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INHIBITORY AND NON-INHIBITORY MONOCLONAL ANTIBODIES TO HUMAN CYTOCHROME P450 3A3/4

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Abstract—Cytochromes P450 3A3/4 are inordinately important P450 enzymes catalyzing the metabolism of a large variety of clinically useful drugs, steroids, and carcinogens. Two monoclonal antibodies, MAb 3-29-9 and MAb 275-1-2, were prepared to human P450 3A4 from mice immunized with baculovirus-expressed human P450 3A4. MAb 3-29-9 was a powerful inhibitor of the enzymatic activity of P450 3A3/4/5. MAb 3-29-9 inhibited the P450 3A3, 3A4, and 3A5 catalyzed metabolism of substrates of divergent molecular weights, e.g., *p*-nitroanisole, phenanthrene, diazepam, testosterone, taxol, and cyclosporin. However, MAb 3-29-9 did not give a western blot with P450 3A3 or 3A4. MAb 275-1-2 was non-inhibitory but yielded a strong western blot with P450 3A3 and 3A4 but not with 3A5, and thus distinguished between 3A3/4 and 3A5. The two MAbs did not cross-react with human 2E1, 1A2, 2B6, 2C8, and 2C9; rat 2A1, 3A1/2, 4A1, 4A3, and 2B1; and mouse 1A1 and 1A2. MAb 3-29-9 has been used successfully to measure the quantitative contribution of P450 3A3 and 3A4 to the metabolism of the above-designated substrates in human adult liver. MAb 3-29-9 and MAb 275-1-2 are precise and sensitive reagents for P450 3A studies.

Key words: human P450 3A4; inhibitory monoclonal antibodies; western blot; *p*-nitroanisole; phenanthrene; diazepam; taxol; cyclosporin; testosterone

Cytochromes P450 3A3/4 are inordinately important members of the P450 class of enzymes. P450 enzymes are the primary instruments of the metabolism of xenobiotics and several classes of endobiotics. These include: drugs, carcinogens, mutagens, pesticides, sundry environmental chemicals, and steroids [1, 2]. Human P450 3A3/4 assumes an especially important place among the human P450s in that it shows broadly based function in the metabolism of a large variety of drugs, steroids, and carcinogens [3]. This study reports the isolation of an MAb§ made to human P450 3A4, MAb 3-29-9, which strongly inhibited the enzyme activity of human P450 3A3/4/5. A second MAb, MAb 275-1-2, was non-inhibitory and yielded a strong western blot with P450 3A3/4 but not with P450 3A5.

The P450s exist in multiple isozymic forms that direct the metabolic flow of individual substrates. Thus, P450s are a paradigm for multi-isozymic systems whose activity may result in metabolic products with opposing physiological and pathological consequences. The large multiplicity of P450 forms, their differing structures and functions and, in many cases, their poorly defined substrate and product specificity, and their heterogeneous distribution *in vivo* make difficult the determination of the quantitative contribution of each P450 to the metabolism of specific substrates. This determination is ideally suited for study with MAbs that specifically inhibit single P450s [4, 5].

MAbs are products of hybridoma cells formed by the fusion of spleen cells from immunized mice and myeloma cells [6]. MAbs are superior reagents in respect to chemical purity, epitope specificity, and reproducibility for limitless generations of hybridoma cells. MAbs are useful for quantitative measurement of P450 by radioimmunoassay, ELISA, and western blotting, as well as for immunopurification and immunohistochemical analysis [4, 5].

The MAbs that are inhibitory to enzyme function possess a large and additional dimension of usefulness in that they serve to determine the quantitative contribution of an individual epitope-specific P450 to any reaction in a tissue preparation containing multiple P450s. Thus, the amount of inhibition by an MAb defines the quantitative role of a single P450 in total metabolism [7–11].

The production of MAbs to human P450s has been greatly hindered by the inability to obtain human P450s in amounts sufficient for immunization. To obviate this difficulty, we have utilized the cDNA-directed [12] expression of human P450 3A4 using baculovirus. This resulted in successfully obtaining adequate amounts of human P450 3A4 for immunization [12]. We also used vaccinia virus cDNA-directed expression of enzymatically active P450s that were used for inhibition and cross-reactivity studies [13, 14].

The isolated MAb 3-29-9 was highly inhibitory to human P450 3A3/4/5. MAb 3-29-9 inhibited the P450 3A3/4/5 enzymatic activity toward a wide range of substrates of different molecular weights that we have used,

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§ Abbreviations: MAb, monoclonal antibody; DZ, diazepam; NDZ, *N*-desmethyldiazepam; TMZ, temazepam; OQZ, 2-oxoquanzepam; FBS, fetal bovine serum; B[a]P, benzo[*a*]pyrene; HCF, hybridoma cloning factor; PEG, polyethylene glycol; tk, thymidine kinase; DCM, dichloromethane; PCN, pregnenolone 16α-carbonitrile; and KP_i, potassium phosphate.

i.e., *p*-nitroanisole, phenanthrene, diazepam, testosterone, taxol, and cyclosporin. A comprehensive review [3] of P450 3A function contains a list of numerous drug, steroid, and carcinogen substrates.

A second monoclonal antibody, MAb 275-1-2, yielded a strong western blot with P450 3A3/4 but was not inhibitory to P450 3A3/4 enzyme activity. Thus, this western blot yielding MAb may be of complimentary use to MAb 3-29-9. These MAbs are precise and sensitive probes for identification and function analysis of the P450 3A3/4 enzyme.

MATERIALS AND METHODS

Chemicals

DZ was purchased from USP Convention, Inc. (Rockville, MD). NDZ, TMZ, and OQZ were provided by F. Hoffmann-La Roche, Inc. (Nutley, NJ), the Sandoz Pharmaceuticals Corp. (East Hanover, NJ) and the Schering-Plough Corp. (Bloomfield, NJ), respectively. Taxol and cyclosporin were supplied by the Drug Synthesis & Chemistry Branch, NCI. *p*-Nitroanisole and 4-nitrophenol were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Deuterated (2,3,5,6-D_4) nitrophenol was obtained from Cambridge Isotope Laboratories. Testosterone and hydroxytestosterone metabolites were obtained from Steraloids, Inc. (Wilton, NH). NADPH was purchased from Boehringer Mannheim (Indianapolis, IN). B[a]P 9,10-diol was purchased from the NCI Chemical Carcinogen Repository.

Media and cells

RPMI 1640 (GIBCO) supplemented with 20% FBS (BioWittaker), 2 mM L-glutamine, and 1 mM sodium pyruvate was used as complete medium. The myeloma cell line, NS-1, supplied by the Fredrick Cancer Research and Development Center (FCRDC), resistant to 8-azaguanine, was used as a myeloma fusion partner. After fusion, newly formed hybrids were grown in complete medium containing 100 μM hypoxanthine (H), 0.4 μM aminopterin (A), and 16 μM thymidine (T) (HAT medium) supplemented with 10% HCF (IGEN, Inc.). RPMI 1640 (not supplemented) was used as the washing medium [9, 10].

Preparation of human P450 3A4 as immunogen

Human cDNA for P450 3A4 was constructed into a baculovirus vector. SF9 insect cells were infected with the virus that expressed the P450 3A4. Purification of the P450 3A4 was accomplished by a series of extraction and column chromatography procedures that are fully described in Ref. 12.

Immunization of mice and production of hybridomas

Two female Balb/c mice were immunized by i.p. injection weekly for 3 weeks with 10 μg of purified baculovirus-expressed human 3A4 protein emulsified in 0.2 mL of complete Freund's adjuvant for the first week, and then with incomplete Freund's adjuvant thereafter [15]. Three days after the third injection, one mouse was killed and the spleen removed. The fusion of myeloma cells with primed, dissociated spleen cells was carried out essentially as described [16]. Myeloma cells were washed three times with washing medium with spleen cells added with the third wash (centrifugation 500 *g*). The pellet was loosened and treated with PEG 4000 (Boehringer Mannheim) slowly over a 1-min period and then diluted with 35 mL of washing medium. The PEG-treated cells were centrifuged at 300 *g*, and then resuspended in HAT medium. Cells were counted and further diluted with HAT medium and dispensed into twenty 96-well tissue culture plates at a density of 5000 cells/well in 0.2 mL/well.

Screening for the production of antibodies

ELISA was performed by the generally accepted method [17] using alkaline phosphatase-conjugated goat F(ab')2 fragment to mouse IgG(H + L), mouse IgG (Fc specific), and mouse IgM (μ chain specific). Beckman immunoassay plates were coated with baculovirus-expressed 3A4 in 1× coating solution (KPI Laboratories) at 1.5 pmol/well (100 μL). Due to the low yield of highly purified expressed protein, we used partially purified baculovirus-expressed 3A4 for purposes of screening hybrids; therefore, we also used lysate from cells infected with wild-type baculovirus, which also went through the same partial purification scheme to check for specificity of the antibodies. As the hybrids began to grow, the spent medium from each individual well with hybrid growth was screened for the presence of antibody to 3A4 as described above. Each individual well on the 96-well plates that had cell growth was assigned a number. Each positive well was rescreened and then cloned using complete medium with 10% HCF. Hybrids of interest were cloned at least three times.

Isotyping of mouse Ig

Isotyping was carried out using the Ouchterlony immunodiffusion technique as described and provided by the mouse monoclonal antibody typing kits (The Binding Site).

Cross-reactivity of MAbs

cDNAs coding for the following P450s were vaccinia virus expressed in tk^- cells: mouse 1A1 and 1A2; rat 2A1, 2B1, 3A1, 4A1, and 4A3; human 1A2, 2B6, 2C8, 2C9, 2E1, 3A3, 3A4, and 3A5. The cross-reactivities of MAbs 3-29-9 and 275-1-2 were determined by ELISA [17].

Western blot analysis

Expressed P450 from tk^- cells (described above) and liver microsomes prepared from rats induced with dexamethasone, and human liver microsomes were electrophoresed on SDS gels, transferred onto nitrocellulose [9], and probed with MAb 3-29-9 and 275-1-2 in culture fluid and ascites. MAb binding was detected using alkaline phosphatase conjugated goat anti-mouse antibodies as described above.

Preparation of human and rat liver microsomes

Human liver specimens were obtained from four healthy human organ donors. Liver microsomes from humans and dexamethasone-induced rats were prepared. Cytochrome P450 content and protein concentration were determined according to the procedures as described in Refs. 18 and 19.

Preparation of baculovirus- and vaccinia virus-expressed P450

cDNAs coding for different isozymes were constructed into vaccinia virus vectors [13, 14]. The en-

zymes were expressed by infecting tk^- or Hep G2 cells for 24–48 hr. The cell lysates were used as the source of P450, and the content was determined by spectral analysis. Cell lysate from infected tk^- cells was used in ELISA and western blotting, and cell lysate from Hep G2 cells was used for metabolic studies. Baculovirus-expressed P450s were prepared as described [12].

Preparation of MAbs in culture fluids and ascites

Cloned hybrids producing MAbs were grown in flasks containing serum-free medium (UltraDOMA–BioWittaker) at a concentration of 5×10^5/mL for 3–5 days. Cells were removed by centrifugation, and the resulting supernatant was concentrated with a Filtron Macrosep concentrator (mol. wt cutoff 30,000). Concentrated MAbs were dialyzed in PBS (pH 7.2). Ascites fluid was prepared as previously described [16].

MAb 3-29-9 inhibition of P450 3A3/4/5 catalyzed metabolism

MAb 3-29-9 (concentration ranged from 10 to 500 μg) was preincubated with 10–50 pmol of P450 3A4, 3A3, 3A5, or human or dexamethasone-induced rat liver microsomes in 100 μL of 50 mM potassium phosphate buffer (pH 7.4) at 37°C for 5 min. The mixture was then diluted with buffer to 1 mL as a final volume. Substrate dissolved in 10 μL of methanol (250 μM testosterone, 80 μM diazepam, 100 μM taxol, 10 μM cyclosporin, 100 μM phenanthrene, or 150 μM *p*-nitroanisole as final concentration) was added, and the reaction was initiated by the addition of NADPH (1 mM) at 37°. Anti-lysozyme MAb HyHel or MAb 1-68-11 against rat 2C11 with an amount equivalent to 3A4 MAb was used as a control for nonspecific binding. Reactions were followed for 20–30 min and terminated with 5 vol. of DCM except for the metabolism of cyclosporin. 6β-OH-progesterone, OQZ and deuterated nitrophenol were used, respectively, as internal standards in the metabolism of testosterone [20], diazepam [21], and *p*-nitroanisole [22], and B[a]P 9,10-diol was used as an internal standard in the metabolism of taxol [23], cyclosporin [24], and phenanthrene [25] for quantitation of the products formed. Extracts of products were dissolved in methanol and analyzed immediately by reversed-phase HPLC. The metabolites formed were identified by comparing their retention times with authentic standards. Metabolically formed nitrophenol (D_0) from *p*-nitroanisole and internal standard (D_4-nitrophenol) were further derivatized and analyzed by GC–MS as previously reported [22]. For achieving higher recoveries of cyclosporin and its metabolites, a modified procedure was utilized. Three milliliters of a mixture of 20% acetonitrile, 30% methanol, and 5% zinc sulfate in water was added to precipitate the proteins. Samples were centrifuged at 600 *g* in a Sorvall RT600 centrifuge (Du Pont Co., Wilmington, DE) after the addition of B[a]P 9,10-diol (internal standard), and the supernatant was loaded onto a Sep-Pak cartridge C_{18} column (Millipore Corp., Milford, MA), washed with 2 mL of water, and eluted with 4 mL of methanol. The eluent was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 50% methanol in water for further HPLC analysis.

High performance liquid chromatography

HPLC was performed on a Hewlett–Packard Mode HP1050 liquid chromatograph equipped with an HP model 1050 autosampler, a ternary solvent delivery system, and a multiple-wavelength detector, which are controlled by Hewlett–Packard HPLC^{2D} ChemStation software installed in a Compaq Prolinea 4/66 personal computer.

DZ and metabolites (TMZ and NDZ) were separated on a Zorbax SB-C_{18} column (4.6 mm × 15 cm, MAC-MOD Analytical Inc. Chadds Ford, PA) eluted isocratically with acetonitrile:methanol:water (10:40:50, by vol.) at a flow rate of 1 mL/min and a detection of 232 nm. Cyclosporin and metabolites were injected onto an Ultrasphere™-octyl column (5 μm, 25 cm × 4.6 mm, ALTEX). The elution of metabolites was used with a 65:30:5 mixture of diluted phosphoric acid (pH = 3): acetonitrile:tetrahydrofuran for 5 min and a 23-min linear gradient to 60:35:5 and 12 min to 38:57:5 at a flow rate of 1 mL/min and a detection of 230 nm. The metabolites of taxol [23], testosterone [20], or phenanthrene [25] were separated and identified as previously described.

RESULTS

Monoclonal antibody production, screening, and cross-reactivity

Hybridoma cells were obtained by immunization of mice with purified baculovirus-expressed human P450 3A4 followed by the fusion of the mouse spleen cells with myeloma cells. ELISA analysis was made of 24 hybridoma clones that were selected from more than 700 clones and examined for their binding to baculovirus-expressed P450 3A4. Of the 24 hybridomas tested, 15 yielded antibodies that were positive for specific binding to P450 3A4 by the ELISA test; 6 were high to moderate positive, and 9 were moderately or negligibly positive. Of the 24 hybridomas, 13 were subcloned and one of the hybridoma clones, MAb 275-1-2, yielded antibodies that gave an especially strong western blot. Seven of the hybridomas produced MAbs that were tested for inhibition of P450 3A4 catalyzed testosterone metabolism. Of all the hybridoma clones tested, the MAb 3-29-9 strongly inhibited (by 85%) the P450 3A4 enzyme activity. MAbs of interest were isotyped using the Ouchterlony immunodiffusion technique as described in Materials and Methods. MAb 3-29-9 and MAb 275-1-2 were found to be mouse IgM types. The other remaining hybridomas were either slightly inhibitory or non-inhibitory. The ascites fluid containing MAb 3-29-9 or MAb 275-1-2 was prepared after each of the latter hybridoma clones were subcloned three times to guarantee monoclonality.

Figure 1 shows a western blot analysis of MAb 3-29-9 and MAb 275-1-2 with baculovirus-expressed human P450 3A4, human P450 2E1, wild-type control, and vaccinia-expressed human P450 3A3, 3A4, and 3A5. The MAb 275-1-2 showed strong western blot activity toward baculovirus-expressed human 3A4 and vaccinia-expressed human 3A3 and 3A4, but was negative toward human 3A5. Thus, MAb 275-1-2 detected a common epitope in human 3A3 and 3A4 that was not present in 3A5 and hence MAb 275-1-2 can distinguish between the closely related human 3A3/4 and 3A5. The MAb 275-1-2 also detected P450 3A3/4 in three different samples of human liver with one of the livers giving a second weaker band. The MAb 275-1-2 did not detect any

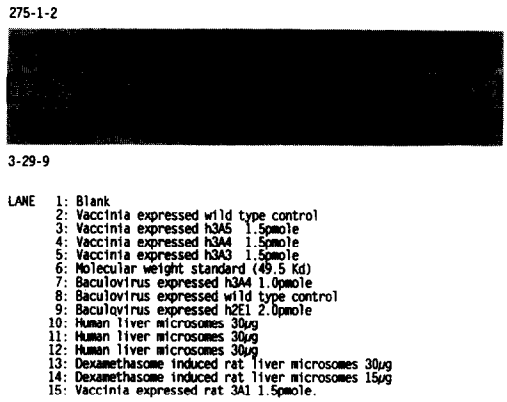

Fig. 1. Analysis of MAbs 3-29-9 and 275-1-2 by western blotting. Western blot analysis of human and rat liver microsomes and human expressed P450s using MAbs 3-29-9 and 275-1-2. Lanes were loaded with corresponding protein (see below), were separated electrophoretically on SDS–polyacrylamide gels (10%), were transferred to nitrocellulose, and then were incubated with MAb. Lanes 10, 11, and 12 were three separate samples of human liver microsomes, 30 µg each.

bands in the livers of dexamethasone-treated rats (Fig. 1), although it showed slight cross-reactivity with the ELISA test (Table 1). These are likely P450 3A1 and 3A2 of rat. The inhibitory MAb 3-29-9 did not yield a western blot with any of the above samples. Vaccinia or baculovirus wild-type and human P450 2E1 were negative to western blotting with either MAb 275-1-2 or MAb 3-29-9.

Table 1 shows the cross-reactivity of MAb 3-29-9 and MAb 275-1-2 with a variety of vaccinia-expressed and baculovirus-expressed human P450s, as measured by the ELISA assay. Both MAb 3-29-9 and MAb 275-1-2 made to baculovirus-expressed human 3A4 was, as expected, positive to baculovirus-expressed human 3A4 but was negative to baculovirus-expressed control human 2E1. MAb 3-29-9 also showed cross-reactivity with vaccinia-expressed human 3A4, 3A3, and, to a lesser extent, human 3A5. The MAb 275-1-2 showed cross-reactivity with human 3A3 and 3A4 and only negligibly with human 3A5, which gave slightly greater cross-reactivity than wild-type. Both the MAb 3-29-9 and MAb 275-1-2 showed no cross-reactivity with a variety of vaccinia-expressed human P450s other than 3A3, 3A4, and 3A5. These included human 2C8, 2C9, 2B6, 2E1, and 1A2. Thus, the MAb 3-29-9 and MAb 275-1-2 exhibited a high specificity for the human 3A3 and 3A4 and to a considerably lesser extent to the 3A5 and no cross-reactivity with five other human P450s. This lack of cross-reactivity of MAb 3-29-9 and MAb 275-1-2 with other major human P450s indicates that these new MAbs are of high specificity and will be of great use in P450 research.

Table 2 shows that the MAb 3-29-9 and MAb 275-1-2 did not cross-react with vaccinia-expressed rat 3A1, rat 2A1, rat 4A1, rat 4A3, rat 2B1, mouse 1A1, and mouse 1A2. There was slight cross-reactivity of MAb 275-1-2 with dexamethasone-induced rat liver microsomes. Thus, the MAbs were highly specific to the epitope of human 3A3/4 and did not recognize this epitope in the mouse, rat, and other human P450s examined.

Inhibitory effect of the monoclonal antibody 3-29-9 on P450 3A3/4

A large number of MAbs from different hybridoma clones were examined for their inhibitory effects on the catalytic activity of human P450 3A3 and 3A4 toward testosterone. The highly inhibitory MAb 3-29-9 was tested with six different substrates with divergent molecular sizes. These were *p*-nitroanisole (K_d 153), phenanthrene (K_d 178), diazepam (K_d 284), testosterone (K_d 288), taxol (K_d 854) and cyclosporin (K_d 1203). MAbs made from a large number of hybridoma clones were examined for their inhibitory effects on human P450 3A3 and 3A4. One hybridoma clone produced MAb 3-29-9 that exhibited potent inhibitory activity toward metabolism catalyzed by P450 3A3/4, as measured

Table 1. Cross-reactivity of MAbs 3-29-9 and 275-1-2 to human 3A3/4 with expressed human P450s

	O.D.	
P450	MAb 3-29-9	MAb 275-1-2
bv 3A4	0.89	1.34
bv 2E1	0.05	0.07
bv Wild	0.09	0.13
vv 3A4	0.31	0.56
vv 3A3	0.41	0.71
vv 3A5	0.24	0.18
vv Wild	0.09	0.15
vv 2C8	0.08	0.15
vv 2C9	0.09	0.16
vv 2B6	0.08	0.14
vv 2E1	0.09	0.17
vv 1A2	0.07	0.16

Assay: ELISA using expressed P450s from vaccinia virus protein (vv) and/or baculovirus protein (bv), 2–5 pmol/well of each P450. The MAbs (10 µg/mL) used were from culture fluid or serum-free medium. Two experiments were performed.

Table 2. Cross-reactivity of MAbs 3-29-9 and 275-1-2 against rat and mouse expressed P450s by ELISA

	O.D.	
P450	MAb 3-29-9	MAb 275-1-2
bv h2E1	0.03	0.07
bv h3A4	0.41	0.62
bv Wild	0.05	0.05
DEX microsomes	0.02	0.24
vv r3A1	0.03	0.11
vv r2A1	0.03	0.15
vv r4A1	0.02	0.05
vv r4A3	0.02	0.07
vv Wild	0.02	0.06
vv m1A1	0.03	0.16
vv m1A2	0.02	0.05
vv r2B1	0.02	0.06

Abbreviations: bv = baculovirus-expressed protein; vv = vaccinia virus-expressed protein. P450: 2.5 pmol/well; MAb: 10 µg/mL in concentrated serum-free medium. Two experiments were performed.

by the metabolic conversion to products of the six substrates listed above. A very large number of substrates exist for the P450 3A3/4 enzyme [3]. Different molecular weight substrates were used to examine the possibility that the MAb 3-29-9 inhibition of P450 3A3/4 was limited to substrates of unique molecular size, which could reflect a unique binding of the substrate or the MAb with the P450 3A4 epitope. The results we obtained, however, showed that the MAb 3-29-9 inhibited the metabolism of substrates with very diverse molecular size. The inhibition of P450 3A3/4 activity by MAb 3-29-9 ranged between 80 and 90% for the metabolism of all the substrates except for the metabolism of *p*-nitroanisole, which was metabolized by both cDNA-expressed P450 3A3 and 3A4. The MAb 3-29-9 inhibited P450 3A3-catalyzed *p*-nitroanisole metabolism by 45% and P450 3A4 by 60% (Fig. 2). The metabolism of *p*-nitroanisole by P450 3A5 was inhibited by only 26%. *p*-Nitroanisole is not considered a good substrate for 3A3/4 and, therefore, it may be uniquely insensitive to inhibition. Rat microsomes induced for P450 3A1/3A2 activity by dexamethasone were not inhibited by the MAb 3-29-9. Thus, the MAb 3-29-9 that inhibits human P450 3A3/4 does not cross-react by inhibition with rat P450 3A1/2. Human P450 2E1, used as a control, is an enzyme highly active for *p*-nitroanisole metabolism and was not inhibited by MAb 3-29-9. Human liver microsomes displayed metabolic activity toward *p*-nitroanisole. MAb 3-29-9 however, did not inhibit *p*-nitroanisole metabolism in human liver. These results indicate that all of *p*-nitroanisole metabolism in human liver is a function of P450s other than P450 3A3/4. These results demonstrate the usefulness of inhibitory MAbs for defining the role or lack of role of individual P450 enzymes in the metabolism of specific substrates in tissues containing many P450s.

The metabolism of phenanthrene by P450 3A3/4/5 and its inhibition by MAb 3-29-9 were examined (Fig. 3). Phenanthrene is known to be metabolized by P450 3A3/4/5 and by microsomes from dexamethasone-treated rats and by human liver microsomes. MAb 3-29-9 inhibited phenanthrene metabolism catalyzed by P450 3A4 by 97%. P450 3A3-catalyzed metabolism was inhibited by 86% and the P450 3A5-catalyzed metabolism was inhibited by about 78%. Human liver microsomes were inhibited 30%, which indicates that the human P450 3A3/4/5 complex contributes not less than 30% to the metabolism of phenanthrene in human liver. In rat liver microsomes from dexamethasone-treated rats, no inhibition by MAb 3-29-9 was observed. This is consistent with our finding that MAb 3-29-9 does not cross-react with dexamethasone-induced P450 3A1/2 in rat liver. The 30% inhibition in human liver demonstrates the value of the MAbs for determining the quantitative role of an individual P450 in a tissue containing a variety of P450s. The difference in sensitivity to MAb 3-29-9 of 3A3/4 in human liver and the P450 3A1/2 in rat suggests that species differences can be sensitively elucidated with MAbs.

Diazepam is one of the most common clinically used drugs in the world. We examined its metabolism catalyzed by human P450 3A3/4 and the inhibitory effects of the MAb 3-29-9. Diazepam was converted by P450 3A3/4 to the metabolite TMZ by hydroxylation of the ring and to metabolite NDZ by demethylation. The results (Fig. 4 A and B) showed that MAb 3-29-9 is a strong inhibitor of both human P450 3A3 and 3A4 catalyzed diazepam metabolism. The formation of the two

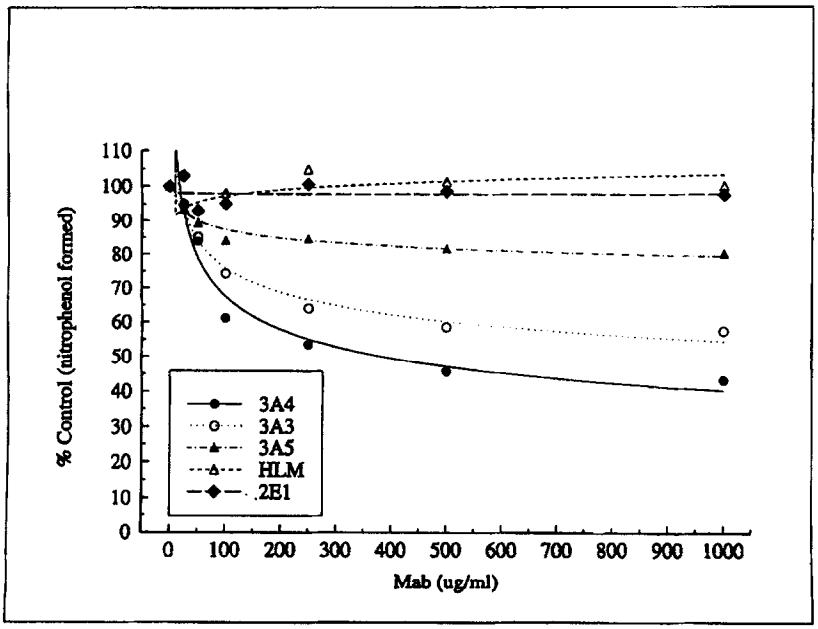

Fig. 2. Inhibitory effect of MAb 3-29-9 on *p*-nitroanisole metabolism. MAb 3-29-9 and P450 (20 pmol) were preincubated at 37° for 5 min and diluted to 1 mL with KP_i buffer. *p*-Nitroanisole (150 μM) and NADPH (1 mM) were used. Incubations were carried out for 20 min and terminated with 4 mL DCM. 4-Nitrophenol (5 μM, D4), was added as an internal standard. Nitrophenol (D_0) and the internal standard (D_4-nitrophenol) were derivatized for GC–MS [22]. Specific activities (nmol/min per nmol of P450) in the formation of nitrophenol for 3A4, 3A3, 3A5, HLM, and 2E1 without MAb 3-29-9 were 0.20, 0.21, 0.14, 4.93, and 7.80, respectively.

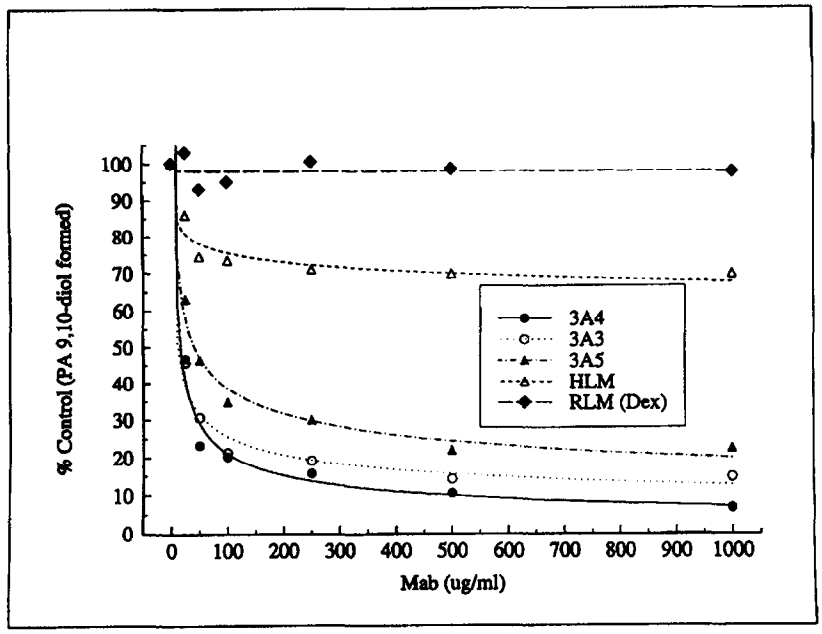

Fig. 3. MAb 3-29-9 inhibition of phenanthrene metabolism. P450 (20 pmol) and MAb 3-29-9 were preincubated in 100 μL of 50 mM KP_i for 5 min. Then 200 μM phenanthrene, 1 mM NADPH, and 50 mM KP_i were added in 1 mL final volume and incubated for 25 min. The reaction was terminated with 4 mL DCM, and 1.5 μM B[a]P 9,10-diol, as internal standard, was added. Extracts were dried down and analyzed by reversed-phase HPLC [25]. The percent of the control in the formation of 9,10-diol metabolite by 3A3, 3A4, 3A5, HLM, and dexamethasone-induced RLM was expressed by a comparison of the ratio of metabolite formed to internal standard in the presence and absence of MAb 3-29-9.

major diazepam metabolites, TMZ and NDZ, by P450 3A4 was inhibited by MAb 3-29-9 by 92 and 87%, and the metabolites formed by P450 3A3 were inhibited by approximately 80% for TMZ and 52% for NDZ. Thus, there was a distinct preference of MAb 3-29-9 inhibition of TMZ (by 80%) relative to NDZ, which was inhibited by 52%. The metabolism catalyzed by 3A5 was inhibited 73% by MAb 3-29-9 for TMZ and 48% for NDZ. With another substrate, *p*-nitroanisole, however, the MAb 3-29-9 was inactive toward P450 3A5 activity. Examination of the metabolism of diazepam by the human liver microsomes showed that the TMZ and NDZ metabolite formation were due largely to P450 3A3/4/5 since MAb 3-29-9 in human liver inhibited diazepam to TMZ conversion by 75% and NDZ by 27%. The difference in inhibition of TMZ and NDZ in human liver by MAb 3-29-9 indicates that there are at least two P450 enzymes in liver responsible for diazepam metabolism. One is P450 3A3/4 and the other is due to an unknown P450(s).

Human cytochrome P450 3A3/4 metabolizes a large diverse number of steroids, a prototype of which is testosterone [20]. MAb 3-29-9 was found to be a potent inhibitor of human P450 3A3, 3A4, and 3A5 catalyzed conversion of testosterone to 6β-hydroxy testosterone (Fig. 5). Thus, all three of the vaccinia-expressed P450s were inhibited by MAb 3-29-9. Similar inhibition was observed for the formation of 15β-OH testosterone (data not shown). The addition of MAb 3-29-9 to human liver microsomes inhibited 6β-OH formation more than 90%, indicating that P450s 3A3/4/5 are essentially the sole P450s responsible for testosterone metabolism by human liver, a fact that may have large therapeutic importance.

With certain substrates, e.g., *p*-nitroanisole, MAb 3-29-9 showed little inhibition of 3A5. However, with 3A5 we did observe 80% inhibition with testosterone, 70% inhibition with diazepam, and 77% inhibition with phenanthrene. The difference in the behavior of the MAb in respect to inhibition of different metabolite formation may also be due to the positioning of the substrates, the MAb, and the active site of the enzyme. The positioning may allow certain metabolites to be formed in spite of the presence of the inhibitory MAb.

Taxol is known to be metabolized [23] by the human P450 3A3/4 system and is a very important compound currently used in the therapy of certain kinds of cancer. Taxol phenol has been characterized to be a major product of taxol metabolism by human P450 3A4 [23]. Taxol conversion to phenol by human P450 3A3/4 was inhibited by the MAb 3-29-9 by about 90% (Fig. 6). The metabolism of taxol by human liver microsomes was inhibited 84% by the MAb 3-29-9, which indicates clearly the very dominant and important roles of P450 3A3/4 for taxol metabolism in human liver. Thus, 84% of taxol metabolism in human liver is due to P450 3A3/4. The rat liver microsomes obtained from the PCN-treated rats showed total resistance to inhibition by MAb 3-29-9 and retained 97% of its usual activity in the presence of the MAb. This is similar to the results obtained with other substrates.

Cyclosporin [24] is a very important drug for suppressing the immune response and is crucial for organ transplants. It has a relatively high molecular weight (see above). We examined the conversion of cyclosporin by 3A4 to four different metabolites. The HPLC analysis of cyclosporine metabolism (Fig. 7) shows that the formation of all four metabolites was inhibited by approximately the same extent by MAb 3-29-9. This inhibition ranged from 70 to 85% for the formation of each of the

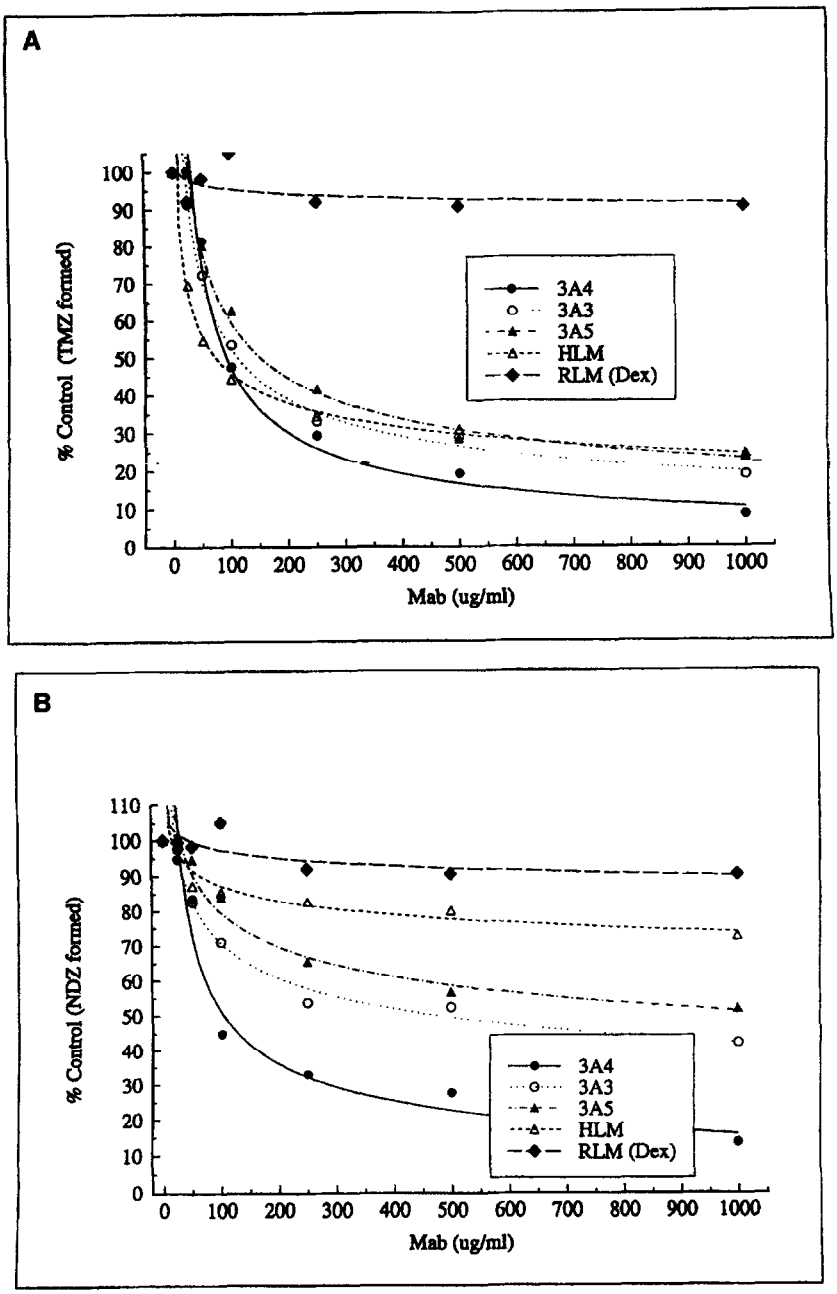

Fig. 4. MAb 3-29-9 inhibition of diazepam conversion to TMZ (A) and NDZ (B). P450 (20 pmol) and MAb were preincubated for 5 min, and 200 μM diazepam and 1 mM NADPH were added. Incubations were carried out for 20 min and terminated with 4 mL DCM, OQZ was added as an internal standard. Extracts were dried and dissolved in 50% methanol for HPLC analysis. Specific activities in the formation of TMZ (nmol/min per nmol of P450) for 3A3, 3A4, 3A5, HLM, and dexamethasone induced RLM without MAb 3-29-9 were 20.7, 15.4, 19.6, 9.2, and 50 and in the formation of NDZ were 4.1, 2.7, 2.1, 2.0, and 8.5, respectively.

four metabolites. Thus, the metabolism of a very large molecule such as cyclosporin is catalyzed by P450 3A3/4 and also is inhibited effectively by MAb 3-29-9.

DISCUSSION

The P450s 3A3/4 are key instruments in the metabolism of a very large variety of substrates. The role of 3A3/4/5 in this metabolism has been reviewed in an article in which a large and multifunctional list of substrates for P450s 3A3/4/5 was enumerated [3]. In addition to the substrates examined here, i.e., *p*-nitroanisole, phenanthrene, diazepam, testosterone, taxol, and cyclosporin, other substrates for P450 3A3/4 include: warfarin, quinoline, sulfentamil, erythromycin, benzphetamine, dapsone, codeine, and imipramine. Some steroid substrates in addition to testosterone are cortisol, progesterone, and 17β-hydroxy estradiol. The carcinogen substrates include aflatoxin, aminochrysene, 1-nitropyrene, B[a]P, and B[a]P 7,8-diol. The types of reaction that are catalyzed are diverse and include hydroxylation, ring dehydrogenation, and N-dealkylation. Herein we

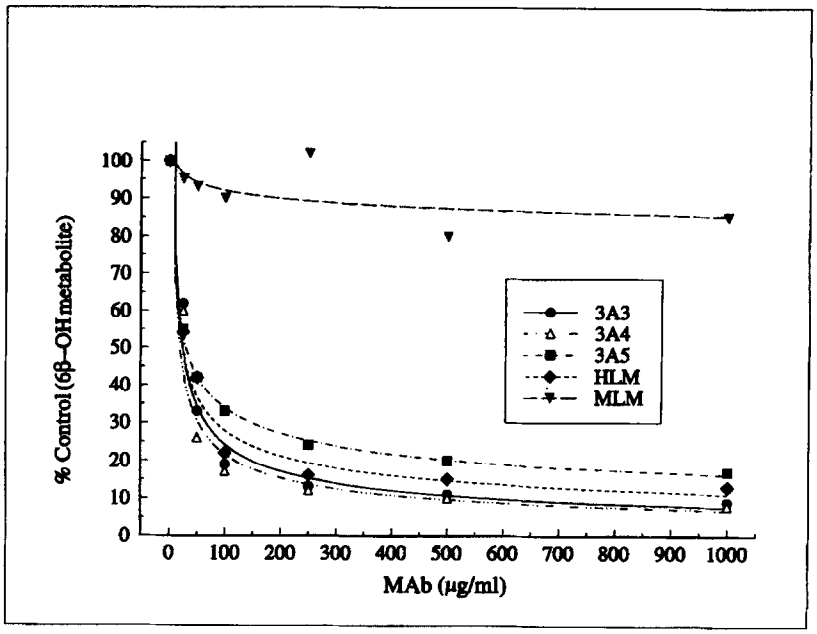

Fig. 5. Inhibition by MAb 3-29-9 of testosterone conversion to the 6β-OH metabolite. Enzyme (15 pmol) and MAb were preincubated. Testosterone (250 μM), NADPH (1 mM), and KP_i (50 mM) were added in 1 mL and incubated for 20 min. Metabolites were extracted with DCM and analyzed by HPLC [23]. Specific activities for 3A3, 3A4, 3A5, HLM, and MLM in the formation of 6β-OH testosterone without MAb 3-29-9 were 25, 25, 10, 2.0, and 65, respectively.

describe the successful isolation of two monoclonal antibodies to the inordinately important human cytochrome P450 3A3/4. Human P450 3A4 was prepared and used as the immunogen by cloning the cDNA for human 3A4, expressing the cDNA with a baculovirus vector with subsequent purification of the active enzyme [12]. This purified P450 3A4 was used as a source of immunogen. After immunization, the dissociated cells from the

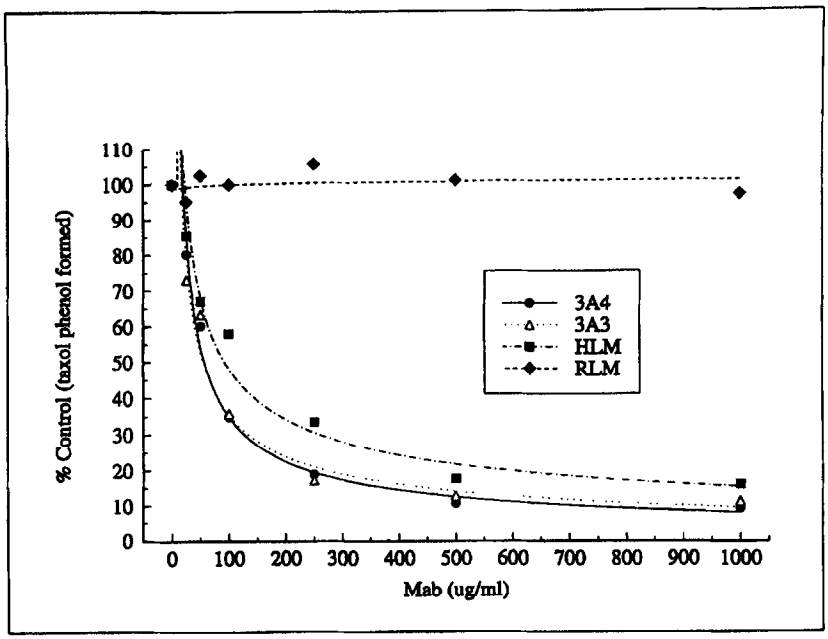

Fig. 6. Inhibition of taxol metabolism by MAb 3-29-9. P450 (20 pmol) was preincubated with MAb 3-29-9 for 5 min at 37°. Taxol (100 μM), NADPH (1 mM), and KP_i (50 mM) were added in a final volume of 1 mL and incubated for 30 min. Incubation was terminated with DCM, and 1.5 μM B[a]P 9,10-diol, an internal standard, was added. The extracts were analyzed by HPLC [21]. The percent of control in the formation of taxol phenol metabolite by 3A3, 3A4, HLM, and dexamethasone-induced RLM was expressed by a comparison of the ratios of metabolites to internal standard in the presence and absence of MAb 3-29-9.

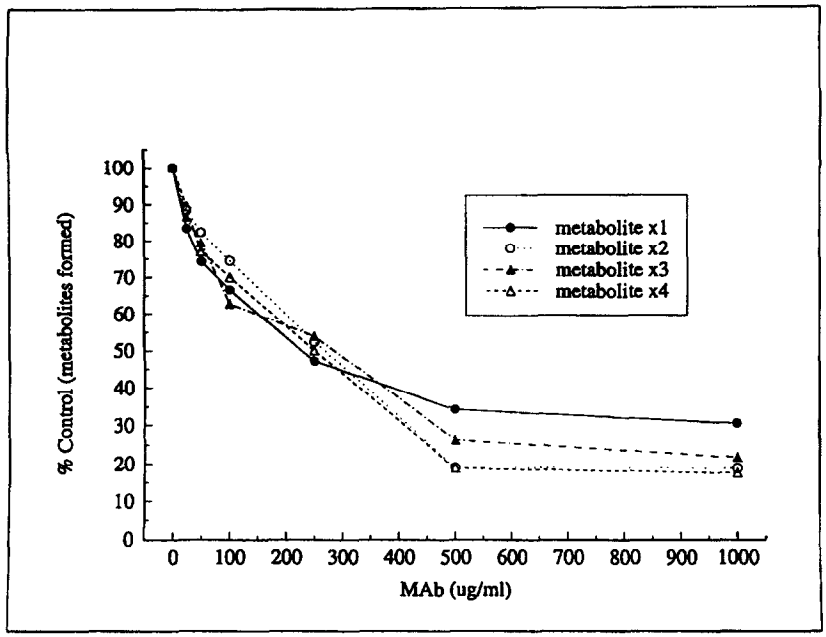

Fig. 7. MAb 3-29-9 inhibition of cyclosporin metabolism by 3A4. P450 (30 pmol) was preincubated in 100 μL of 50 mM KP_i with MAb 3-29-9 for 5 min at 37°. Cyclosporin (10 μM) and NADPH (1 mM) were added, and incubations were carried out for 20 min. Extraction and analysis are described in Materials and Methods. The percent of control in the formation of individual metabolites by 3A4 was expressed by a comparison of the ratios of metabolites to internal standard in the presence and absence of MAb 3-29-9.

spleens of the immunized mice were isolated and fused with myeloma cells, which produced a very large number of fused cells or hybridomas. These were examined for their ability to bind to the P450 3A4 antigen. Of the more than 700 hybridomas screened, only a single hybridoma MAb 3-29-9 produced a MAb that strongly inhibited the enzyme activity of P450 3A3/4 metabolism. The MAb from this clone, MAb 3-29-9, did not cross-react with rat P450 3A1 and six other rodent P450 or with six other human P450s prepared by expression from a vaccinia vector [2, 13, 14]. Although highly inhibitory to P450 3A3/4, MAb 3-29-9 did not form a western blot with P450 3A3/4/5. A second MAb, 275-1-2, which was not inhibitory, yielded a strong western blot with P450 3A3/4 but not with P450 3A5. Thus, MAb 275-1-2 will be helpful for use in conjunction with and complementary to the inhibitory MAb 3-29-9 for quantitatively assessing the tissue amount of P450 3A3/4. The enzyme activity of the P450 3A3/4/5 and inhibition by the MAb 3-29-9 were performed with vaccinia-expressed human cytochrome P450 3A3/4/5. Thus, MAb 3-29-9 is an extraordinarily useful reagent for measuring the contribution of human P450 3A3/4 to metabolic activity in human tissue. MAbs that bind to human P450 3A3/4 and rat P450 3A1/2 have been reported, but in each of these studies inhibitory MAbs were not obtained [16, 26, 27]. The inhibitory activity of MAb 3-29-9 on P450 3A3/4 was examined with a number of P450 3A3/4 substrates. There was moderate inhibition of the metabolism of *p*-nitranisole, and very strong inhibition of phenanthrene, diazepam, testosterone, taxol, and cyclosporin. These substrates cover a wide range of molecular weights. In each case, the monoclonal antibody 3-29-9 was a highly-effective inhibitor. The monoclonal antibody also was used successfully to determine the contribution of 3A3/4 to the metabolism of the five substrates in human liver and human fetal liver.

Analysis of individual P450 enzymes purified from tissues or derived from expression vectors yields important information on the P450 enzyme in respect to its substrate specificity and the formation of individual products [2]. The contribution of the individual P450 to the total metabolism of the substrates cannot be determined by this method. With the use of an inhibitory MAb, the degree of inhibition of a P450 reaction in a tissue preparation determines the contributions of the individual P450 to the total metabolism of the substrate [7, 8]. In the wide scope of drug metabolism studies, identification and quantification of the role of individual P450s are of paramount importance for understanding drug disposition, activation, and therapeutic potential. Knowing the nature and contribution of individual P450s will also permit studies of drug–drug interactions based on competitive metabolism by a common P450. An inhibitory MAb such as we have described will be a useful and precise reagent for these studies. We found that 3A3/4 was responsible for differing amounts of *p*-nitroanisole, diazepam, phenanthrene, testosterone, taxol, and cyclosporin metabolism in human liver. The inhibitory MAb 3-29-9 will be an important and major reagent for cytochrome P450 research, and in particular for the determination of the role of the important human P450 3A4 family in the metabolism of the large variety of clinically used drugs, steroids, and carcinogens.

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