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Use of inhibitory monoclonal antibodies to assess the contribution of cytochromes P450 to human drug metabolism

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Abstract

Three inhibitory monoclonal antibodies specific to cytochrome P450 3A4/5 (CYP3A4/5), CYP2C8/9/19 and CYP2E1, respectively, were used to assess the contribution of the P450s to the metabolism of seven substrates in liver microsomes from 18 human donors, as measured by monoclonal antibody inhibition phenotyping of the substrate conversion to product(s). Metabolism of seven substrates by recombinant cytochromes P450 and human liver microsomes was performed in the presence of monoclonal antibodies and their metabolites were analyzed by high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrophotometry (GC-MS) to measure the magnitude of inhibition. Our results showed that CYP3A4/5 contributes to testosterone 6β-hydroxylation, taxol phenol formation, diazepam 3-hydroxylation, diazepam *N*-demethylation, and aflatoxin B1 3-hydroxylation in human liver by 79.2%, 81.5%, 73.2%, 34.5% and 80%, respectively. CYP2E1 contributes to chlorzoxazone 6-hydroxylation, *p*-nitroanisole *O*-demethylation, and toluene hydroxylation by 45.8%, 27.7% and 44.2% respectively, and CYP2C8/9/19 contribute to diazepam *N*-demethylation by 30.6%. The additive contribution (75.3%) of human CYP3A and CYP2C to diazepam *N*-demethylation was also observed in the presence of both anti-CYP3A4/5 and anti-CYP2C8/9/19 monoclonal antibodies. The contribution of individual P450s to the specific metabolic reaction in human liver varies greatly in the individual donors and the substrates examined. Thus, inhibitory monoclonal antibodies could play a unique role in defining the single or subfamily of cytochrome P450 that is responsible for the metabolism of specific drugs.

Keywords: Cytochrome P450; Monoclonal antibody; cDNA-expression; Inhibition; Liver microsome, human; Drug metabolism

1. Introduction

The cytochromes P450 (CYPs) collectively catalyze the oxidation of a multitude of xenobiotic and endobiotic substrates (Gonzalez 1988; Gonzalez et al., 1990; Guengerich, 1994; Guengerich et al., 1990, 1991a,b). The capacity to metabolize this large number of substrates is due, in part, to multiplicity of P450 enzymes, many of which possess overlapping substrate specificities. P450 structural homology varies greatly and large differences in substrate and product specificity among cytochrome P450s make it difficult to determine the precise role of each cytochrome P450 in the metabolism of individual substrates. One of the essential questions relating to cytochrome P450 function concerns the ability of measuring the contribution of each individual form or class of cytochrome P450 to the metabolism of an individual substrate. This measurement in different tissues, organs, and individuals would yield information concerning the metabolic routing by cytochrome P450s that determine their role in metabolism. The reagents that fulfill these requirements are the monoclonal antibodies (Gelboin, 1993). The monoclonal antibodies that are inhibitory to enzyme function possess a large and additional dimension of usefulness in that they serve to determine the quantitative contribution of individual epitope-specific cytochrome P450s to any reaction in a tissue preparation containing multiple cytochrome P450s.

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Thus, the amount of inhibition by a monoclonal antibody defines the quantitative role of a single cytochrome P450 in the overall metabolism of a given substrate.

Recent advances in molecular biology have led to the cloning and expression of a large number of rodent and human cytochrome P450 cDNAs (Gonzalez, 1988). Many of these cDNAs were expressed using different vectors (Gonzalez et al., 1991a,b,c; Gonzalez and Korzekwa, 1995). The expressed cytochrome P450 proteins were used as immunogens for production of monoclonal antibody (Park et al., 1989; Gelboin et al., 1995, 1996, 1997). Monoclonal antibodies are pure, chemically defined reagents that recognize a single determinant or epitope on the antigen. The specificity remains for the lifetime of the monoclonal antibody-producing cell and its progeny. Monoclonal antibodies can measure individual or epitope related cytochrome P450s with radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), Western blot or by immunohistochemistry. Monoclonal antibodies that are highly inhibitory to a specific form of cytochrome P450 make them useful for the quantitative determination of the epitope-specific or class-specific cytochrome P450 contribution to the metabolism of a substrate in a tissue preparation containing multiple cytochrome P450s (Park et al., 1989; Gelboin, 1993; Gelboin et al., 1995, 1996, 1997). This analysis, utilizing an inhibitory monoclonal antibody, has been termed reaction phenotyping because it identifies the phenotype of the responsible cytochrome P450. Furthermore, monoclonal antibody reaction phenotyping in combination with the cDNA expression offers a unique advantage to the study of human cytochrome P450s. Thus, the two techniques are complementary, cDNA expression defining individual cytochrome P450 specificity and the inhibitory monoclonal antibody determining the quantitative contribution of the specific cytochrome P450 to the enzyme reaction, and permit a more precise assessment of the inhibitory ability of the monoclonal antibody with respect to its target cytochrome P450, which in turn aids in the elucidation of the role of that form towards the total metabolism of the substrate in question. In the present study, we have used the vaccinia virus vector to express human cDNAs into active cytochrome P450 proteins that were targeted by specific monoclonal antibodies to obtain the maximal inhibition of metabolism. Highly inhibitory monoclonal antibodies to CYP3A4/5, CYP2C8/9/19 and CYP2E1, respectively, at the optimal concentrations of inhibition were employed to determine cytochrome P450 contribution to the metabolism of seven designated substrates in a panel of normal human liver microsomes.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study were obtained from the following sources: diazepam, temazepam, nordiazepam, aflatoxin B1, aflatoxin G, 3-hydroxy-aflatoxin B1 (aflatoxin Q1), and chlorzoxazone from Sigma (St. Louis, MO); oxazepam from Schering-Plough (Bloomfield, NJ); 6-hydroxychlorzoxazone from Salford Ultrafine Chemicals and Research (Manchester, UK); testosterone and hydroxytestosterone metabolites from Steraloids (Wilton, NH); taxol from the Drug Synthesis and Chemistry Branch, National Cancer Institute; *p*-nitroanisole, 4-nitrophenol and toluene from Aldrich (Milwaukee, WI); deuterated (2,3,5,6-D4) nitrophenol from Cambridge Isotope Laboratories; benzo[*a*]pyrene *trans*-9,10-dihydrodiol from the NCI Chemical Carcinogen Repository and NADPH from Boehringer Mannheim (Indianapolis, IN); and *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide (MtBSTFA), and *N,O*-bis(trimethylsilyl)-acetamide (BSA) from Regis Technologies (Morton Grove, IL).

2.2. Inhibitory monoclonal antibodies

Inhibitory monoclonal antibodies 3-29-9 for CYP3A4/5, 1-73-18 for CYP2E1, and 1-68-11 for CYP2C8/9/19 were developed and characterized as previously reported (Park et al., 1989; Gelboin et al., 1995, 1996, 1997).

2.3. Expression of cytochrome P450s in vaccinia virus

The construction and characterization of recombinant vaccinia viruses containing CYP2C8, CYP2C9, CYP2E1, and CYP3A4 were described (Gonzalez et al., 1991a). Human TK 143 (thymidine kinase-deficient human embryoblast) or Hepatoma G2 cells (HepG2) infected with recombinant vaccinia viruses were used to express cytochrome P450 proteins. The cells were grown to confluence on 175 cm^2 plastic flask, infected with the recombinant vaccinia viruses, and harvested 24 h after infection. Cytochrome P450 contents were measured by Fe^{2+}–CO versus Fe^{2+} difference spectroscopy. For metabolism studies, the cells were lysed by sonication and centrifuged at $500{,}000 \times g$ for 12 min. The membrane fractions (pellets) of HepG2 cells were resuspended in 0.1 M potassium phosphate buffer. The protein concentration was determined by using a bicinchoninic acid procedure according to the manufacturer's directions (Pierce Chemical, Rockford, IL). The membrane protein of HepG2 cells infected with wild-type vaccinia virus was used as control for metabolic experiments.

2.4. Human liver microsomal preparation

Normal liver specimens from 18 human subjects aged from 19 to 53 were provided by National Cancer Institute Cooperative Human Tissue Network (Philadelphia, PA). Donors of liver specimens are 8 females and 10 males. Clinical information on the donors was provided and included 12 cases of automobile accident, two cases of

coronary artery disease, two cases of respiratory failure, one case of breast cancer and one case of hepatic adenoma. They were non-smokers and non-alcoholics. Microsomes were prepared to contain 0.4–0.9 nmol of cytochrome P450/mg protein according to the procedure as previously reported (Wang et al., 1983), and stored at −80°C until further used.

2.5. Monoclonal antibody inhibition of cDNA expressed cytochrome P450- and human liver microsome-catalyzed reactions

Incubation mixture of 1 ml contained the membrane proteins adjusted to 20–100 pmol of cytochrome P450 expressed from HepG2 cells or from human liver microsomes, 0.1 M potassium phosphate buffer (pH = 7.5) and 50 nM of the monoclonal antibody. After a 5-min preincubation of enzyme(s) with the specific monoclonal antibody at room temperature, the reaction was initiated at 37°C for 20 min by adding a substrate and 1 mM NADPH. The substrate concentrations used were 200 μM testosterone, 400 μM diazepam, 200 μM chlorzoxazone, 100 μM taxol, 200 μM aflatoxin B1, 500 μM *p*-nitroanisole and 500 μM toluene, respectively. Anti-lysozyme monoclonal antibody HyHel (IgG) with an amount equivalent to the corresponding monoclonal antibodies was employed as a control for nonspecific binding. The reactions were terminated by adding eight volumes of methylene chloride and respective internal standards for metabolite quantitation. Monoclonal antibody inhibition was determined by comparing the product(s) formed between the presence and the absence of the monoclonal antibody, which reflects the percentage of contribution of the cytochrome P450 responsible for the metabolism.

2.6. High-performance liquid chromatography (HPLC)

Separation of metabolites was accomplished using a Hewlett Packard model HP1050. Separation and quantitation of the metabolites of testosterone (Hanioka et al., 1990), diazepam (Gelboin et al., 1995), chlorzoxazone (Gelboin et al., 1996), and taxol (Harris et al., 1994) were previously described. Aflatoxin B1 and its product (3-hydroxy-aflatoxin B1 or aflatoxin Q1) as well as aflatoxin G (internal standard) were separated on 20/20 ODS column (Thomson, Chantilly, VA) eluted isocratically with 30% methanol in water (v/v) for 10 min and then a 20-min linear gradient to 90% at a flow rate of 1 ml/min. The metabolite was quantitated by comparing the peak area under chromatography with internal standard at a detection of 365 nm.

2.7. Gas chromatography-mass spectrophotometry (GC-MS)

GC-MS analysis was performed on a Hewlett-Packard 5890 instrument with a 5971 mass selective detector. Analysis and derivatization of *p*-nitroanisole and toluene metabolites were previously reported (Gelboin et al., 1996).

3. Results

3.1. Monoclonal antibody inhibition of expressed cytochrome P450-catalyzed reaction

The structures of seven substrate molecules used in the present study and their sites of oxidation by the specific P450s are shown in Fig. 1. The titration of monoclonal antibody inhibition for individual cDNA-expressed CYP3A4-, 2C8-, 2C9-, 2C19- and 2E1-catalyzed metabolism of specific substrates was achieved, respectively, with a series of the monoclonal antibody concentrations (1–100 nM, data not shown). At 50 nM and above of monoclonal antibody, maximal inhibition was observed (> 85 %). Monoclonal antibodies were then selected to assess single cytochrome P450 contributions to the microsomal metabolism of the substrates in 18 human livers. The percentage inhibitions, as an average with standard error for 18 microsomal samples, for each reaction is shown in Table 1. The turnover of the cytochrome P450 for the product formation by human liver microsomes and the distributions of the individual cytochrome P450 contribution to the metabolism of each substrate are shown in Figs. 2–11.

3.2. Contribution of CYP3A4 in human liver

Diazepam is widely used worldwide as an anxiolytic and hypnotic drug. Diazepam 3-hydroxylation to temazepam was catalyzed primarily by CYP3A4/5 and *N*-demethylation to nordiazepam was catalyzed mainly by CYP3A4/5, CYP2C8/9/19 and CYP2B6 as previously reported (Fig. 1) (Reilly et al., 1990; Hooper et al., 1992; Ono et al., 1996; Yang et al., 1998). The inhibitory effect of the monoclonal antibody 3-29-9 on diazepam metabolism catalyzed by CYP3A4 was examined. The results showed that monoclonal antibody 3-29-9 inhibited the formation of temazepam and nordiazepam catalyzed by CYP3A4 by 92% and 91%, respectively (Figs. 2 and 3). Examination of diazepam metabolism by human liver microsomes indicated that temazepam and nordiazepam formation in the presence of the monoclonal antibody 3-29-9 were inhibited by 73.2% (63–86%) and 34.5% (28–46%), respectively, with a wide range between individuals, suggesting that CYP3A contributes 73.2% to the 3-hydroxylation and 34.5% to the *N*-demethylation of diazepam in the metabolism of diazepam in the human liver population. Thus, the remaining activities for temazepam and nordiazepam production in human liver are a function of P450s other than CYP3A.

Testosterone conversion to 6β-hydroxytestosterone is used commonly as a probe substrate for CYP3A4 activity

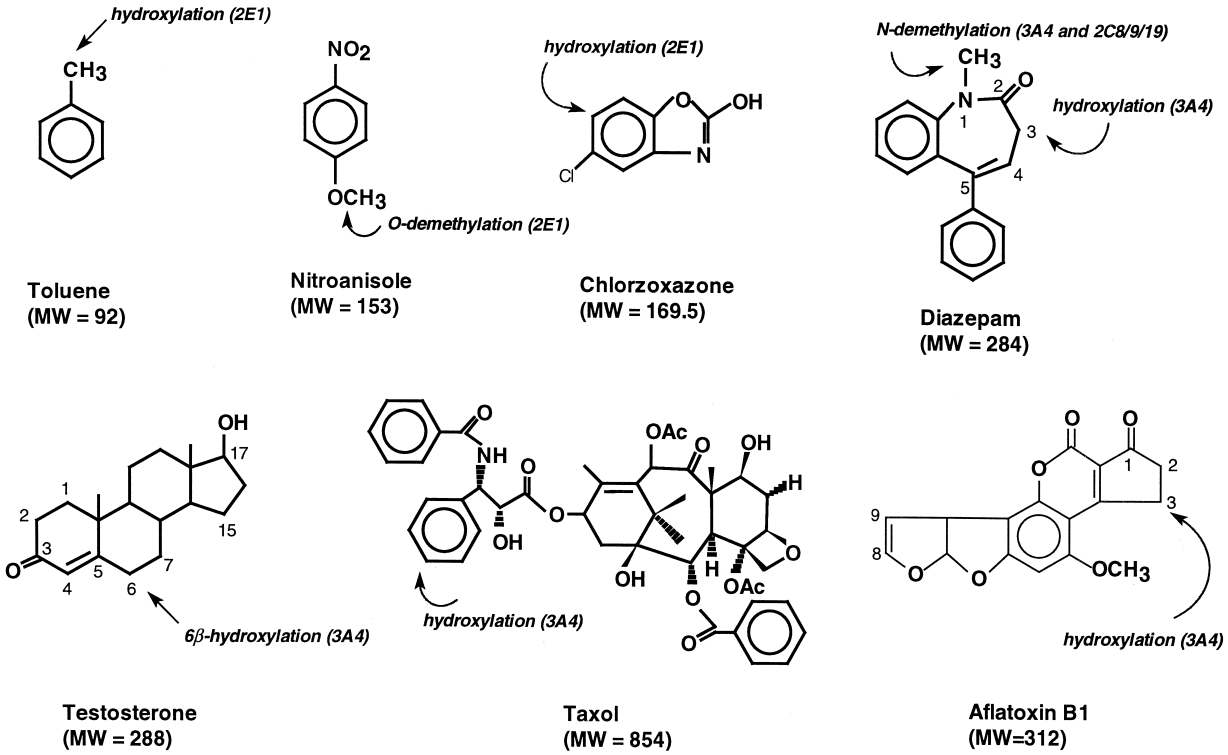

Fig. 1. Structures of substrate molecules used in the present study and the positions metabolized by individual P450s.

in human tissue preparation. Monoclonal antibody 3-29-9 was found to potently inhibit the recombinant CYP3A4-catalyzed 6β-hydroxylation of testosterone by 90% (Fig. 4). The addition of the monoclonal antibody to the microsomal incubation of human liver resulted in 79% inhibition (58–89%) of the 6β-hydroxylation reaction, suggesting that CYP3A4 is the primary catalyst in the liver for the formation of 6β-*OH*-testosterone (Table 1 and Fig. 4).

Taxol, a potent clinically used anticancer agent, is metabolized almost exclusively in the liver and excreted with bile in all species. Taxol phenol was characterized to be a major product of taxol metabolism by CYP3A4 in human liver microsomes (Harris et al., 1994). Monoclonal antibody 3-29-9 inhibited the recombinant CYP3A4-catalyzed taxol conversion to phenol by 90% (Fig. 5). When the monoclonal antibody was applied to liver microsomal

Table 1
Contribution of individual P450s to the metabolism of substrates in human liver

Substrate[a]	Reaction	Sample size[b]	Monoclonal antibody[c]	Percentage contribution of individual P450s[d]
Testosterone	6*β*-hydroxylation	18	anti-CYP3A4/5	79.2 ± 6.8
Taxol	phenol formation	18	anti-CYP3A4/5	81.5 ± 8.6
Aflatoxin B1	3-hydroxylation	18	anti-CYP3A4/5	80.0 ± 6.3
Diazepam	3-hydroxylation	18	anti-CYP3A4/5	73.2 ± 6.7
Diazepam	*N*-demethylation	18	anti-CYP3A4/5	34.5 ± 7.8
Diazepam	*N*-demethylation	18	anti-CYP2C8/9/19	30.6 ± 11.4
Diazepam	*N*-demethylation	18	anti-CYP3A and anti-CYP2C	75.3 ± 9.5
Chlorzoxazone	6-hydroxylation	18	anti-CYP2E1	45.8 ± 13.7
p-Nitroanisole	*O*-demethylation	18	anti-CYP2E1	27.7 ± 19.2
Toluene	hydroxylation	18	anti-CYP2E1	44.2 ± 14.6

[a] 1-ML incubation of individual substrates with the specific P450 and the monoclonal antibody was carried out as shown in the text.
[b] Eighteen human liver microsomes.
[c] Anti-CYP3A4 = monoclonal antibody 3-29-9; anti-CYP2C8/9/19 = monoclonal antibody 1-68-11 and anti-CYP2E1 = monoclonal antibody 1-73-18.
[d] Percentages of the cytochrome P450 contribution are the means ± SE of those observed from 18 individuals and are reflected by the amount of inhibition (percentage inhibition) of the metabolism of a given substrate by the monoclonal antibody for a single or class of cytochrome P450 present in human liver microsomes.

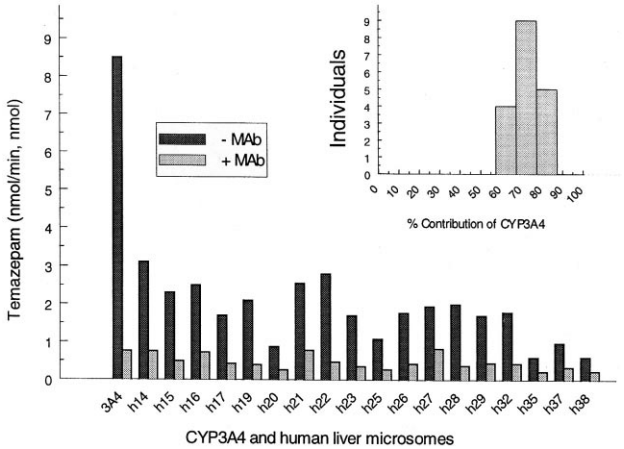

Fig. 2. Monoclonal antibody 3-29-9 inhibition (anti-CYP3A4/5) of the metabolism of diazepam by cDNA-expressed CYP3A4 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for temazepam formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP3A4 contribution was measured by that of the percentage inhibition of diazepam 3-hydroxylation observed from individual human livers.

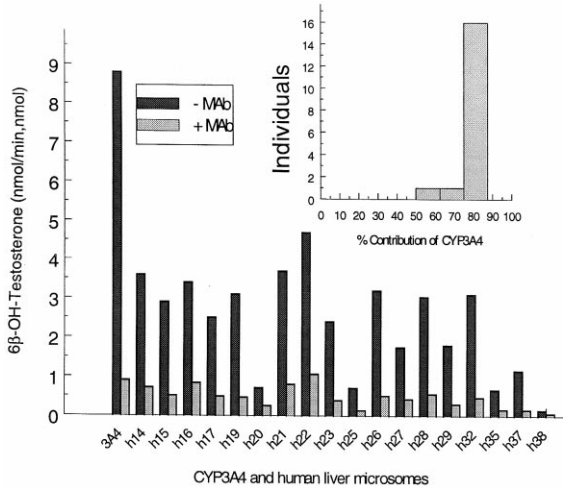

Fig. 4. Monoclonal antibody 3-29-9 inhibition (anti-CYP3A4/5) of the metabolism of testosterone by cDNA-expressed CYP3A4 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for testosterone 6β-hydroxylation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP3A4 contribution was measured by the percentage inhibition of 6β-*OH*-testosterone formation observed from individual human livers.

incubation, 81% inhibition (73–94%) of taxol metabolism was observed, indicating that CYP3A4 plays a very dominant role in taxol phenol production in the human liver (Table 1 and Fig. 5).

Aflatoxin B1 is a strong carcinogen and is activated by CYP3A4 to the 8,9-oxide, the ultimate carcinogenic form (Ueng et al., 1995). Hydroxylation of aflatoxin B1 at the 3-position is a major inactivation pathway that is catalyzed by CYP3A4, forming aflatoxin Q1. Monoclonal antibody 3-29-9 at 50 nM inhibited the activity of cDNA-expressed CYP3A4 in aflatoxin Q1 formation by 91%. The contribution of CYP3A4 in human liver microsomes, as probed with the monoclonal antibody was found to be 80% (66–88%) (Table 1 and Fig. 6). Thus, it appears that CYP3A4 is responsible for at least 80% of the aflatoxin B1 3-hydroxylation in human liver.

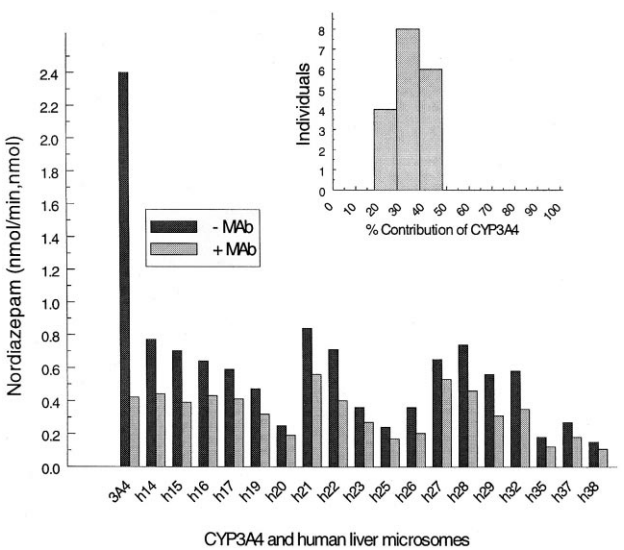

Fig. 3. Monoclonal antibody 3-29-9 inhibition (anti-CYP3A4/5) of the metabolism of diazepam by cDNA-expressed CYP3A4 and liver microsomes from 18 human donors. The turnover numbers of the cytochrome P450 for nordiazepam formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP3A4 contribution was measured by the percentage inhibition of diazepam *N*-demethylation observed from individual human livers.

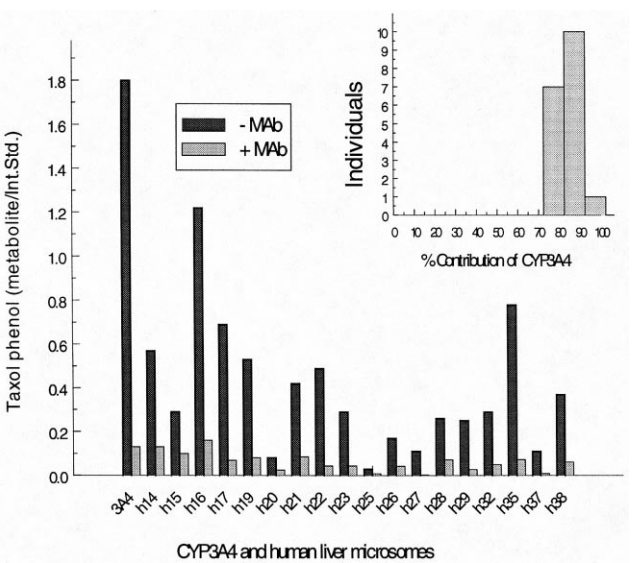

Fig. 5. Monoclonal antibody 3-29-9 inhibition (anti-CYP3A4/5) of the metabolism of taxol by cDNA-expressed CYP3A4 and liver microsomes from 18 human donors with and without the monoclonal antibody 3-29-9 (anti-CYP3A4/5). The turnovers of the cytochrome P450 for taxol phenol formation were expressed by the ratio of product formed to internal standard. The distribution of CYP3A4 contribution was measured by the percentage inhibition of taxol metabolism observed from individual human livers.

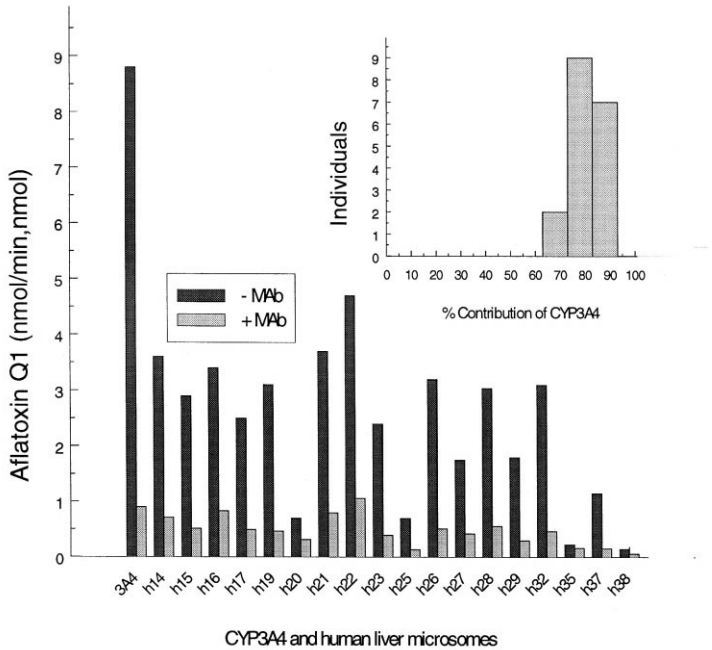

Fig. 6. Monoclonal antibody 3-29-9 inhibition (anti-CYP3A4/5) of the metabolism of aflatoxin B1 by cDNA-expressed CYP3A4 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for aflatoxin Q1 formation (aflatoxin B1 3-hydroxylation) were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of contribution of CYP3A4 was measured by the percentage inhibition of aflatoxin B1 3-hydroxylation observed from individual human livers.

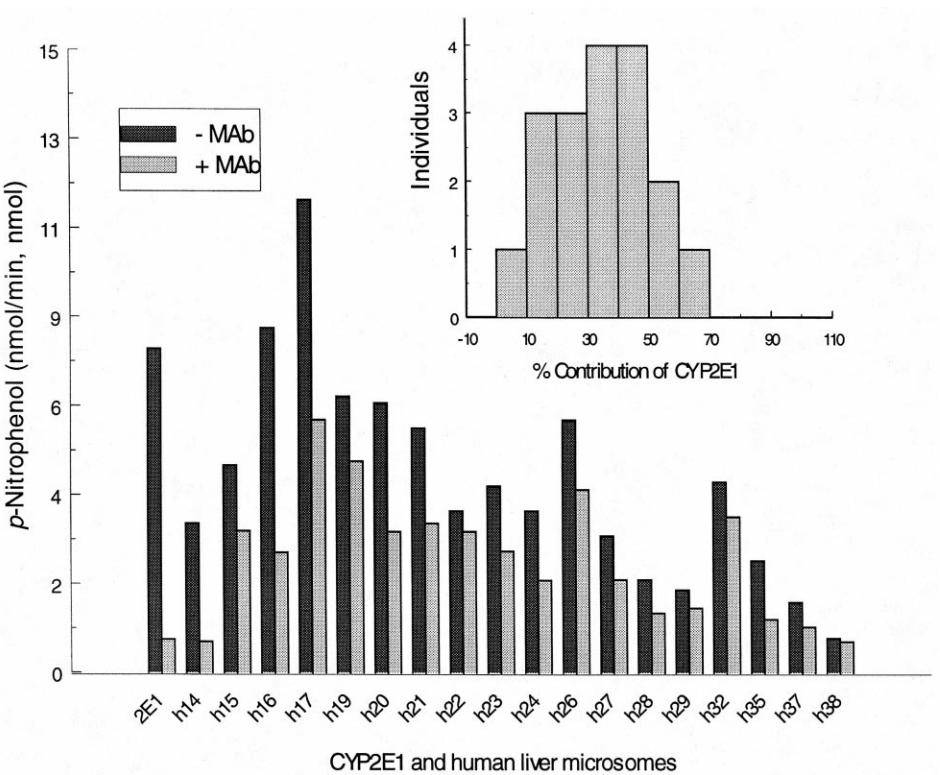

Fig. 7. Monoclonal antibody 1-73-18 inhibition (anti-CYP2E1) of the metabolism of *p*-nitroanisole by cDNA-expressed CYP2E1 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for nitrophenol formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450 and the percentage contribution of CYP3A4 was measured by the percentage inhibition of *p*-nitroanisole *O*- demethylation observed from individual human livers.

3.3. Contribution of CYP2E1 in human liver

Human CYP2E1 is the most important cytochrome P450 involved in the metabolism of a variety of environmentally important small molecules, some of which are carcinogenic in humans, as well as drugs of therapeutic value (Guengerich et al., 1991; Patten et al., 1993; Tassaneeyakul et al., 1993; Yamazaki et al., 1995). We employed monoclonal antibody 1-73-18, the highly specific inhibitor of CYP2E1 to determine the contribution of CYP2E1 to the metabolism of *p*-nitroanisole, toluene and chlorzoxazone in human liver microsomes (Peter et al., 1990; Ono et al., 1995; Gorski et al., 1997). Fig. 7 shows the effect of the monoclonal antibody 1-73-18 on the CYP2E1-catalyzed conversion of *p*-nitroanisole to nitrophenol (*p*-nitroanisole *O*-demethylation). The oxidation of *p*-nitroanisole catalyzed by CYP2E1 was inhibited by 88% following the use of the monoclonal antibody 1-73-18. However, human liver microsomal activity was inhibited by 28% with a broad range from 6% to 67% depending on the individuals. Therefore, CYP2E1 is believed to be one of the P450s responsible for *p*-nitroanisole metabolism in human liver. The residual activity (72%) is due to the cytochrome P450s, which lack the epitope recognized by the monoclonal antibody 1-73-18 or are insensitive to inhibition by the monoclonal antibody 1-73-18.

Fig. 8 shows inhibitory activity of the monoclonal antibody 1-73-18 in the toluene conversion to benzyl alcohol by both cDNA-expressed CYP2E1 and human liver microsomes. Our results showed that 18 liver microsomes are capable of catalyzing toluene oxidation with various turnovers. The presence of the monoclonal antibody 1-73-18 resulted in 44.2% inhibition of toluene metabolism with a range between 19% and 74%.

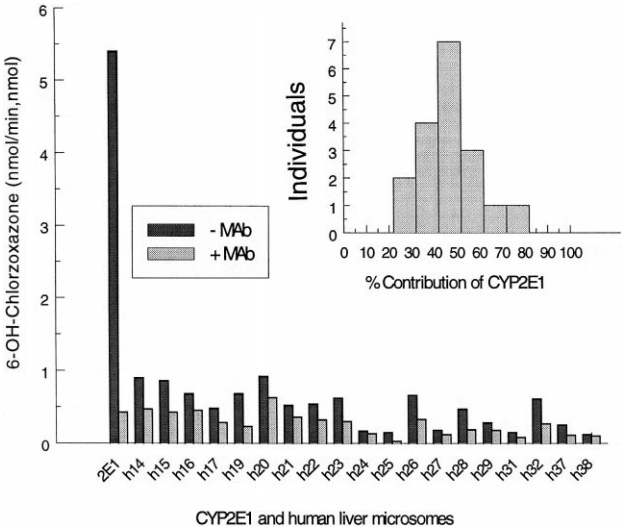

Fig. 9. Monoclonal antibody 1-73-18 inhibition (anti-CYP2E1) of the metabolism of chlorzoxazone by cDNA-expressed CYP2E1 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for the formation of 6-*OH*-chlorzoxazone were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP2E1 contribution was measured by the percentage inhibition of the metabolism of chlorzoxazone observed from individual human livers.

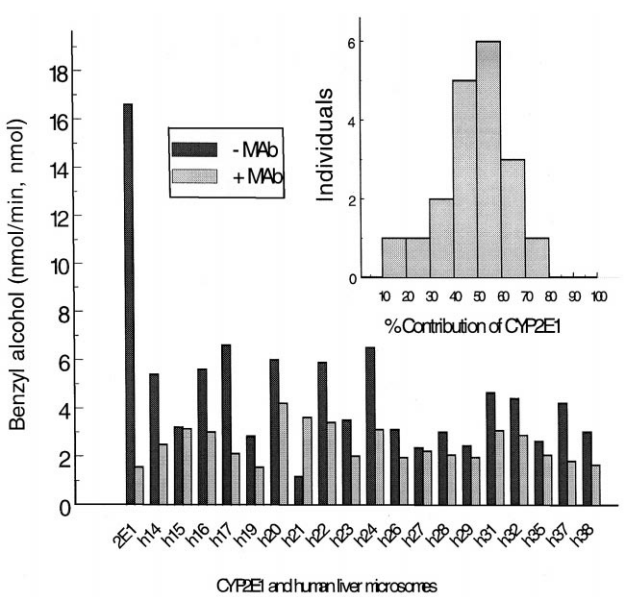

Fig. 8. Monoclonal antibody 1-73-18 inhibition (anti-CYP2E1) of the metabolism of toluene by cDNA-expressed CYP2E1 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for benzyl alcohol formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP2E1 contribution was measured by the percentage inhibition of toluene metabolism observed from individual human livers.

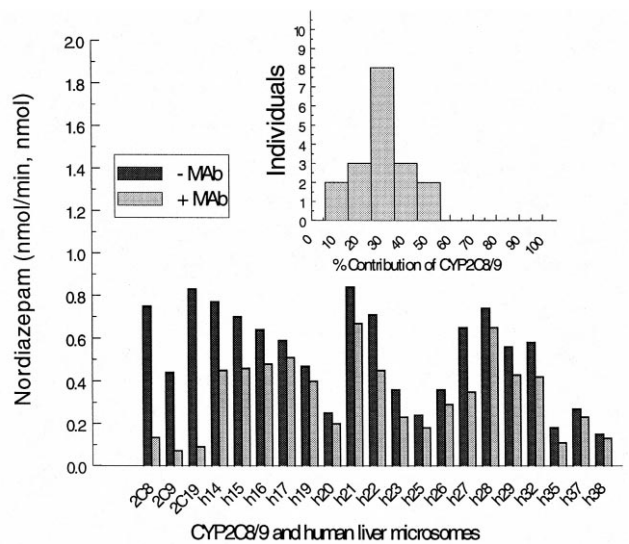

Fig. 10. Monoclonal antibody 1-68-11 inhibition (anti-CYP2C8/9/19) of the metabolism of diazepam by cDNA-expressed CYP2C8/9/19 and liver microsomes from 18 human donors. The turnovers of the cytochrome P450 for nordiazepam formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of CYP2C contribution was measured by the percentage inhibition of diazepam *N*-demethylation observed from individual human livers.

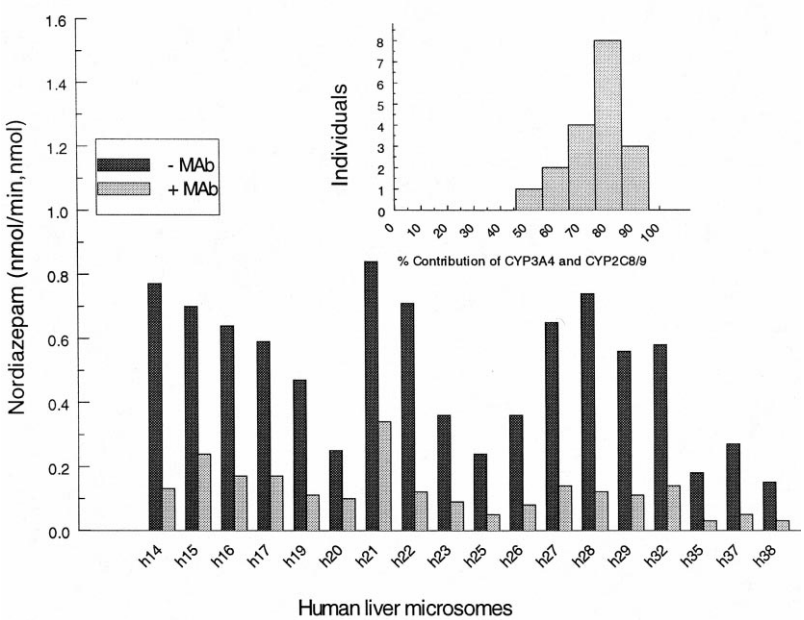

Fig. 11. The combined inhibition of monoclonal antibodies 3-29-9 and 1-68-11 of the diazepam metabolism by liver microsomes from 18 human donors. The turnovers for the cytochrome P450s for nordiazepam formation were expressed as nanomole product formed per minute per nanomole of cytochrome P450. The distribution of both CYP3A and CYP2C contribution was measured by the percentage inhibition of diazepam *N*-demethylation observed from individual human livers.

Chlorzoxazone, a muscle relaxant is oxidized by CYP2E1, CYP1A and CYP3A to form the 6-hydroxyl product (Peter et al., 1990; Ono et al., 1995; Yamazaki et al., 1995; Gorski et al., 1997). Monoclonal antibody 1-73-18 was shown to inhibit the expressed CYP2E1 activity in the chlorzoxazone 6-hydroxylation by 92% (Fig. 9). When an equivalent amount of the monoclonal antibody was applied to the human liver samples, 45.8% inhibition was observed and the variability of inhibition ranged from 20% to 80% (Table 1 and Fig. 9), suggesting that other cytochrome P450s (such as CYP1A and CYP3A) may be involved in chlorzoxazone metabolism.

3.4. Contribution of CYP2C8 / 9 / 19 to diazepam metabolism

In previous reports, diazepam *N*-demethylation was found to be metabolized by various cytochrome P450s, with activities ranked as CYP3A4/5 > CYP2B6 > CYP2C8/9/19 (Ono et al., 1996; Jung et al., 1997; Yang et al., 1998). As mentioned above, CYP3A4 catalyzed about 34.5% of nordiazepam formation in the 18 livers studied as shown in Fig. 3. To investigate the activity of CYP2C subfamily in diazepam metabolism by human liver microsomes, the monoclonal antibody 1-68-11, that specifically inhibits CYP2C8/9/19 enzymes (Fig. 10) was employed (Gelboin et al., 1997). Our results (Table 1 and Fig. 10) showed that the monoclonal antibody caused a partial inhibition of nordiazepam formation by 31% (8–57%). The inhibition was enhanced markedly by the combination of the two individual monoclonal antibodies specific to CYP3A and CYP2C as shown below.

3.5. Additive contribution of CYP3A and CYP2C to diazepam metabolism

Additive inhibition by the two monoclonal antibodies specific for CYP3A4/5 (monoclonal antibody 3-29-9) and CYP2C8/9/19 (monoclonal antibody 1-68-11), respectively, in diazepam metabolism was examined to determine the contribution of two cytochrome P450 subfamilies to the *N*-demethylation of the diazepam metabolism. As seen above, CYP3A and CYP2C provided 34.5% and 30.6% inhibition, respectively, for 18 liver samples to diazepam *N*-demethylation. When both monoclonal antibodies were used together, an increase in inhibition (75%) was found (Table 1 and Fig. 11). Thus, 75% contribution to the *N*-demethylation is attributed to the CYP3A and CYP2C enzymes contained in human liver, suggesting that the application of more than one monoclonal antibodies can determine the role of multiple cytochrome P450s that metabolize one substrate molecule in a specific reaction.

4. Discussion

Multiplicity and overlapping substrate specificity of cytochrome P450 have greatly complicated efforts at understanding the precise role of individual cytochrome P450 forms in the metabolism, activation, and detoxification of different drugs and environmental pollutants. Monoclonal antibodies that specifically inhibit the activity of single or class of cytochrome P450 can be undertaken to assess the contribution of individual cytochrome P450 isoforms to drug metabolism in a human tissue, e.g. human liver

microsomes. The specificity of monoclonal antibodies, their interaction with individual epitopes on proteins, homogeneity as defined chemical reagents, immortality, and ease of handling, all contribute to their extraordinary utility. Monoclonal antibodies that are highly inhibitory to a specific form of cytochrome P450 make them especially useful for the quantitative determination of the contribution of an epitope-specific single or class of cytochrome P450 to metabolism when the cytochrome P450 is contained within a mixture of cytochrome P450s. Thus, in common tissue preparations, the inhibitory monoclonal antibodies can measure the contribution of a single cytochrome P450 to the metabolism of a specific substrate. If the cytochrome P450 to which the monoclonal antibody binds is involved in the catalysis of a given substrate, cytochrome P450 activity in the tissue can be reduced relative to the control that has been preincubated with non-specific antibodies. If two cytochrome P450s are involved in the metabolism, one of which the monoclonal antibody binds to, a partial inhibition can be observed. The accuracy of results is dependent on the specificity and the inhibition potency of the monoclonal antibodies employed, both of which can be determined by the approaches of the cDNA-expressed cytochrome P450-catalyzed reaction and the reaction of a probe substrate with human liver microsomes.

The results presented in this report demonstrate the utility of the combined use of monoclonal antibodies and cDNA-expressed cytochrome P450s in determining the role of individual cytochrome P450s in the microsomal metabolism of a drug. The contribution of the individual cytochrome P450s in total liver microsomal metabolism of a substrate relies on the specific activity and expression level of each enzyme in the tissue. The specific activity is determined by the kinetic characteristics of the enzyme for a specific drug, e.g. K_m and V_{max}. If a substrate is metabolized by more than one cytochrome P450s, the contribution of the enzymes involved in the metabolism is accounted for by not only the specificity but also the expression level of individual cytochrome P450s. The cDNA-expressed cytochrome P450s allow a precise definition of the substrate and product specificity of a given cytochrome P450 form but does not represent specific cytochrome P450 contribution towards substrate metabolism in a crude tissue. Thus, the inhibitory monoclonal antibodies have the advantage of measuring quantitatively the minimum contribution of the cytochrome P450(s) that comprises an epitope(s) recognized by the monoclonal antibody, to the metabolism of a designated substrate. In the present study, we selected the seven compounds that are known to be substrates for one or more cytochrome P450s to evaluate the metabolic contribution of individual cytochrome P450s in a panel of human liver microsomes.

The complementary approach was demonstrated in the report with respect to the metabolism of seven compounds. As shown in Fig. 2–10, the three individual monoclonal antibodies yielded the maximum inhibition of the cDNA-expressed cytochrome P450-mediated reaction of individual substrates by 85–95%. Thus, 5–15% of remaining activity that was not inhibited at the optimal monoclonal antibody concentrations is probably due to the epitope on the antigen, which cannot protect completely the active site on the enzyme from the substrate access after binding, suggesting that the monoclonal antibody–enzyme–substrate complex could be productive even if its rate is much slower in the product formation than that of the substrate–enzyme form (Sai et al., 1998). When the monoclonal antibodies were applied to human liver microsomal metabolism, the quantitative contribution of individual cytochrome P450s was determined as seen in Table 1. The monoclonal antibody 3-29-9, a specific monoclonal antibody to CYP3A inhibited the formation of 6β-*OH*-testosterone, 3-*OH*-aflatoxin B1, taxol phenol, and temazepam by 79%, 80%, 81% and 73%, respectively, indicating that CYP3A4/5 are major cytochrome P450 catalysts of the reactions in liver microsomes and a minor amount is attributed to the cytochrome P450s rather than CYP3A4/5. The monoclonal antibody 1-73-18 for CYP2E1 inhibited the metabolism of chlorzoxazone, *p*-nitroanisole and toluene by 46%, 28% and 44%, respectively, with a large variability among individual livers, implying that other P450s except for CYP2E1 are also responsible for the residual activities in liver microsomes. It has been reported that cDNA-expressed CYP3A4 and CYP1A2 are involved in the 6-hydroxylation of chlorzoxazone (Peter et al., 1990; Ono et al., 1995 Gorski et al., 1997) and, thus, both enzymes contained in liver microsomes may also be contributors of chlorzoxazone metabolism. Interestingly, the *N*-demethylation of diazepam is believed to be catalyzed by multiple cytochrome P450s, at least CYP3A4/5, CYP2B6, and CYP2C8/9/19 by using a cDNA-expression system. The activities of individual cytochrome P450s were ranked by an order of CYP2B6 > CYP3A5 > CYP3A4 > CYP2C8/9 ≫ CYP1A2 and CYP2E1 (Yang et al., 1998) or of CYP3A4 > CYP2B6 > CYP3A5 > CYP2C19 (Ono et al., 1996). We employed the combination of two monoclonal antibodies separately to CYP3A and CYP2C, to determine the additive contribution of the CYP3A and CYP2C subfamilies to the *N*-demethylation of diazepam in human liver. The results showed that monoclonal antibodies 3-29-9 and 1-68-11 inhibited respectively the *N*-demethylation reaction by 34.5% and 30.6%. When both monoclonal antibodies were used together, an additive inhibition (75%) was observed, indicating that CYP3A and CYP2C contribute to at least 75% of the diazepam *N*-demethylation in human liver. This implies that the combined use of more than one monoclonal antibodies could determine the contribution of multiple cytochrome P450s that involve the same metabolic reaction of a drug in a tissue preparation. It is noted that specific activities of 18 human liver samples for a substrate, expressed as the

nanomole of product formed per minute per nanomole of total microsomal cytochrome P450s, varied markedly and depend heavily on individuals (Figs. 2–11). The degrees of the monoclonal antibody inhibition observed in different individuals were largely variable. The cause of the individual variability in metabolism is obviously due to the different expression level and relative activities of the cytochrome P450s involved in the human liver for a given substrate.

Although monoclonal antibody has some advantages in cytochrome P450 identification and quantitation studies, there are a number of concerns in application of monoclonal antibodies. First, a library of specifically inhibitory monoclonal antibodies for all human individual cytochrome P450s is not commercially available. Second, no monoclonal antibodies were obtained to achieve 100% inhibition as seen in Figs. 2–11, which is different from chemical inhibitors. The reason for this could be that monoclonal antibody–enzyme–substrate complex is productive (Sai et al., 1998). Third, many present monoclonal antibodies cannot distinguish between closely related cytochrome P450 subfamily members.

Chemical inhibitors of cytochrome P450s have been proven to be a useful tool in defining the role of individual cytochrome P450s involved in drug metabolism. These compounds have a number of inherent advantages over antibodies, which include that (1) many chemical inhibitors are readily available from chemical synthesis, (2) can be used with intact cells and in vivo, thus making it possible to link a particular cytochrome P450 with a specific toxicological or pharmacological response. Although chemical inhibitors can be very useful in the elucidation of catalytic specificity of human cytochrome P450s, full information about the selectivity of an inhibitor may not be known. The selectivity of many chemicals for particular cytochrome P450s has not been evaluated directly and completely, however, but is based primarily on the observed preferential inhibition of liver microsomal enzyme activities that are associated with the particular cytochrome P450 form or cytochrome P450 subfamily. For instance, disulfiram is a selective inhibitor of human CYP2E1 (Guengerich et al., 1991) but was later found to inhibit CYP2A6 (Yamazaki et al., 1992). α-Naphthoflavone is known to be a potent inhibitor for CYP1A1/2 but also a good substrate and a modulator for CYP3A4, which stimulates or sometimes inhibit many of the CYP3A4-mediated reactions (Friedman et al., 1985; Raney et al.,1992). α-Naphothoflavone inhibits not only CYP1A1/2 (IC_{50} = 0.4–0.5 μM), but is also similarly effective against CYP2C8/9. Increasing the concentration of α-naphthoflavone to 10 μM also causes inhibition of CYP2A6 and CYP2B6. Although diethyldithiocarbamate is used widely as a selective inhibitor of CYP2E1, diethyldithiocarbamate also inhibits CYP1A1/2, 2A6, 2B6, 2C8 and 3A4 at a concentration of IC_{50} required to inhibit CYP2E1 (Chang et al., 1994). Thus, diethyldithiocarbamate cannot be employed as a diagnostic inhibitory probe for CYP2E1. Hence, any characterization of a new inhibitor should include tests with diagnostic substrates for as many P450s as possible.

Our studies indicate that the use of monoclonal antibodies appears obviously to be one of several combined approaches that provides the most convincing evidence for the involvement and contribution of a particular cytochrome P450 in the metabolism of therapeutic agents. Monoclonal antibodies are generally of greater specificity than either polyclonal antibodies or most chemical inhibitors. The extent of inhibition affected by the monoclonal antibody is a quantitative measure of the contribution of the target cytochrome P450 to the metabolic reaction studied, which includes the content and intrinsic activity of the cytochrome P450(s) in the tissue, and nature of substrates for the cytochrome P450. The contribution to the metabolism of single or multiple drugs by individual cytochrome P450s is important for understanding the regulation of the pathway and rate of drug metabolism, the drug interaction as a result of the cytochrome P450 inhibition or activation, and the consequences of pharmacology, toxicology and therapeutics of a drug or multiple drugs.

References

Chang, T.K.H., Gonzalez, F.J., Waxman, D.J., 1994. Evaluation of triacetyloleandomycin, α-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. Arch. Biochem. Biophys. 311, 422–437.

Friedman, F.K., West, D., Dewick, P.M., Gelboin, H.V., 1985. Specificity of medicarpin and related flavones in inhibition of rat hepatic mixed function oxidase activity. Pharmacology 31, 289–293.

Gelboin, H.V., 1993. Cytochrome P450 and monoclonal antibodies. Pharmacol. Rev. 45, 413–453.

Gelboin, H.V., Goldfarb, I., Krausz, K.W., Grogan, J., Korzekwa, K.R., Gonzalez, F.J., Shou, M., 1996. Inhibitory and noninhibitory monoclonal antibodies to human cytochrome P450 2E1. Chem. Res. Toxicol. 9, 1023–1030.

Gelboin, H.V., Krausz, K.W., Goldfarb, I., Buters, J.T., Yang, S.K., Gonzalez, F.J., Korzekwa, K.R., Shou, M., 1995. Inhibitory and non-inhibitory monoclonal antibodies to human cytochrome P450 3A3/4. Biochem. Pharmacol. 50, 1841–1850.

Gelboin, H.V., Krausz, K.W., Shou, M., Gonzalez, F.J., Yang, T.J., 1997. A monoclonal antibody inhibitory to human P450 2D6: a paradigm for use in combinatorial determination of individual P450 role in specific drug tissue metabolism. Pharmacogenetics 7, 469–477.

Gonzalez, F.J., 1988. The molecular biology of cytochrome P450s. Pharmacol. Rev. 40, 243–288, [published erratum appears in Pharmacol Rev 1989 Mar; l41(1):91–92].

Gonzalez, F.J., Aoyama, T., Gelboin, H.V., 1990. Activation of promutagens by human cDNA-expressed cytochrome P450s. Prog. Clin. Biol. Res. 340b, 77–86.

Gonzalez, F.J., Aoyama, T., Gelboin, H.V., 1991a. Expression of mammalian cytochrome P450 using vaccinia virus. Methods Enzymol. 206, 85–92.

Gonzalez, F.J., Crespi, C.L., Gelboin, H.V., 1991b. DNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. Mutat. Res. 247, 113–127.

Gonzalez, F.J., Kimura, S., Gelboin, H.V., 1991c. Expression of mam-

malian cytochrome P450 using baculovirus. Methods Enzymol. 206, 93–99.

Gonzalez, F.J., Korzekwa, K.R., 1995. Cytochromes P450 expression systems. Annu. Rev. Pharmacol. Toxicol. 35, 369–390.

Gorski, J.C., Jones, D.R., Wrighton, S.A., Hall, S.D., 1997. Contribution of human P4503A subfamily members to the 6-hydroxylation of chlorzoxazone. Xenobiotica 27, 243–256.

Guengerich, F.P., 1994. Catalytic selectivity of human cytochrome P450 enzymes: relevance to drug metabolism and toxicity. Toxicol. Lett. 70, 133–138.

Guengerich, F.P., Shimada, T., Bondon, A., Macdonald, T.L., 1991. Cytochrome P-450 oxidations and the generation of biologically reactive intermediates. Adv. Exp. Med. Biol. 283, 1–11.

Guengerich, F.P., Shimada, T., Iwasaki, M., Butler, M.A., Kadlubar, F.F., 1990. Activation of carcinogens by human liver cytochromes P-450. Basic Life Sci. 53, 381–396.

Hanioka, N., Korzekwa, K., Gonzalez, F.J., 1990. Sequence requirements for cytochromes P450IIA1 and P450IIA2 catalytic activity: evidence for both specific and non-specific substrate binding interactions through use of chimeric cDNAs and cDNA expression. Protein Eng. 3, 571–575.

Harris, J.W., Rahman, A., Kim, B.R., Guengerich, F.P., Collins, J.M., 1994. Metabolism of taxol by human hepatic microsomes and liver slices: participation of cytochrome P450 3A4 and an unknown P450 enzyme. Cancer Res. 54, 4026–4035.

Hooper, W.D., Watt, J.A., McKinnon, G.E., Reilly, P.E., 1992. Metabolism of diazepam and related benzodiazepines by human liver microsomes. Eur. J. Drug Metab. Pharmacokinet. 17, 51–59.

Jung, F., Richardson, T.H., Raucy, J.L., Johnson, E.F., 1997. Diazepam metabolism by cDNA-expressed human 2C P450s: identification of P4502C18 and P4502C19 as low K(M) diazepam *N*-demethylases. Drug Metab. Dispos. 25, 133–139.

Ono, S., Hatanaka, T., Hotta, H., Tsutsui, M., Satoh, T., Gonzalez, F.J., 1995. Chlorzoxazone is metabolized by human P4501A2 as well as by human P4502E1. Pharmacogenetics 5, 143–150.

Ono, S., Hatanaka, T., Miyazawa, S., Tsutsui, M., Aoyama, T., Gonzalez, F.J., Satoh, T., 1996. Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: role of P4502B6, 2C19 and the 3A subfamily. Xenobiotica 26, 1155–1166.

Park, S.S., Waxman, D.J., Lapenson, D.P., Schenkman, J.B., Gelboin, H.V., 1989. Monoclonal antibodies to rat liver cytochrome P450-2c/RLM5 that regiospecifically inhibit steroid metabolism . Biochem. Pharmacol. 38, 3067–3074.

Patten, C.J., Thomas, P.E., Buy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P., Yang, C.S., 1993. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem. Res. Toxicol. 6, 511–518.

Peter, R., Bocker, R., Beaune, P.H., Iwasaki, M., Guengerich, F.P., Yang, C.S., 1990. Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. Chem. Res. Toxicol. 3, 556–573.

Raney, K.D., Shimada, T., Kim, D.H., Groopman, J.D., Harris, T.M., Guengerich, F.P., 1992. Oxidation of aflatoxins and sterigmatocystin by human liver microsomes: significance of aflatoxin Q1 as a detoxication product of aflatoxin B1. Chem. Res. Toxicol. 5, 202–210.

Reilly, P.E., Thompson, D.A., Mason, S.R., Hooper, W.D., 1990. Cytochrome P450IIIA enzymes in rat liver microsomes: involvement in C3-hydroxylation of diazepam and nordazepam but not *N*-dealkylation of diazepam and temazepam. Mol. Pharmacol. 37, 767–774.

Sai, Y., Dai, R., Yang, T.J., Rushmore, R.H., Baillie, T.A., Gonzalez, F.J., Gelboin, H.V., Shou, M., 1998. Inhibition kinetics of monoclonal antibodies against cytochrome P450-mediated drug metabolism. FASEB J. 12, A146.

Tassaneeyakul, W., Birkett, D.J., Veronese, M.E., McManus, M.E., Tukey, R.H., Quattrochi, L.C., Gelboin, H.V., Miners, J.O., 1993. Specificity of substrate and inhibitor probes for human cytochromes P4501A1 and 1A2. J. Pharmacol. Exp. Ther. 265, 401–407.

Ueng, Y.F., Shimada, T., Yamazaki, H., Guengerich, F.P., 1995. Oxidation of aflatoxin B1 by bacterial recombinant human cytochrome P450 enzymes. Chem. Res. Toxicol. 8, 218–225.

Wang, P.P., Beaune, P., Kaminsky, L.S., Dannan, G.A., Kadlubar, F.F., Larrey, D., Guengerich, F.P., 1983. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. Biochemistry 22, 5375–5383.

Yamazaki, H., Guo, Z., Guengerich, F.P., 1995. Selectivity of cytochrome P4502E1 in chlorzoxazone 6-hydroxylation. Drug Metab. Dispos. 23, 438–440.

Yamazaki, H., Inui, Y., Yun, C.H., Guengerich, F.P., Shimada, T., 1992. Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of *N*-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. Carcinogenesis 13, 1789–1794.

Yang, T.J., Sai, Y., Krausz, K.W., Gonzalez, F.J., Gelboin, H.V., 1998. Inhibitory monoclonal antibodies to human cytochrome P450 1A2: analysis of phenacetin *O*-deethylation in human liver. Pharmacogenetics 8, 375–382.

Yang, T.J., Shou, M., Korzekwa, K.R., Gonzalez, F.J., Gelboin, H.V., 1998. Role of cDNA-expressed human cytochromes P450 in the metabolism of diazepam. Biochem. Pharmacol. 55, 889–896.