$-axis, lateral) in the presence of MAb$_{2D6-184}$ ($y$-axis, horizontal). The meshed plot represents predicted results, which yield estimates of kinetic parameters as shown in Table 2. The goodness of the fitting can be expressed by RSS (8.13) and $R^2$ (0.988), indicative of an excellent agreement of proposed model with actual measurements. The $K_S$ and $V_{max}$ in the model were calculated to be 7.8 μM and 17.5 nmol/min/nmol, respectively, similar to apparent $K_m$ (7.5 μM) and $V_{max}$ (17.3 nmol/min/nmol) observed in the absence of the MAb. However, $K_I$ value (0.055 μM), which represents dissociation constant of the MAb for CYP2D6, has been shown to be very low with respect to $K_m$ (or $K_S$). Because the presence of the MAb may alter both apparent $K_m$ and $V_{max}$ for the substrate, factors $\alpha$ and $\beta$, which represent maximal changes in $K_m$ and $V_{max}$ in the presence of the MAb, were taken into consideration. Factors $\alpha$ and $\beta$ in the study were predicted to be 0.99 and 0.05, respectively, implying that addition of MAb had no significant effect on the $K_S$ for the substrate ($\alpha$ = 1) but resulted in an inhibition of the maximal velocity ($V_{max}$) by 95%, similar to that observed ($\sim$98%).

**Testosterone 6β-Hydroxylation.** Varying concentrations of MAb$_{3A4}$ (0–0.1 μM) and testosterone (6.25–200 μM) were combined to generate 36 measurements for 6β-OH testosterone formation. Apparent $K_m$ and $V_{max}$ for testosterone 6β-hydroxylation in the absence of MAb$_{3A4}$ were determined to be 31.1 μM and 44.1 nmol/min/nmol, respectively (Fig. 8). However, the addition of the MAb did not significantly change apparent $K_m$ but dramatically decreased $V_{max}$ with regard to the increase of the MAb concentra-
tion (Fig. 8). The decrease in $V_{\text{max}}$ can be achieved by up to 6% of that in the absence of the MAb. The fitting of the actual data points to the kinetic model is shown in Fig. 9 and the predicted kinetic values that result in $K_m$ (30.4 μM), $V_{\text{max}}$ (43.8 nmol/min/nmol), $K_I$ (0.05 μM), $\alpha$ (0.41), and $\beta$ (0.06) are listed in Table 2, respectively. In comparison, the observed kinetic parameters were consistent with that predicted with the equation. The goodness of the fit to the data was stated by the RSS (16.7) and $R^2$ (0.996) values.

### Aflatoxin B1 3-Hydroxylation

Apparent $K_m$ (240.1 μM) and $V_{\text{max}}$ (64.1 nmol/min/nmol) for the 3-OH-aflatoxin B1 formation in the presence of the MAb 3A4 were determined as shown in Fig. 10. The changes in $K_m$ and $V_{\text{max}}$ with increasing MAb concentrations (Fig. 10) exhibited a similar pattern to that observed with testosterone 6β-hydroxylation. The prediction of kinetic parameters was also performed by the three-dimensional fitting of the experimental data to the model (Fig. 11; Table 2) and again displayed the results almost identical with the observed values. In comparison with the results of the three sets between predicted and observed values, the model seems to be reliable for use in predicting kinetic constants. The trend of the $K_m$ and $V_{\text{max}}$ changes for the MAb-induced inhibition exhibits a fashion similar to noncompetitive inhibition except for $\alpha$ and $\beta$ values in most cases ($\alpha < 1$ and $\beta \neq 0$; Table 2). The $\alpha$ values ($\alpha < 1$) obtained suggest that the binding of the MAb to the P450 enzyme can alter the binding affinity of the substrate ($K_m$) for the enzyme, and the $\beta$ values ($\beta \neq 0$) suggest that the triple-bound complexes of the MAb, enzyme, and substrate are catalytically active, which can break down to form product(s).

### Discussion

CYP2D6 is among the most well-characterized P450s and is the first P450 to be identified as a genetically polymorphic enzyme in the human population (Alvan et al., 1990; Kalow, 1991; Bertilsson et al., 1992; Greenblatt, 1993; Meyer, 1994; Kroemer and Eichelbaum, 1995; Gonzalez, 1996; Gaedigk et al., 1999; Ramamoorthy et al., 2001; Wan et al., 2001). Although CYP2D6 is expressed at a low concentration in tissues, it metabolizes a large array of clinically used drugs (i.e., antiarrhythmics, β-blockers, psychotropics, antihypertensives, neuroleptics, and analgesics) and contributes to more than 25% of the total drug metabolized by P450 (Benet et al., 1996). Approximately 5 to 10% of the Caucasian and 1% of the Asian populations carry the autosomal recessive trait for a poor metabolizer (Alvan et al., 1990; Kalow, 1991; Bertilsson et al., 1992; Greenblatt, 1993; Meyer, 1994; Kroemer and Eichelbaum, 1995; Gonzalez, 1996; Gaedigk et al., 1999; Ramamoorthy et al., 2001; Wan et al., 2001).
FIG. 11. Inhibition kinetics of MAbA44 in the metabolism of aflatoxin B1 by recombinant CYP3A4.

Scatter points were actual measurements of the experiment and meshed plot was a fitting of the kinetic model (eq. 1) with experiment data. Predicted kinetic parameters are shown in Table 2.

The in vitro studies demonstrated that addition of the MAb to the human liver microsomes containing multiple P450s in the metabolism of a given drug (Krausz et al., 1997; Gelboin et al., 1996; Andreassen et al., 1997; Sellers et al., 1997; Coutts and Urichuk, 1999). Because the P450s, in many cases, have overlapping substrate specificity with CYP2D6, it is necessary to measure the contribution of CYP2D6 to the total metabolism of a given drug (Krausz et al., 1997; Gelboin et al., 1999), and, thus, anti-CYP2D6 MAbs as a tool are extremely suitable for this purpose.

MAb3A4 and MAb3A6.184, which are inhibitory to CYP2D6 activity, are very suitable and of tremendous utility for identifying and quantifying CYP2D6 phenotype in the field of drug metabolism and drug-drug interaction. The two MAbs exhibited potent inhibitory activity in bufuralol 1′-hydroxylation and dextromethorphan O-demethylation by CYP2D6, resulting in 98% inhibition. The MAbs also inhibited, to a lesser extent (90%), activity of CYP2D6*10, a variant with lower overall CYP2D6 activity possessing Pro44Ser and Ser46Thr amino acid mutations, present in approximately 75% of Asians (Wang et al., 1993; Johansson et al., 1994). In addition, the MAbs did not display any significant cross-inhibition with any of the other 11-human recombinant CYP450s, suggesting that these MAbs are highly epitope specific. The advantages of the MAbs allow us to investigate selectively and quantitatively the role of CYP2D6 in human liver microsomes containing multiple P450s in the metabolism of a designated drug. The approach is to measure the inhibition of CYP2D6 activity for a specific reaction in a human tissue (i.e., human liver). The inhibition values represent the contribution of CYP2D6 to the metabolism of a drug. Our study has shown that when MAbs were added to the reaction, the bufuralol 1′-hydroxylation was inhibited by a range of 47 to 93%, depending on the individuals (Fig. 5). These results suggest that CYP2D6 contributes to the total bufuralol 1′-hydroxylase in human liver by 47 to 93%, and the remaining activity of the enzyme is attributed to other P450s (i.e., CYP2C19) (Man-kowski, 1999). Interestingly, HL16 exhibited a very low activity in the formation of 1′-OH bufuralol in which CYP2D6 played the least role in the reaction among all liver donors, suggesting the donor may express the CYP2D6 level lower than that observed in normal population. With regard to the cross-species of the MAbs, inhibition of bufuralol 1′-hydroxylase in liver microsomes from other species (mouse, rat, dog and rhesus monkey) showed that the two MAbs are poorer inhibitors of bufuralol 1′-hydroxylase than that of CYP2D6, although their sequence homology of the P450s in the subfamily is highly reserved in some species.

To better understand kinetic nature of MAb inhibition in P450-mediated reactions, a kinetic model of the MAb inhibition is needed. Because an IgG immunoglobulin has two identical binding domains for CYP2D6, the model (Scheme 1) was designed to account for all of the possible interactions among MAb, enzyme, and substrate. The velocity equation (eq. 1) was derived according to this model. The common features in kinetics are seen in Figs. 6, 8, and 10, showing that apparent K_m values remain statistically unchanged but V_max decrease with increase of the MAb concentration (multiple t tests). These behave as kinet in a similar fashion to a noncompetitive inhibition. However, using the model derived from noncompetitive inhibition to predict kinetic parameters of the MAb inhibition is inappropriate due to the existence of the two binding domains on an MAb molecule. Thus, taking this into consideration, a model in the present study was developed. The resulting equation was used to predict kinetic parameters. Our results have shown that after the data fitting, the predicted kinetic constants (K_m, V_max, and β) are comparable with those observed (K_m, V_max, and maximal inhibition). Factor α, which represents a change in K_m in the presence of the MAb, was predicted to be 0.99 for MAb3A6.184 and 0.41 to 0.48 for MAb3A4, respectively, suggesting that the addition of the MAP2D6.184 does not change the binding affinity of the enzyme for the substrates (α = 1) but the MAb3A4 increases the binding affinity of CYP3A4 for the substrates (α = 0.41–0.48). The latter is probably due to a conformational change in the enzyme bound with the MAb that alters the K_m for the substrate. Factor β, which reflects a change in V_max for the substrate when the MAb is introduced, was predicted to have all values less than 0.06 (Table 2), meaning that the presence of the MAb can inhibit V_max by at least 94% (inhibition potency). The model also predicts that the K_m values for the two MAbs (0.05–0.24 μM) are critical in the understanding of the MAb binding affinity for the enzyme. The kinetic model for MAb inhibition can help us to accurately estimate kinetic parameters and to better understand the nature of MAb (binding affinity and inhibitory potency) in P450-catalyzed reactions.

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References

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of cytochrome P450 2D6 (CYP2D6) phenotype assignment using a genotyping algorithm based on allel frequency data. *Pharmacogenetics* **9**:699–822.


