Role of a Potent Inhibitory Monoclonal Antibody to Cytochrome P-450 3A4 in Assessment of Human Drug Metabolism

QIN MEI, CUYUE TANG, CAROL ASSANG, YUH LIN, DONALD SLAUGHTER, A. DAVID RODRIGUES, THOMAS A. BAILLIE, THOMAS H. RUSHMORE, and MAGANG SHOU

Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania

Accepted for publication July 25, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Cytochrome P-450 (CYP) 3A4 is an inordinately important CYP enzyme that catalyzes the metabolism of a vast array of clinically used drugs. Microsomal proteins of Spodoptera frugiperda (S21) insect cells infected with recombinant baculoviruses encoding CYP3A4 cDNA were used to immunize mice and to develop a monoclonal antibody (mAb3A4a) specific to CYP3A4 through the use of hybridoma technology. The mAb is both a potent inhibitor and a strong binder of CYP3A4. One and 5 μM (0.5 and 2.5 μM IGp) of the mAb mouse ascites in 1-ml incubation containing 20 pmol of CYP3A4 strongly inhibited the testosterone 6β-hydroxylation and diazepam 3-hydroxylation activity. mAb3A4a exhibited no cross-reactivity with any of the other recombinant human CYP isoforms (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1) in the course of CYP reaction phenotyping and Western immunoblot analyses. The potency of mAb-induced inhibition is insensitive to substrate concentration in human liver microsomes. Therefore, mAb3A4a was used to assess the quantitative role of CYP3A4/5 to the metabolism of testosterone and diazepam in five human liver microsomes. The results showed that CYP3A4 and CYP3A5 contribute >95% to both testosterone 6β-hydroxylation and diazepam 3-hydroxylation and 52 to 73% to diazepam N-demethylation, respectively. In addition, mAb3A4a significantly inhibited testosterone 6β-hydroxylase activity in rhesus monkey liver microsomes to a degree equal to that observed with CYP3A4A in human liver microsomes. By comparison, no inhibition of testosterone 6β-hydroxylase activity was observed in the presence of dog, rat, and mouse liver microsomes. The selectivity of ketoconazole, a chemical inhibitor of CYP3A4, was probed with mAb3A4a and was shown to be highly concentration dependent in the diazepam N-demethylation by human liver microsomes. The results demonstrate that inhibitory and immunoblotting mAb3A4a can offer a precise and useful tool for quantitative identification of CYP3A4A/5 in the metabolism of drugs in clinical use and drugs in development.

The cytochrome P-450 enzymes (CYPs) represent the key enzyme system responsible for the oxidative metabolism of drugs and environmental chemicals, as well as for the metabolism of certain classes of endobiotics. (Gonzalez, 1988; Guengerich et al., 1991; Coon et al., 1992; Guengerich, 1992; Nelson et al., 1996). Because CYPs are present in multiple forms, with some expressed at very low concentrations in tissues, their substrate and product specificity often overlap. In addition, the levels of many CYPs change in response to environmental, hormonal, or nutritional influences or are determined by genetic polymorphism. These factors have greatly limited our understanding of the precise role of individual CYPs in the metabolism, activation, and detoxification of different substrates and have impeded our understanding of the relationship between CYP phenotype in various tissues and an individual's responsiveness and sensitivity to drugs. Thus, the development of reagents that can precisely define the role of a single member or a class of CYP in the metabolism of a given drug would be of tremendous benefit to the field of drug metabolism.

Monoclonal antibodies (mAbs) inhibitory to individual CYPs are ideally suited for investigation of the properties and molecular diversity of CYPs. mAbs are chemically pure reagents that recognize a single antigenic determinant or epitope on the surface of a CYP molecule, resulting in the inhibition of catalytic activity (Thomas et al., 1986; Gelboin, 1993; Ryan et al., 1993; Gelboin et al., 1995; Edwards et al., 1998; Ekins et al., 1998). Therefore, the measurement of CYP

ABBREVIATIONS: CYP, cytochrome P-450; OR, cytochrome P-450 oxidoreductase; mAb, monoclonal antibody; DDC, diethylidithiocarbamate; DZ, diazepam; TMZ, temazepam; NDZ, nordiazepam; B[α]P t-4,5-diol, benzo[α]pyrene trans-4,5-diisohydrodiol; BA t-5,6-diol, benz[a]anthracene trans-5,6-diisohydrodiol; m.o.i., multiplicity of infection; KPi, potassium phosphate buffer; ELISA, enzyme-linked immunosorbent assay; IgG (H + L), IgG (H chain + L chain).
inhibition in different tissues would allow one to estimate the contribution of a particular CYP to the metabolism of the compound in question. If a compound is metabolized by more than one CYP, the contribution of multiple CYPs in a tissue would be assessed by the coinoculation of several specific mAbs. Because mAbs can be produced in limitless quantity from hybridoma cells that combine the characteristics of the B-lymphocytes (commitment to the continuous and stable production of a specific antibody) with those of the myeloma tumor cells (imparted of immortal properties to the hybridoma cells), a library of inhibitory mAbs would be valuable for the characterization of the multiple forms of CYP involved in the metabolism of a drug and for the quantitation of respective CYP contributions to overall metabolism (Gelboin, 1993; Gelboin et al., 1995). In addition, binding activity of mAb can be used to determine the expression level of a specific CYP in various tissues with a variety of immunoassays (Shimada et al., 1994; Shou et al., 1998), including enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemical methods.

CYP3A4 represents one of the best studied members of the human CYP superfamily. Interest has focused on this CYP isoform due to its high abundance in human liver (Wrighton and Stevens, 1992); inducibility by numerous agents, including glucocorticoids and phenobarbital (Hostetler et al., 1987); and prominent role in the metabolism of a large number of clinically relevant drugs. Phenotyping of CYP3A4 in preclinical and clinical drug metabolism currently represents a major goal towards generating a full understanding of the pharmacological, toxicological, and pharmacokinetic consequences of drug metabolism, which has been aided in part by the development of an mAb that is specific and inhibitory toward CYP3A4. Although a hybridoma clone producing mAbs (mAb-A334, IgM) has been developed to inhibit the enzymatic activity of CYP3A4 probed with various substrates (Gelboin et al., 1995), these mAbs display only partial inhibition (45–90%) of most recombinant CYP3A4-mediated reactions (up to 100 μl ascites/ml incubation) and have no immunoblotting activity for CYP3A4 antigen. Furthermore, because the inhibitory cross-reactivity of the mAb with other subfamilies of CYP has not been investigated fully by using individual recombinant CYPs, or CYP-marker assays with human liver microsomes, the quantitative role of CYP3A4 in the metabolism of a given drug examined by the mAb could not be well characterized. In the present study, an mAb -bufuralol hydrochloride salt, and (45–90%) of most recombinant CYP3A4-mediated reactions (up to 100 μl ascites/ml incubation) and have no immunoblotting activity for CYP3A4 antigen. Furthermore, because the inhibitory cross-reactivity of the mAb with other subfamilies of CYP has not been investigated fully by using individual recombinant CYPs, or CYP-marker assays with human liver microsomes, the quantitative role of CYP3A4 in the metabolism of a given drug examined by the mAb could not be well characterized. In the present study, an mAb (mAb-A334, IgG2a) specific to CYP3A4 was developed to be both a very powerful inhibitor and a strong binder (Western blot) of the enzyme with no cross-reaction toward any of other CYP subfamily members. The results demonstrate that mAb-A334 can be used as a precise and sensitive probe for functional analysis of CYP3A4 in vitro studies of human drug metabolism.

Materials and Methods

Chemicals. (±)-4-Hydroxymephenytoin, (S)-mephenytoin, 6-hydroxychloroxazone, (±)-bufuralol hydrochloride salt, and (±)-1'-hydroxybufuralol maleate salt were obtained from Ultracefine Chemicals (Manchester, UK). Diazepam (DZ), temazepam (TMZ), nordiazepam (NDZ), flurbiprofen, chloroxazone, taxol, coumarin, 7-hydroxycumarin, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Testosterone, 6β-hydroxytestosterone, estrone, 2-hydroxyestrone, and 6β-hydroxyprogesterone were obtained from Steraloids Inc. (Wilton, NH). mAb-A334 (catalog no. A334; anti-CYP3A4 mAb), human CYP3A4 plus CYP oxidoreductase (OR) supernatants, 6-hydroxychloroxazone, and 6α-hydroxydiazepam were obtained from Genetest Corp. (Woburn, MA). Benzyl[α]pyrene trans-4,5-dihydrodiol (BioP t-4,5-diol), phenanthrene, 9-hydroxyphenanthrene, and benz[α]anthracene trans-5,6-dihydrodiol (BA t-5,6-diol) were obtained from the National Cancer Institute Chemical Carcinogen Repository (Kansas, MO). SF-9001 II serum-free medium, fetal bovine serum, and hypoxanthine/aminopterin/thymidine supplement were obtained from Life Technologies (Rockville, MD). 8-Asaguanine-resistant myeloma cell line NS-1 was obtained from Frederick Cancer Research and Development Center (Frederick, MD).

Preparation of Human CYPs. Plasmids containing the full-length cDNAs for CYPs 3A4, 1A1, 1A2, 2A6, 2D6, 2C8, 2C9, 2C19, 2D6, 2E1, and OR were provided by Dr. Frank J. Gonzalez of the National Cancer Institute. The entire coding region of each cDNA was excised from the vectors through digestion with respective enzymes and inserted into baculovirus shuttle vector pBlueBac 4.5 (Invitrogen, Carlsbad, CA), downstream of the polyhedrin promoter. Recombinant virus was constructed according to the manufacturer’s procedure (Gonzalez et al., 1991) and isolated using Blue-Gal for color selection of recombinant virus. After two runs of 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 SF900 serum-free medium (Life Technologies) to a density of 1 to 2 × 10⁶ cells/ml (400 ml in total) in 1-liter spinner flasks (Belco Glass, Inc., Vineland, NJ) with enlarged bladders at 90 rpm. Cells were infected at approximately 1.0 multiplicity of infection of virus encoding individual CYPs and 0.1–1 multiplicity of infection of virus encoding OR. Then, 1 μg hemin/ml medium in the form of a hemin-albumin complex was added. After 72 h, cells were harvested by centrifugation and resuspended in 20% glycerol in 0.1 M potassium phosphate buffer (KP) and stored at −70°C until microsomal preparation.

The total CYP content was measured by the CO-difference spectrum at 450 nm. Microsomes were prepared as described below, and the resulting protein concentration was determined with bicinchoninic acid according to the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL). The activities of individual CYPs coexpressed with OR were determined by the assays as indicated in Table 1.

Microsomal Preparations from Human, Animal Livers, and Sf21 Cells Expressing Individual CYPs. Normal liver specimens from five subjects aged 20 to 55 (two female and three male Caucasians) were provided by the National Cancer Institute Cooperative Human Tissue Network (Philadelphia, PA). Clinical information on the donors was provided, indicating the cause of death to be automobile accidents (three cases), coronary artery disease (one case), and respiratory failure (one case). The donors were nonsmokers and nonalcoholics, and their renal and hepatic function tests were normal. Microsomes from Sf21 cells and from human, rhesus monkey, dog, rat, and mouse livers were prepared by two centrifugation steps (9,000g and 105,000g), as described previously (Wang et al., 1983) 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 0.1 M KPi and stored at −80°C until used. Sf21 cell microsomes containing individual CYPs with and without OR were used as a source of enzyme for metabolism studies and as immunogens for mAb production, respectively.

Procedure for mAb Development. Three female BALB/c mice were immunized i.p. with 50 μg of Sf21 cell microsomes containing baculovirus-expressed CYP3A4 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 taken for fusion with nonsecreting myeloma cells P3/NS1-Ag4-1 (NS-1). Fusion of the spleen cells with the NS-1 cells was performed with PEG 5000, the fused cells were plated onto microtiter wells in 96-well plate at a density of 1 × 10⁴ cells/well, and
TABLE 1

<table>
<thead>
<tr>
<th>P-450&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Substrate</th>
<th>Concentration (nM)</th>
<th>mAb&lt;sup&gt;b&lt;/sup&gt; Concentration (μM/μl)</th>
<th>Internal Standard&lt;sup&gt;c&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>200</td>
<td>0.1</td>
<td>60-OH-Testosterone</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>3A5</td>
<td>Testosterone</td>
<td>200</td>
<td>0.1</td>
<td>60-OH-Testosterone</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>3A7</td>
<td>Testosterone</td>
<td>200</td>
<td>0.1</td>
<td>60-OH-Testosterone</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>1A1</td>
<td>Phenanthrene</td>
<td>300</td>
<td>0.1</td>
<td>9.10-Dihydrodiol</td>
<td>Shou et al. (1994)</td>
</tr>
<tr>
<td>1A2</td>
<td>Estrone</td>
<td>100</td>
<td>0.1</td>
<td>2-OH-Estrone</td>
<td>Shou et al. (1994)</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin</td>
<td>100</td>
<td>0.1</td>
<td>7-OH-Coumarin</td>
<td>Yun et al. (1991)</td>
</tr>
<tr>
<td>2B6</td>
<td>Diazepam</td>
<td>200</td>
<td>0.1</td>
<td>Norepinephrine</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>2C8</td>
<td>Taxol</td>
<td>25</td>
<td>0.1</td>
<td>6-OH-Taxol</td>
<td>Rahman et al. (1994)</td>
</tr>
<tr>
<td>2C9</td>
<td>Fluoribuprofen</td>
<td>20</td>
<td>0.1</td>
<td>4′-OH-Flurbiprofen</td>
<td>Tracy et al. (1995)</td>
</tr>
<tr>
<td>2C19</td>
<td>Mephenytoin</td>
<td>100</td>
<td>0.1</td>
<td>4′-OH-Mephenytoin</td>
<td>Goldstein et al. (1994)</td>
</tr>
<tr>
<td>2D6</td>
<td>Bufuralol</td>
<td>25</td>
<td>0.1</td>
<td>1′-OH-Bufuralol</td>
<td>Crespi et al. (1995)</td>
</tr>
<tr>
<td>2E1</td>
<td>Chlorozoxone</td>
<td>200</td>
<td>0.1</td>
<td>6-OH-Chlorozoxone</td>
<td>Peter et al. (1990)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Baculovirus-expressed individual P-450s were coexpressed with OR in SF21 insect cells as described in the text.  
<sup>b</sup> mAb concentration was expressed as μl of mouse ascites/ml incubation mixture.  
<sup>c</sup> Internal standard was used for metabolite quantitation.

Inhibitory and Immunoblotting mAbs against CYPs

Isotyping of Mouse Ig. Isotyping of mAbs was conducted by Ouchterlony immunodiffusion using the mouse mAb typing kits containing anti-mouse IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, and IgM from Binding Site, Inc. (Birmingham, AL).

mAb Inhibition of CYP-Catalyzed Metabolism. To determine the inhibitory activity of the mAb, 10 μl of ascites (or diluted ascites containing inhibitory mAbs) was added to 950 μl of 100 mM KPi (potassium phosphate buffer, pH 7.5) containing 10 to 50 pmol of each recombinant CYP or 50 to 100 pmol of total CYP from liver microsomes of humans, monkeys, dogs, rats, and mice. The mixture was vortexed gently and preincubated at room temperature for 5 min. The reaction was initiated by the addition of each substrate and NADPH (1 mM) in a total volume of 1 ml and incubated at 37°C for 15 to 30 min depending on the substrates used. Incubations were terminated by the addition of 8 volumes of dichloromethane and respective 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 residue was dissolved in a mixture of methanol or acetonitrile and water and analyzed immediately by reversed phase HPLC (HP1100; Hewlett Packard, Avondale, PA) or liquid chromatography-mass spectrometry for quantitation of the desired mAbs excreted from cloned hybridomas. Hybridoma cells producing inhibitory mAbs were subcloned at least three times, and their media were tested each time.

ELISA. ELISA was used to test the binding activity of hybridoma supernatants collected from hybridoma screening. Alkaline phosphatase-conjugated goat F(ab′)2 fragment to mouse IgG (H chain + L chain) [IgG (H + L)] was purchased from Cappel Laboratories (Durham, NC), and that to mouse IgG (<i>μ</i> chain specific) or to mouse IgM (<i>μ</i> chain specific) was purchased from Jackson ImmunoResearch (West Grove, PA).

Immunoblot Assay. To determine the specificity and binding activity of mAbs, 11 baculovirus-expressed CYPs and liver microsomes from human donors, rhesus monkeys, dogs, rats, and mice were subjected to SDS-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel for 2 h at room temperature. The residue was dissolved in a mixture of methanol or acetonitrile and water and analyzed immediately by reversed phase HPLC (HP1100; Hewlett Packard, Avondale, PA) or liquid chromatography-mass spectrometry for quantitation of the desired mAbs excreted from cloned hybridomas. Hybridoma cells producing inhibitory mAbs were subcloned at least three times, and their media were tested each time.

Hybridoma cells were obtained through immunization of mice with the microsomal fraction recovered from SF21 cells expressing human CYP3A4 (99% purity of CYP3A4) followed by the fusion of the mouse spleen cells with myeloma cells. ELISA assay was used to screen the positive hybridoma clones that were selected on a basis of the absorption difference at 405 nm between the microsomes in the presence and absence of CYP3A4. One hundred twenty-eight hybridoma clones, selected from approximately 500 wells that yielded positive antibodies by ELISA for specific binding to CYP3A4 antigen compared with those of baculovirus wild type microsomes, were chosen for examining their inhibitory activities toward CYP3A4-catalyzed testosterone metabolism. Of the 128 clones, one hybrid-
oma clone (mAbAαa) resulted in a potent inhibition of CYP3A4-catalyzed 6β-hydroxylation of testosterone (>93%). This clone was further subcloned at least three times to ensure monoclonality. Subsequently, the hybridoma cells that produced the mAbs were injected into mice for the preparation of ascites fluid. The murine Ig type of the mAbAαa was identified as IgG2α by the Ouchterlony immunodiffusion technique, and protein concentration of this Ig preparation was found to be 74 mg/ml mouse ascites.

Cross-Immunoreactivity by Western Blotting. Ascites mAbAαa was tested by immunoblotting for its specificity toward 11 baculovirus-expressed human CYPs (CYP3A4, CYP3A5, CYP3A7, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1) at a gel loading of 1 pmol. As shown in Fig. 1, mAbAαa exhibited a strong binding activity with CYP3A4 and, to a lesser extent, with CYP3A5 and CYP3A7. In the lane containing CYP3A4, two weak bands other than that of CYP3A4 were also recognized by mAbAαa, and these remain unclear. The sensitivity for CYP3A4 detection was 0.1 pmol. However, mAbAαa did not cross-react with any of the other eight CYPs tested. The results indicate that three human CYP3A isoforms share a common epitope (identical or similar) recognized by the mAbAαa that yields different responses in immunoblotting activity. Different binding activities may result from the unique structural features of the epitope on three individual CYP3As (conformational, sequential, or combination). Similarly, CYP3A4/5/6 proteins were also detected at a loading of 1 pmol of total CYP present in seven human liver microsomal preparations (Fig. 2). Thus, mAbAαa with Western blotting characteristics can be used to probe CYP3A4/5 in human tissue and CYP3A7 in fetal liver. To determine the cross-reactivity of the mAb, 5 and 20 pmol of a total CYP in the liver microsomes of monkey, dog, rat, and mouse was probed with the mAbAαa on Western blotting, respectively. The results showed that mAbAαa cross-reacted with CYP proteins contained in monkey liver but not with any of other species (Fig. 3).

mAbAαa Inhibition of CYP3A4/5/7-Catalyzed Metabolism. mAbAαa was examined for its inhibitory effect on CYP3A4-, CYP3A5-, and CYP3A7-catalyzed metabolism of two known substrates, testosterone and DZ. Figures 4 and 5 show the titration curve of mAb-mediated inhibition of metabolism versus the amount of mouse ascites. mAbAαa was found to be very inhibitory to all three CYP3A forms. As seen in Fig. 4, the addition of mAbAαa at 1 μl ascites/1-ml incubation of testosterone containing 20 pmol of each enzyme inhibited CYP3A4/5/7-catalyzed testosterone 6β-hydroxylation by 95, 52, and 58%, respectively, whereas at 10 μl/ml, metabolism was inhibited by 99, 90, and 90%, respectively. Similarly, mAbAαa (10 μl) inhibited the CYP3A4-mediated conversion of DZ to TMZ and NDZ by 99 and 98%, respectively (Fig. 5). In a comparative study, mAb-A334 (Genetest Corp.) was examined for its ability to inhibit CYP3A4-mediated 6β-hydroxylation of testosterone. As seen in Fig. 6, mAbAαa and mAb-A334 at 5 μl ascites/1-ml incubation inhibited CYP3A4 activity by 99 and 24%, respectively. When 80 μl ascites of mAb-A334 was used, only 79% inhibition was observed. These results demonstrate that mAbAαa is more effective than mAb-A334 as an inhibitor of testosterone 6β-hydroxylase activity.

Cross-Inhibition with Other Human CYPs. To confirm the specificity of the mAbAαa assays for individual cDNA-expressed CYPs were performed as shown in Table 1. Then, 10 μl of mAbAαa ascites was added to 1 ml of incubation with individual CYPs (15–30 pmol/incubation) to examine cross-inhibition. Figure 7 shows that mAbAαa strongly inhibited CYP3A4/5/7-catalyzed testosterone 6β-hydroxylation by 99, 93, and 94%, respectively, but did not significantly display any inhibitory effects toward cDNA-expressed human CYP-mediated phenanthrene 9,10-epoxidation (CYP1A1), estrone 2-hydroxylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), DZ-N-demethylation (CYP2B6), taxol 6a-hydroxylation (CYP2C8), flurbiprofen 4’-hydroxylation (CYP2C9), (S)-mephenytoin 4’-hydroxylation (CYP2C19), bufuralol 1’-hydroxylation (CYP2D6), and chlorzoxazone 6-hydroxylation (CYP2E1), respectively. In each case, enzyme activity in the
presence of mAb3A4a ranged from 86 to 108% of the corresponding values in the absence of the mAb. These results suggest that the CYPs in question may lack the epitope that is recognized by mAb3A4a.

The selectivity of mAb 3A4a toward CYP3A4 also was evaluated in human liver microsomal preparations (Fig. 8). The results showed that mAb3A4a almost exclusively inhibited testosterone 6β-hydroxylation (95–98%), suggesting that the 6β-position is an exclusive site catalyzed only by CYP3A isoforms present in human liver. In contrast to its effects on testosterone hydroxylation, mAb3A4a did not exhibit any significant effect on coumarin 7-hydroxylation (CYP2A6; 76–110% of control), flurbiprofen 4'-hydroxylation (CYP2C9; 87–111%), (S)-mephenytoin 4'-hydroxylation (CYP2C19; 80–104%), bufuralol 1'-hydroxylation (CYP2D6; 92–115%), and chlorzoxazone 6-hydroxylation (CYP2E1; 89–111%; Fig. 8). The minor inhibition exhibited by the mAb3A4a for some marker assays (up to 24%) may be attributed to the overlapping substrate specificity of CYP3A4 in human liver.

To determine whether mAb3A4a inhibition is dependent on substrate concentration ([S]), pooled human liver microsomes were incubated at varying concentrations of testosterone (relative to $K_m$). Table 2 shows that mAb3A4a inhibited strongly CYP3A4 activity (95–98%) but had no effect on the reactions catalyzed by other CYPs. This implies that the mAb-induced inhibition of testosterone 6β-hydroxylation was insensitive to [S] and $K_m$ at a saturating concentration of mAb3A4a. As a result, mAb inhibition has an advantage over competitive chemical inhibitors, wherein the inhibitory specificity and potency often depend on the [S] and $K_m$ of substrates studied.

**Cross-Inhibition in Different Species.** As shown in Fig. 9, mAb3A4a almost completely inhibited testosterone 6β-hydroxylase activity (94–98%) in liver microsomes of humans and rhesus monkeys; however, no inhibition was observed for dog, rat, and mouse liver microsomes, which is consistent with the specificity of mAb3A4a observed with immunoblotting assays (Fig. 3).

**Quantitative Assessment of CYP3A4/5 Contribution to Metabolism of Probe Substrates in Human Liver Microsomes.** Testosterone is known to be a prototype substrate for CYP3A. As mentioned above, mAb3A4a inhibited 6β-hydroxy-testosterone formation by more than 95% in all five microsomal preparations, indicating that CYP3A4/5 are essentially the sole CYP isoforms responsible for testosterone-6β-hydroxylation in human liver (Fig. 8). DZ is hydroxylated to TMZ mainly by CYP3A4/5 and N-demethylated to NDZ by multiple CYPs (e.g., CYP3A4/5, CYP2B6, and CYP2C8/9/19), as previously reported (Scheme 1). We used mAb3A4a and mAb2C (anti-CYP2C8/9/19 mAb) to assess the additive contribution of the CYP3A and CYP2C subfamilies to TMZ and NDZ formation in human livers. mAb2C, an inhibitory mAb developed recently in our laboratory, was derived from CYP2C9 immunogens in a similar way to the development of mAb3A4a, and at 20 µl/ml incubation, it potently inhibited the CYP2C8 (taxol 6α-hydroxylation)-.
CYP2C9 (flurbiprofen 4′-hydroxylation)-, and CYP2C19 (me-
phenytoin 4′-hydroxylation)-mediated reactions, respectively,
to an equal extent. The data indicated that mAb3A4a, at 20 μl of
ascites in 1-ml of liver microsomal incubation containing 100
pmol of CYP and 100 μM DZ, inhibited the conversion of DZ to
TMZ by more than 95% but that mAb2C had no apparent effect
on TMZ formation (Fig. 10). This indicated that CYP3A4/5 are
responsible for the 3-hydroxylation of DZ in human liver (reac-
tion specific). However, when the production of NDZ was mon-
itored, the reaction was inhibited partially by mAb3A4a (52–
73%) and mAb2C (9–37%; Fig. 11). Interestingly, additive
inhibitions were observed in each of five liver microsomal sam-
ples when both mAbs were used together, showing that 86 to
93% of the N-demethylation of DZ relied on both CYP3A and
CYP2C isoforms (Fig. 11). Thus, when a reaction is suspected to
be catalyzed by multiple CYPs, two or more mAbs can be coin-
cubated to evaluate the combined contribution of the enzymes
in question to the metabolism of a particular drug.

Use of mAb3A4a to Determine Selectivity of Ketocon-
azole. The N-demethylation of DZ to NDZ is known to be
catalyzed by various CYPs in human liver microsomes con-
taining multiple CYPs (Yang et al. 1998). As shown in Fig.
12A, mAb3A4a was optimized to block almost exclusively the
activity of recombinant CYP3A4 but inhibited only partially
those of the two microsomal preparations (L16 and L40) in
DZ oxidation to NDZ by 79 and 67%, respectively, suggesting
that 21 and 33% of remaining activities were attributed to
the CYPs other than CYP3A4 in human liver microsomes.
However, when ketoconazole was present in the metabolism,
the activities in both recombinant CYP3A4 and human liver
microsomes were ultimately eliminated with an increase in
concentration. These results demonstrate that the selectivity
of ketoconazole is highly dependent on the concentration
used.

Discussion

The correct assignment and quantitative contribution of
individual cytochrome CYP isoforms to particular metabolic
routes of a given drug represent an area of considerable
importance in the prediction of potential drug/drug interac-
tions. Many different strategies are currently used for the
identification of the CYP isoforms responsible for the bio-
transformation of therapeutic agents, including the wide-
spread exploitation of chemical inhibitors and CYP-probe
substrates (Halpert et al., 1994; Rodrigues, 1999). However,
there are some limitations in the use of the former approach,
predominantly in terms of the specificity of inhibitors, the over-
lapping substrate specificity of CYP, and the large multiplicity
of homologous CYP structures. One of the essential ques-
tions relating to CYP function concerns the ability to
accurately measure the contribution of each individual iso-
form or class of CYP to the metabolism of a designated drug
or candidate in different tissues.

mAbs are directed at single epitopes on the antigenic CYP.
The singularity of the epitope-mAb combination confers on
CYP3A4 structurally related peptide (21 amino acids) was forms. Recently, a rabbit antiserum derived from the cross-reacts with the closely related human CYP3A5/7 iso-

Hydroxylation catalyzed by recombinant CYP3A4 and by human, mon-
mAb3A4a-mediated inhibition of the metabolism of marker substrates by human liver microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration(^a)</th>
<th>% Control(^b) (+ mAb)</th>
<th>Substrate</th>
<th>Concentration</th>
<th>% Control(^b) (+ mAb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>(250) (\mu M)</td>
<td>1.97 ± 0.05</td>
<td>Bufuralol</td>
<td>(50) (\mu M)</td>
<td>115.2 ± 0.23</td>
</tr>
<tr>
<td>(CYP3A4/7)</td>
<td>75</td>
<td>2.60 ± 0.00</td>
<td>(CYP2D6)</td>
<td>15</td>
<td>111.9 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.55 ± 0.06</td>
<td></td>
<td>5</td>
<td>106.8 ± 14.5</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>(500) (\mu M)</td>
<td>104.7 ± 6.37</td>
<td>Diclofenac</td>
<td>(100)</td>
<td>108.3 ± 1.16</td>
</tr>
<tr>
<td>(CYP2E1)</td>
<td>100</td>
<td>103.6 ± 0.56</td>
<td>(CYP2C9)</td>
<td>30</td>
<td>113.1 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>119.8 ± 0.3</td>
<td></td>
<td>(10)</td>
<td>125.7 ± 0.15</td>
</tr>
<tr>
<td>(S)-Mephenytoin</td>
<td>(400) (\mu M)</td>
<td>99.5 ± 1.41</td>
<td>Phenacetin</td>
<td>(1000)</td>
<td>101.9 ± 1.75</td>
</tr>
<tr>
<td>(CYP2C19)</td>
<td>120</td>
<td>94.6 ± 4.33</td>
<td></td>
<td>(150)</td>
<td>107.5 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>96.4 ± 1.99</td>
<td></td>
<td>(25)</td>
<td>113.9 ± 0.91</td>
</tr>
</tbody>
</table>

\(^a\) Concentration of substrate used for metabolism.

\(^b\) Percentage of control was determined as the difference of activity values between the presence and absence of mAb3A4a.

Individual P-450 activities were probed with respective marker substrates.

The effect of mAb 3A4a (0, 5, and 15 \(\mu \text{g}\)) on testosterone 6β-hydroxylation catalyzed by recombinant CYP3A4 and by human, monkey, dog, rat, and mouse liver microsomes.

The mAb its high specificity and renders mAbs extraordinarily suitable for investigation of the properties and molecular diversity of CYPs (Gelboin, 1993). This specificity is retained throughout the lifetime of the cell and its progeny. In terms of its chemical purity, stability, precision, specificity, and reproducibility for limitless generations of hybridomas, mAbs are considered to be superior reagents to most chemical inhibitors and polyclonal antibodies. In conjunction with cDNA-expressed CYPs for which substrate and product specificity of an individual CYP are well defined, mAbs can be used successfully to measure the contribution of each individual form or class of CYP to the metabolism of an individual substrate (Gelboin et al., 1988). It is true, however, that various features of the protein immunogen may govern the mAb interaction with the various domains of CYP, as well as with the CYP active site. A limitation of mAb-directed methods exists when the mAb target epitope is common to a large number of other CYPs, especially for the CYPs within a subfamily. mAb3A4a is no exception in this regard in that it cross-reacts with the closely related human CYP3A5/7 isoforms. Recently, a rabbit antiserum derived from the CYP3A4 structurally related peptide (21 amino acids) was prepared in our department and shown to discriminate CYP3A4 from CYP3A5 in inhibition phenotype (Wang and Lu, 1997; Wang et al., 1999). This may provide a challenge in the development of peptide-designed mAbs that can be used to identify the subfamily members of CYP in in vitro metabolism (e.g., CYP3A and CYP2C subfamilies).

Due to the simplicity of use of chemical inhibitors that are commercially available, characterization of the catalytic specificity of individual CYP isoforms involved in drug metabolism has been facilitated greatly and has proved to be valuable. In comparison with mAbs, chemical inhibitors can be used with intact cells and in vivo, even with human subjects, thus making it possible to link a particular CYP with a specific toxicological or pharmacological response. However, many available chemical inhibitors used to target individual CYP forms are poorly or incompletely characterized. Indeed, some chemicals are no longer considered suitable for use as diagnostic probes due to their considerable overlapping inhibition for CYP isoforms. For example, disulfram is a selective inhibitor of human CYP2E1 (Guengerich et al., 1991) but was found to inhibit CYP2A6 (Yamazaki et al., 1992). α-Naphthoflavone is known to be a potent inhibitor for CYP1A2 but is also a good substrate and a modulator for CYP3A4, which stimulates (or sometimes inhibits) many of the CYP3A4-mediated reactions (Friedman et al., 1985). α-Naphthoflavone not only inhibits CYP1A1/2 (IC\(_{50}\) = 0.4–0.5 \(\mu \text{M}\)) but also is similarly effective against CYP2C8/9. Increasing the concentration of α-naphthoflavone to 10 \(\mu \text{M}\) also causes inhibition of CYP2A6 and CYP2B6. Although diethyldithiocarbamate (DDC) is used widely as a selective inhibitor of CYP2E1, DDC also inhibits CYP2C9 and CYP3A4 at a concentration required to inhibit CYP2E1 (Eagling et al., 1998). Thus, DDC cannot be used as a diagnostic inhibitory probe for CYP2E1. Studies on the overlapping selectivity of chemical inhibitors are reported frequently in the literature; the selectivity of chemical inhibitors must be fully characterized by using as many cDNA-expressed individual human CYPs as possible.

mAb-induced inhibition kinetics have been shown to exhibit mixed-typed inhibition in which a noncompetitive component predominates, resulting in little change in \(K_a\) and a marked change in \(V_{\text{max}}\) for many CYP-mediated oxidations (Sai et al., 1998). The \(K_a\) values are usually less than \(10^{-8} \text{ M}\), which suggest that mAbs exhibit an inhibition potency greater than those associated with most chemical inhibitors (\(10^{-3}–10^{-6} \text{ M}\)). The kinetic behavior of the mAb inhibition is

**Table 2**

mAb3A4a-mediated inhibition of the metabolism of marker substrates by human liver microsomes
unlikely to be similar to that of chemical inhibitors, most of which act via a competitive mechanism (Bourrie et al., 1996). A competitive inhibitor acts only to increase the apparent $K_m$ for the substrate but has no effect on $V_{max}$. The degree of inhibition exhibited by a competitive inhibitor depends on $[S]$, $[I]$, $K_m$, and $K_i$:

$$\% \text{ Inhibition} = \frac{[I]}{[I] + K_i \left( 1 + \frac{[S]}{K_m} \right)}$$

This disadvantage can be overcome by the use of a noncompetitive inhibitor. The inhibited velocity ($v_i$) by an mAb is always a constant fraction of $v_0$, regardless of the substrate concentration or the value of the $K_m$, as long as the enzyme is saturated by the mAb:

$$\% \text{ Inhibition} = \frac{[\text{Ab}]}{K_{\text{Ab}} + [\text{Ab}]}$$

Fig. 10. Assessment of the respective contributions of CYP3A and CYP2C to DZ 3-hydroxylation in liver microsomes of five organ donors.

Fig. 11. Assessment of the respective contributions of CYP3A and CYP2C to DZ $N$-demethylation in liver microsomes of five organ donors.

Scheme 1. Metabolic pathways of DZ catalyzed by CYP isoforms (Yang et al., 1998).
Therefore, mAbs, which function largely as noncompetitive inhibitors and are insensitive to $K_{i}$ and $[S]$, can be used as ideal reagents to accurately define the role of the CYPs responsible for drug metabolism (Table 2). In contrast to mAb, mechanism-based inhibitor requires metabolism by the target enzyme into intermediates or products that irreversibly inactivate the enzyme. Thus, the selectivity between the two inhibitors (mAb and suicide inhibitor) is highly based on the recognition of the inhibitor on the functional region of the specific enzyme that relates to the metabolism of drugs. To date, few mAbs have been obtained that achieve complete inhibition of their target enzymes, and many mAbs presently available cannot distinguish between highly conserved CYP subfamily members (Goldfarb et al., 1993; Gelboin et al., 1995). In the present study, the selectivity of mAb$_{3A4a}$ and ketoconazole on the conversion of DZ to NDZ catalyzed by multiple CYPs (e.g., CYP3A, CYP2C, and CYP2B6) present in human liver microsomes was investigated (Fig. 12, A and B). The results showed that mAb$_{3A4a}$ inhibited only the activity exhibited by CYP3A in the pool of multiple CYPs, and the remaining activities clearly reflected the contribution of the CYPs other than CYP3A in liver. However, the selectivity of ketoconazole was highly dependent on the concentration used. At higher concentrations, the activity of all CYPs in liver appeared to be inhibited (Fig. 12B). Thus, when ketoconazole is used to define a role of CYP3A in the metabolism of a suspect drug, it must be used carefully and other approaches may be helpful to guarantee and support the conclusion made.

mAb$_{3A4a}$ was shown to provide both a strong ELISA response and Western blot reaction with CYP3A4 antigen. The positive Western blot result indicates that the epitope on CYP3A4 recognized by mAb$_{3A4a}$ is of sequential dimension of the antigenic protein, which is required for the native conformation of the protein for its integrity as an antigen. Therefore, in addition to its potent inhibitory activity, mAb$_{3A4a}$ can be beneficial as a sensitive probe for the quantification of the specific CYP3A in human tissue with a limit of detection of 0.1 pmol of CYP3A4 on Western blotting using alkaline phosphatase-conjugated anti-mouse IgG (H + L).

In the present study, microsomes of Sf21 cells containing cDNA-expressed CYP3A4 were used directly as immunogens for mouse immunization and mAb screening by ELISA in the production of mAb$_{3A4a}$. Antibodies against CYP3A4 were selected easily by subtracting the absorption at 405 nm raised from nonspecific antibodies generated by native microsomal proteins. Thus, impure antigen preparations as both immunogen and cloning components of the hybridoma technology were allowed in the isolation of the desired clones specific for a single epitope. The technique can be used to obtain mAbs for CYP proteins present at low concentrations or those that may be minor deviants of the primary protein in the immunogen preparation. Our study indicated that solubilized microsomes containing a low portion of immunogenic proteins are useful for immunization of mice and for screening and detecting the hybridomas producing the desired mAb. Thus, the previously reported procedures for the purification of large amounts of CYP proteins for the isolation of the mAbs are no longer necessary (Gelboin et al., 1995).

Although an inhibitory mAb-A334 specific to CYP3A4/5 has been developed (Gelboin et al., 1995), its cross-reactivity with other individual CYPs has not been fully characterized. Eighty microliters of mAb-A334 mouse ascites/1-ml incubation inhibited CYP3A4-mediated testosterone-6β-hydroxylatation by 79%, similar to the value observed with 0.5 μl of mAb$_{3A4a}$ ascites (82%). The weakness of inhibitory activity and the lack of binding activity on Western blotting could be attributed to the recognition site of the mAb on the enzyme, the Ig type, and mAb concentration in the ascites. mAb-A334 was isotypated as IgM, an unusual Ig in response to a secondary immunization (usually IgG type), and was negative in immunoblotting. mAb-A334, when applied to in vitro metabolism experiment, was found not to achieve a complete inhibition (usually about 45–90% inhibition) for substrates of divergent molecular weights (Gelboin et al., 1995). In contrast, mAb$_{3A4a}$ in ascites form (74 mg IgG$_{2a}$/ml) had 100-fold higher concentration than that of mAb-A334 (0.69 mg IgM/ml) as determined by immunodiffusion assay, suggesting that the nature and characteristics in mAb production between the two hybridoma cell lines differed considerably. It has been reported that several immunochemical and functional properties of mAb depend on the isotype which can limit the practical value. In contrast to IgG antibodies, IgM molecules exhibit little or no affinity for protein A antigen that is used in the selection of antibody purification (Ey et al., 1978). The difference in affinity between IgG and IgM may be
due to the structure of the heavy-chain isotype (γ chain for IgG and μ chain for IgM). In the present study, mAb-A334 (IgM) did not provide an immunoblot for CYP3A4 (Gelboin et al., 1995), suggesting that the inhibitory capacity of the mAb can be restricted by its weak binding characteristics reflected by \( K_I \) for the E-mAb complex and/or by the complex of the S-E-mAb that is productive (0 < \( \beta < 1 \)). In addition, the discrepancy in inhibitory activity can be caused by the actual immunoglobulin concentration between mAb-A3a4a and mAb-A334 in ascites fluid. Hybridomas secreting low titer of antibody may be limited to their use as a sensitive reagent to obtain the desired biological assessment.

mAbs are not necessarily monospecific and may cross-react extensively (Gelboin, 1993; Goldfarb et al., 1993). Cross-reacting mAbs reveal truly shared antigenic determinants on the surface of different antigens, especially for proteins with a highly conserved homology. Thus, specificity of the mAb must be determined carefully. As examined, mAb-A3a4a cross-reacts with CYP3A5/7, which are closely similar to CYP3A4 (84 and 88% sequence identity, respectively), but did not cross-react in either inhibition or immunoblotting with any of the other sub-families. mAb-A3a4a that cross-reacts with CYP3A7 is beneficial for the identification of CYP3A7 in human fetal liver in which CYP3A7 is the sole CYP3A subfamily member. The cross-reactivity of mAb-A3a4a with CYP3A5 can be used to quantitatively assess the total contribution of members of the CYP3A subclass to in vitro drug metabolism, although the expression level of CYP3A5 in liver is insignificant. Additionally, mAb-A3a4a immunoblotted microsomal CYPs and strongly inhibited testosterone 6β-hydroxylase activity present in monkey but not in dog, rat, or mouse. The cross-reactivity of mAb-A3a4a in closely related species probably is attributed to the highly conserved CYP3A sequences between humans and monkey, in which CYP3A4 exhibits 93% identity of amino acid sequence with CYP3A8 (monkey) but, to a lesser extent, 80% with CYP3A12 (dog), 73 and 72% with CYP3A12/1 in rat, and 73% with CYP3a11 in mouse. Thus, mAb-A3a4a directed against one CYP3A4 generally cross-reacts not only with other forms (CYP3A5 and CYP3A7) in the same species but also with corresponding CYP3A forms in monkey. Consequently, the cross-reactivity of the mAb with other species can provide an additional use in the identification of CYP3As.

Our study describes methods that have general applications for determining the contribution of a single CYP to drug metabolism. The inhibitory mAb-A3a4a to human CYP3A4 is a precise reagent for the quantitative assessment of the role of CYP3A in the metabolic route of drugs and for the determination of whether a regioselective or stereoselective oxidative reaction of a compound can serve as a probe reaction for CYP identification. The results indicated that testosterone 6β-hydroxylation and D3 3-hydroxylation are reaction selective (>95% inhibition) for CYP3A4, whereas measurements of D3 N-demethylation do not represent a precise approach for assaying CYP3A4 (52–73%) in human liver because the reaction is also attributed to other CYPs. Thus, mAb-A3a4a is a precise tool, not only for quantifying CYP contribution but also for identifying whether a suspect compound can be used as a CYP marker in human tissue.

In conclusion, mAb-A3a4a is uniquely suited for reaction nontyping (i.e., determining the contribution of epothe-specific CYP3A to metabolic reactions and for immunounquantification of CYP3A expression in human tissues). In addition, the cross-reactivity of mAb-A3a4a with other CYP3As in both inhibitory and Western blotting activity can be very useful for the study of CYP3A7 in human fetus and of CYP3A in monkey. Clearly, the use of mAbs is emerging from an infant stage and, with further maturation, is likely to become one of the most powerful tools for the study of CYP structure and function, as well as for drug development and discovery in pharmaceutical industries.

References

Send reprint requests to: Dr. Qin Mei, Department of Drug Metabolism, Merck Research Laboratories, WP75A-203, West Point, PA 19486. E-mail: qin.mei@merck.com