Minireview

Antibodies as a Probe in Cytochrome P450 Research

Magang Shou and Anthony Y. H. Lu

Department of Pharmacokinetics and Drug Metabolism, Amgen Inc., Thousand Oaks, CA91320 (MS) and Department of Chemical Biology, College of Pharmacy, Rutgers University, Piscataway, NJ08854 (AYHL)
Running title: Role of Antibodies against Cytochrome P450 in Cytochrome P450

Address correspondence: Dr. Magang Shou, Department of Pharmacokinetics and Drug Metabolism, Amgen Inc., 30E-2-B, One Amgen Center Drive, Thousand Oaks, CA91320; email: mshou@amgen.com.

Abbreviations used are: PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; 3MC, 3-methylcholantheren; fm, fraction of drug metabolized; DDI, drug drug interaction; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; RIA, radioimmunoassay; ER, endoplasmic reticulum; TAO, triacetyloleandomycin; GST, glutathione S-transferase; PMNs, polymorphic neutrophils; NCE, new chemical entity; CYP-OR, NADPH CYP oxido-reductase; NHBE, normal human bronchial epithelial cells; AFB1, aflatoxin B1; PAH, polycyclic aromatic hydrocarbon.
Abstract

Cytochrome P450 (CYP) is the superfamily of enzymes responsible for biotransformation of endobiotics and xenobiotics. However, their large isoform multiplicity, inducibility, diverse structure, widespread distribution, polymorphic expression and broad overlapping substrate specificity make it difficult the measurement of the precise role of each individual CYP to the metabolism of drugs (or carcinogens) and hamper the understanding of the relationship between the genetic/environmental factors that regulate CYP phenotype and the responses of the individual CYPs to drugs. The antibodies against CYPs have been useful tools for the quantitative determination of expression level and contribution of the epitope-specific CYP to the metabolism of a drug or carcinogen substrate in tissues containing multiple CYP isoforms and for implications in pharmacogenetics and human risk assessment. In particular, the inhibitory antibodies are uniquely suited for reaction phenotyping that helps predict human pharmacokinetics for clinical drug drug interaction (DDIs) potential in drug discovery and development.
I. Introduction

Many enzyme systems exist in the liver and in extrahepatic tissues such as intestine and kidney that can contribute to the clearance of drugs from the systemic circulation. Among the various phase I and phase II drug-metabolizing enzymes, the cytochrome P450 enzymes (CYPs) play a key role in xenobiotic and endobiotic metabolic processing. It has been estimated that about two thirds of marketed drugs are metabolized by this enzyme system (Williams, et al., 2004b; Rendic and Di Carlo, 1997a; Guengerich, 2003c). In fact, members of three P450 subfamilies (CYP1, CYP2 and CYP3) are largely responsible for the metabolic clearance of drugs and xenobiotics (Williams, et al., 2004b; Rendic and Di Carlo, 1997b; Guengerich, 2003b). Multiplicity and overlapping substrate specificity of CYP have greatly complicated efforts at understanding the precise role of individual CYP isoforms in the metabolism of drugs and drug candidates. Over recent years, therefore, a variety of reagents and tools have been developed for in vitro and in vivo studies and it is now possible to determine which specific CYP isoform(s) is (are) involved in the metabolism of a given compound (Rodrigues and Rushmore, 2002a; Rodrigues, 1999b; Lu, et al., 2003d; Williams, et al., 2003b). Given the importance of fraction of drug metabolized (f_m) in assessing the potential of in vivo drug drug interaction (DDI), CYP reaction-phenotyping studies are conducted at multiple stages during drug discovery and development. Irrespective of the strategy, information related to CYP mediated-metabolism is included in regulatory documents and the absorption, distribution, metabolism and elimination (ADME) and DDI sections of the product label, where precautions concerning the co-administration of other drugs are listed, along with recommendations for dose adjustment (Huang, et al., 2007; Obach, et al., 2006). Antibodies that are specific to individual CYPs can be used to determine CYP protein content by immunoreactivity and quantify the role of the target CYP in tissue preparations (i.e. liver microsomes) by their inhibition of substrate-specific metabolism. Therefore, quantitative knowledge of the CYP-specific metabolism of a drug and the individual’s phenotype by the antibodies in drug discovery and development are important in prediction of efficacy, dosage and toxicity of the drug candidate, individual variability of patients in response to the drug and potential of DDI. In this paper we will
briefly overview types and generation of CYP-directed antibodies and their applications in P450 research, drug development and human risk assessment etc.

II. Antibodies against CYPs

a. Polyclonal antibodies

Polyclonal antibodies isolated from rabbits immunized against purified hepatic CYPs from either 3-methylcholanganthen (3MC)- and phenobarbital (PB)-treated rats were first developed by Thomas et al. (Thomas, et al., 1976b; Thomas, et al., 1976c). Using these antibodies, six different isoforms of rat liver CYPs were detected by Ouchterlony immunodiffusion. In addition, the antibodies raised from 3MC- and PB-treated rats exhibited inhibitory effects on purified rat CYP1A (MC-P448)-mediated benzo[a]pyrene hydroxylation and CYP2B (PB-P450)-mediated benzphetamine \( N \)-demethylation reactions, respectively. The antibody inhibition patterns of the various substrates were also observed in native liver microsomes of rats treated with 3MC, PB and pregnenolone-16\( \alpha \)-carbonitrile (PCN), respectively (Thomas, et al., 1977b). These antibodies inhibitory to marker substrate metabolism helped identify one or multiple CYP isoforms in rat liver microsomes.

b. Monoclonal antibodies

The hybridoma technology (Kohler and Milstein, 1975) used to generate monoclonal antibodies (MAbs) against individual CYPs was a major achievement in antibody development. With this technology each B-lymphocyte antibody-forming cell and its progeny is committed to the production of a single, unique type of antibody molecule (Yelton and Scharff, 1981). The procedures include (1) immunization of mice by injection of pure CYP antigens; (2) hybridoma production by fusion of spleen and myeloma cells; (3) cloning of the hybridomas to a monoclonal hybridoma cell that produces antibodies directed to a single epitope; (4) screening for the produced antibodies using ELISA (enzyme-linked immunosorbent assay) and inhibition of marker CYP
activity; and (5) production of the antibodies by cell culture of the hybridomas or in ascites fluid of mice injected intraperitoneally with the hybridomas (Gelboin, et al., 1998). The generated MAb molecule specifically recognizes and binds a single “epitope” on the CYP protein antigen. The hybridoma cell line serves as an immortal source of producing the limitless epitope—specific antibody proteins. MAbs have been widely used in the study of multiple CYP isoforms and are far superior to polyclonal antibodies which are likely to exhibit considerable undesired cross-reactivity.

Production and characterization of MAbs against individual CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 etc.) has been published by Gelboin’s laboratory (Gelboin, 1993d; Gelboin, et al., 1995c; Gelboin, et al., 1996; Gelboin, et al., 1997; Gelboin, et al., 1998; Gelboin, et al., 1999c; Krausz, et al., 2001; Sai, et al., 1999; Yang, et al., 1998c; Yang, et al., 1998a). These antibodies are specific to the CYPs with immunoblot and inhibitory properties which can be utilized to assess expression level of the individual CYPs in tissues in response to the treatment of an inducer and to measure quantitatively contribution of the CYPs to the metabolism of a drug or chemical including toxins, mutagens and carcinogens (Gelboin, 1993c; Gelboin, et al., 1998; Gelboin, et al., 1999b; Yang, et al., 1998d; Yang, et al., 1999b; Mei, et al., 1999a; Shou, et al., 2000a). Although MAbs have some advantages in CYP identification and quantification studies, there are a number of concerns in their application of MAbs. First, a library of inhibitory MAbs for all human individual CYP isoforms is not commercially available. Second, some MAbs may not achieve a complete inhibition. The reason for this could be that MAb-enzyme-substrate complex is still productive, leading to a partial inhibition (Gelboin, et al., 1995b). Third, some current MAbs can not distinguish between closely related CYP subfamily members (i.e. CYP3A4 and CYP3A5) (Gelboin, 1993b).

c. Peptide-directed CYP antibodies

Antibodies against CYPs can be produced by immunizing animals with synthetic peptides that mimic small regions of the apoprotein. As the amino-acid sequences of a
number of CYPs are known, this approach to generate antibodies can be applied to most of the major CYPs in human and other species. This is a relatively simple, rapid and effective method of producing antibodies. The anti-peptide approach to antibody production overcomes the need to use purified CYPs as immunogens. A high degree of binding specificity can be achieved by directing anti-peptide antibodies towards unique regions of the target antigen (Edwards, et al., 1995; Edwards, et al., 1993). These antibodies are particularly suited for immunoblotting, immunocytochemistry and immunoinhibition of enzyme activity (Edwards, et al., 1991; Edwards, et al., 1990; Wang, et al., 1999).

Unlike antibodies produced by other techniques, the epitopes of these antibodies are predetermined, allowing them to be directed toward specific regions of P450 isoforms. Detailed practical information was described by Edwards (Edwards, 2006) on the selection of regions to target, the synthesis and conjugation of peptides to carrier protein, immunization, and the assessment of the resultant antisera. Pepetide-directed antibodies against human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11 and an antibody that binds to CYP2C8, CYP2C9 and CYP2C19 have been produced by immuno-raising rabbits with synthetic peptides representing small regions of each of these P450 enzymes. The specificity of the antibodies was confirmed by immunoblotting using recombinant P450 enzymes and samples of human hepatic microsomal membranes. The results demonstrated the utility of the anti-peptide approach for producing specific antibodies against human P450 enzymes, enabling a comprehensive panel of antibodies against human CYPs to be produced (Edwards, et al., 1998). The major drawback of this approach was that most of the anti-peptide antibodies were non-inhibitory. Even when inhibitory antibodies can be produced in some rabbits, consistent productions of inhibitory antibodies in all rabbits remain a technical challenge (Wang, et al., 1999).

III. Application

a. Probing the membrane topology of CYP
The liver microsomal CYPs were first characterized in the early 1960’s (OMURA and SATO, 1964). One of the areas of special interest to investigators at that time was the spatial arrangement and localization of both CYP enzyme and NADPH-CYP reductase in the microsomal membrane since this is important for the understanding of the interaction of these two components and the mechanism of microsomal electron transport. The hydrophilic portion of the reductase can be cleaved and released from the microsomal membrane by protease treatment, while the hydrophobic tail of the reductase is embedded in the lipid bilayer of the microsomal membrane. The bulk of the protein containing the active site of the enzyme was exposed to the cytosolic side of the lipid bilayer based on protease digestion and inhibition of metabolism by antibodies (Black and Coon, 1982). In contrast, either CYP or its denatured form, P420, was not solubilized by protease treatment, raising the possibility that CYP may be deeply embedded in the microsomal membrane.

Antibodies produced against CYPs play a prominent role in the investigation of membrane topology of CYP. Initially, inhibition of liver microsomal metabolism of drugs and other substrates was used to probe the accessibility of CYP in the microsomal membrane (Thomas, et al., 1977a). Strong antibody inhibition of the microsomal metabolism indicated that CYP was at least partially exposed to the hydrophilic environment on the exterior of liver microsomal membrane. Subsequently, antibodies against the N-terminal amino acid sequences were used to examine if they can bind to the specific region of CYP in the microsomal membrane. Using antibodies prepared against peptides in various lengths from the N-terminus to residue 38 of CYP2B1, De Lemos-Chiarandini et al. (De Lemos-Chiarandini, et al., 1987) found that an antibody raised to the first 31 residues of CYP2B1 bound well to the purified enzymes but poorly to the microsomal preparations. In contrast, an antibody prepared against residues 24-38 showed strong binding to intact liver microsomes. These results strongly suggested that approximately the first 20 amino acids from the amino terminal of CYP2B1 are embedded in the phospholipid bilayer and the rest of the enzyme is exposed to the cytoplasmic surface of the membrane.
Based on proteolysis study, chemical modification and theoretical considerations, two topological models were proposed for CYP (Black, 1992). One model proposes a single trans-membrane anchor peptide (approximately the first 20 amino acids) with the amino terminus of CYP projected into the lumen of the endoplasmic reticulum. The other model suggested a hairpin loop of the first approximately 46 residues inserted into the membrane with the amino terminus located on the cytosolic side of the lipid bilayer. To distinguish between these two possibilities, Black and coworkers (Black, et al., 1994) used monoclonal antibodies prepared against residues 18-29 of CYP2B4, corresponding to the halt-transfer signal of the enzyme. Based on these two models, the cationic halt-transfer signal peptide of CYP2B4 (residues 21-29) should be located at the cytosolic surface of the membrane in the first model but at the luminal side of the membrane in the second model. ELISA assays showed that all of the antibodies exhibited strong binding to intact liver microsomes. No enhanced binding by any of the antibodies could be observed in detergent-solubilized microsomes. Thus, the halt-transfer signal of CYP2B4 was localized at the cytosolic side of the microsomal membrane and no fraction of the peptide segment was found in the lumen, consistent with the model that a single trans-membrane peptide at the N-terminus was embedded in the microsomal membrane, while the rest of CYP2B4 was exposed to the cytosolic side of the lipid bilayer. This CYP membrane topology model has been widely accepted (Johnson, 2003).

Antibodies are extraordinarily suitable for investigation of the molecular and functional diversity of CYPs. The CYPs are present in multiple forms, and the levels of each isoform vary largely in response to drugs, endogenous substances, physiological and environmental states. Therefore, antibodies can be used for identification and quantification of the CYP proteins under the different conditions. Detection of epitope-specific CYPs in organs, tissues, cells and sub-cellular organelles can be accomplished with immunoassays, such as RIA (radioimmunoassay), ELISA and immunoblotting. RIA assays are rapid and sensitive and can detect CYP at a level of < 100 ng of microsomal protein (Cheng, et al., 1984; Song, et al., 1985). The MAb-based immunochemistry can be applied to localize and examine the distribution of individual
cytochrome CYPs after different inducer administration. The first application to the
analysis of CYP1A1/2 in liver cells showed detection of the epitope containing CYPs in
3MC-induced rat liver and is localized in both the rough and smooth endoplasmic
reticulums (ERs) as well as the nuclear envelope of hepatocytes (Brands, et al., 1985).
The method provided a variety of utility to examine the cellular distribution of CYPs in
the liver and the extrahepatic tissues (Foster, et al., 1986).

b. Probing the multiplicity of CYP

Enzyme multiplicity was one of the most dominant issues in CYP research in the
1970’s. Before recombinant DNA technology was available, classical biochemical
approaches involving multiple purification steps were used to purify either a single CYP
isoform or a mixture of structurally related P450 enzymes. Antibodies against these
purified enzymes played a prominent role in the demonstration that multiple species of
CYP are involved in the metabolism of drugs and other substrates.

Interaction studies between purified CYPs from PB- as well as 3MC-treated rats
with their respective antibodies illustrate the value of this approach before all individual
rat CYPs were purified and characterized. Even though CYPs purified from these
inducer-treated rats were electrophoretically pure, as judged by a single staining protein
band in sodium dodecyl sulfate-gel electrophoresis under the assay condition,
confirmation of homogeneity of these enzyme preparations was required by a different
experimental approach. Using the classical Ouchterlony double-diffusion technique and
antibodies prepared against the purified CYPs from PB- and 3MC-treated rats, Thomas et
al. (Thomas, et al., 1976a) found four antigenically different forms of CYPs in the
purified CYP preparation from PB-treated rats and two different forms in the purified
preparation from 3MC-treated rats. These results were later confirmed by further
purification studies and the cloning of cDNA encoding various CYPs in rats. A similar
approach has also been used to demonstrate structural relatedness among various CYPs
isolated from the same animal (Reik, et al., 1982; Bandiera, et al., 1985).
c. Probing human CYPs

From the early 1970’s to mid-1980, purification and characterization of CYPs were mostly carried out in animal species. When human liver samples became widely available in the mid-1980’s, investigators, led by F.P. Guengerich and P.S. Guzelian, started to isolate and characterize human CYPs. Antibodies again played a significant role in this investigation. Using a different approach, Guengerich and colleagues (Guengerich, et al., 1986; Butler, et al., 1989) purified a number of CYP isoforms from human liver microsomes by following the catalytic activities of the metabolism of specific substrates such as nifedipine. Again, antibodies prepared against various CYPs purified from rat and human livers were valuable tools to characterize the purified CYP isoforms from human liver microsomes, and laid the foundation of our current knowledge of human CYP.

Initially, PCN was found to induce a CYP isoform in rats different from the PB- or 3MC-inducible CYPs based on spectral and catalytic properties (Lu, et al., 1972). In 1980, Elshourbagy and Guzelian (Elshourbagy and Guzelian, 1980) isolated a CYP species, named P450p, from liver microsomes of PCN-treated rats. Although this CYP species exhibited little catalytic activity toward several substrates when reconstituted with NADPH-CYP reductase and phospholipid, a potent antibody was produced against this CYP. Antibody against P450p strongly inhibited the metabolism of several substrates not only from liver microsomes of PCN- or triacetyloleandomycin (TAO)-treated rats, but also from liver microsomes of human subjects (Wrighton, et al., 1985; Watkins, et al., 1985). This antibody cross-reacted strongly with CYP3A4, the major CYP isoform in human liver and was a valuable tool for CYP3A4 research for many years.

d. Human risk assessment

The association of CYP genetic polymorphism and human cancer risk, and susceptibility to environmental hazards, has received increasing attention. In addition, the current status and perspectives of using CYP genetic polymorphism as a biomarker of
individual susceptibility to cancer and environmental toxicity are research areas of great interest (Hong and Yang, 1997). The expression of CYP genes in target cells is an important determinant of human susceptibility to cancers and other chemically initiated diseases. In these studies, antibodies directed against various human CYPs are valuable tools for the localization and characterization of CYPs in all kinds of human tissues. For example, use of immunohistochemistry allowed investigation on the cellular distribution and localization of CYP1A1 and CYP1B1 in human skin and their induction by ultraviolet-B. Human CYP1A1 was found to be primarily localized in the basal cell layer of the epidermis in non-ultraviolet-B exposed skin, whereas CYP1B1 was localized in the epidermal cells other than the basal cell layer. Thus, localizations of CYP1A1 and CYP1B1 in human skin are different and may be related to keratinocyte differentiation. Ultraviolet-B induction of both enzymes in human skin will probably result in enhanced bioactivation of polycyclic aromatic hydrocarbons (PAHs) and other environmental pollutants to which humans are exposed, which in turn could make the human skin more susceptible to ultraviolet-B-induced skin cancers or allergic and irritant contact dermatitis (Katiyar, et al., 2000).

Chronic pancreatitis and pancreatic cancer have been linked to the exposure of environmental chemicals (xenobiotics), which generally require metabolic activation to highly reactive toxic or carcinogenic intermediates. The primary enzyme system involved is made up of numerous CYPs. A study was performed with immunochemical detection of CYP1A1, 1A2, 2B6, 2C8/9/19, 2D6, 2E1, 3A4, glutathione S-transferase-α (GST-α) GST-μ and GST-π, and NADPH CYP oxido-reductase (CYP-OR) in normal pancreatic tissue specimens (Standop, et al., 2002). In all pancreatic regions, most of the enzymes were expressed in islet cells. However, more islets in the head region expressed CYP2B6, 2C8/9/19, 2E1 and OR, than those in the body and tail. Moreover, the expression of CYP 2B6 and 2E1 was restricted to the pancreatic polypeptide (PP) cells, and the concentration of CYP3A4 was higher in PP cells than in other islet cells. The greater content of xenobiotic-metabolizing and carcinogen-activating CYP enzymes and a lower expression of detoxifying GST enzymes in the head of the pancreas could be one reason for the greater susceptibility of this region for inflammatory and malignant diseases.
Anti-CYP1B1 has been used for the immunohistochemical localization of CYP1B1 in breast cancer (McFadyen, et al., 1999). The lung represents an important target for the toxic effects of chemicals. Using either individual human lung samples or pooled microsomes from different individuals, immunoreactive bands specific for the following CYP enzymes were determined by immunoblotting: CYP1A1, CYP1A2, CYP1B1, CYP2C, CYP2A, CYP2B6, CYP2D6, CYP2E1 and CYP3A4/5 (Bernauer, et al., 2006). Study on expression and induction of CYP3A4 in different peripheral blood cell populations was performed. Rifampicin (600 mg/day during 6 days) was given to 8 healthy subjects. Human polymorphic neutrophils (PMNs) and lymphocytes were isolated by centrifugation of whole white blood cell fractions. PMN and lymphocyte smears and homogenates were subjected to immunostaining and immunoblotting, respectively, with a mouse monoclonal antibody recognizing all CYP 3A proteins. These results demonstrate that CYP 3A proteins are constitutively expressed not only in PMNs but also in lymphocytes. However, in both cell lineages CYP 3A protein expression was not induced by rifampicin (Starkel, et al., 1999).

The expression of CYPs in human esophageal mucosa was investigated in a total of 25 histologically non-neoplastic surgical tissue specimens by using specific antibodies in immunoblots analysis. The presence of CYP1A, 2E1, 3A and 4A enzymes was demonstrated by both techniques; CYP2A reactive protein was also detected by immunoblot. These data demonstrate that a range of CYP enzymes are expressed in human esophageal mucosa and indicate that this tissue has the capacity to activate chemical carcinogens to reactive DNA binding metabolites (Lechevrel, et al., 1999).

Aflatoxin B₁ (AFB₁) is a potent hepatocarcinogen and a risk factor for human lung cancer in certain occupations. To study the potential for AFB activation in human lung, CYP-mediated activation and GST-mediated detoxification of AFB₁ were examined in cultured normal human bronchial epithelial (NHBE) cells. Western immunoblots showed that the primary CYP isoforms responsible for AFB₁ activation in the liver, 1A and 3A4, to be constitutively expressed in NHBE cells. Expression of CYP 1A was
significantly increased in 3MC-pretreated cells, while CYP 3A4 expression increased slightly, but not to the extent of the 1A isoforms. The principal AFB oxide detoxifying enzyme, GST, was constitutively expressed in NHBE cells, and was increased approximately two-fold by 3MC pretreatment. These data support modest CYP-mediated AFB₁ activation in human airways, and indicate that exposure to PAHs, such as 3MC, which induce CYP(s) that specifically activate AFB₁ may increase the harmful effects of AFB₁ exposures in human airways (Van Vleet, et al., 2001). In addition to xenobiotic metabolizing CYPs, monoclonal antibodies to human aromatase (CYP19) have been also isolated and characterized (Turner, et al., 2002). Human aromatase is believed to be a potential therapeutic target for the treatment of certain breast cancers.

e. Reaction phenotyping of individual CYPs

Drug metabolism in human is catalyzed by more than ten microsomal CYPs which are heterogeneously distributed in liver and other tissues. Five of them (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) are involved in ~95% of the CYP-mediated metabolism of drugs (about 75% of all drug substrates)(Williams, et al., 2004). Since each microsomal CYP can metabolize multiple drugs and produce multiple metabolites (Rendic and Di Carlo, 1997;Guengerich, 2003), a general principle in drug development is to avoid the development of new drugs that can cause potent inhibition and induction of drug metabolizing CYPs. To this end, identification of CYPs responsible for the metabolism of new therapeutic agents is pivotal in drug development. Multiple approaches have been used for CYP identification (Lu, et al., 2003) which include uses of cDNA-expressed CYPs in various systems, chemical inhibitors selective and antibodies specific to the individual CYPs etc.. Potent, specific and inhibitory antibodies against various human CYPs represent one of the most valuable approaches for CYP identification (Gelboin, 1993;Gelboin, et al., 1999;Gelboin and Krausz, 2006;Lu, et al., 2003;Shou, et al., 2000;Mei, et al., 1999). If the inhibition potency and isoform specificity of a particular antibody is verified the antibody can be chosen as the idea approach necessary for CYP phenotyping study. Thus within a mixture of CYPs, as is present in common tissue preparations such as microsomes or cell homogenates, the inhibitory antibodies can measure the contribution of a single CYP to the metabolism of a
specific substrate. The amount of conversion of a substrate to a product catalyzed by a specific CYP can be quantitatively measured by the amount of inhibition of activity caused by the inhibitory antibody (Kassahun, et al., 2001; Ngui, et al., 2000; Tang, et al., 2000). Reaction phenotyping is important with respect to the toxicity of a drug, DDIs, and a drug administered to an individual that is polymorphically defective in certain CYP isoforms, such as CYP2D6 (Lin and Lu, 1998; Lin and Lu, 2001). Antibodies that are specific to individual CYPs can be used to determine CYP protein content by immunoblot (Yang, et al., 1998b) and to quantify the role of the target CYP in tissue preparations (liver microsomes) by their inhibition of substrate metabolism (Gelboin, 1993; Gelboin, et al., 1995; Gelboin and Krausz, 2006; Shou, et al., 2000; Yang, et al., 1999a). Drugs primarily metabolized by a single CYP can be utilized as markers to identify the presence and the activity of the target CYP in vivo. In addition, antibodies can be used to study polymorphic CYPs, examine the metabolic consequences of an absent or deficient polymorphic CYP in individual liver microsomes (Gelboin, et al., 1999). If a drug is metabolized by multiple CYPs in HLM the partial inhibition represents the contribution of the inhibited enzyme to the metabolism of the drug. Combined use of multiple antibodies examines the role of the target CYP in the presence of other CYPs competing for the same drug substrate (Gelboin and Krausz, 2006).

To conduct a reaction phenotyping study with inhibitory antibodies, saturating concentrations of antibodies in the incubation containing multiple CYPs (i.e. human liver microsomes, HLM) are necessary to measure the maximum contribution of each CYP for the metabolism of the drug. Thus, to achieve the saturating level of antibody a titration curve needs to be performed to define the maximum inhibition in HLM at known microsomal protein concentration. Kinetic nature of antibody inhibition in CYP-mediated reactions has been characterized to show a non-competitive kinetics which causes increase in $K_m$ but decrease in $V_{max}$ as the antibody concentration increases (Mei, et al., 2002).

f. Clinical implications
Identification of a CYP responsible for the metabolism of a drug is important with respect to clinical DDIs, genetic polymorphism and toxicity (toxicant formation, mutagenesis, carcinogenesis, DNA and protein adduct formation, and immunotoxicity). There are numerous examples in which drug metabolism is implicated in toxicity of therapeutic agents either through impaired clearance in the case of polymorphic mechanisms or increased exposure in the case of reactive or toxic metabolites (Sanderson, et al., 2005; Nakamura, et al., 1985; Hong and Yang, 1997; Song, et al., 2001; Agundez, 2004; Ingelman-Sundberg, 2002; Bartsch, et al., 2000; Knowles, et al., 2003). Impairment of drug metabolism due to enzyme inhibition has obvious clinical implications including toxicity as a result of increased bioavailability and decreased clearance. Reaction phenotyping can help understand the potential mechanism of toxicity and identify individual susceptibility to such toxicity. For instance, drugs metabolized primarily by CYP2D6, CYP2C19 or CYP2C9 are likely to be subject to significant inter-individual variability in their exposure and effects as a result of known genetic polymorphisms. Fluoxetine (CYP2D6), omeprazole (CYP2C19) and warfarin (CYP2C9) are metabolized by the polymorphic enzymes and are still used in clinic; however caution should be taken for the safety margins with patients with known genotype/phenotype (e.g. the CYP2C19 slow metabolizer phenotype is much more prevalent in Asians than in Caucasians) (Goldstein, et al., 1997; Shimoda, et al., 1999; Yokono, et al., 2001; Mizutani, 2003; Niu, et al., 2004; Bertilsson, 2007).

Evaluation of the significance of the CYP reaction-phenotyping data requires the integration of information obtained from various in vitro systems, as well as integration of clinical data (e.g., human radiolabel study and drug interaction studies with probe drugs), allowing one to estimate the fraction of the dose cleared via all CYPs (fm), and the contribution of each CYP to total CYP-dependent clearance (fm,CYP). Integration of the data is key because it is the product of fm and fm,CYP (i.e., fm · fm,CYP) that governs the magnitude of a drug interaction, and the impact of a CYP polymorphism on the PK profile of the victim drug in question. As such information becomes available, more effective clinical studies can be designed and conducted to avoid therapeutic failure (due to the induction of metabolic clearance) and unexpected toxicity (due to overdose...
resulting from impaired metabolic clearance via polymorphic enzymes or enzyme inhibition). Likewise, *in vitro* drug metabolism studies can be valuable in helping medicinal chemists to design out “metabolic liabilities” during the discovery stage (Rodrigues, 1999).

Several polymorphisms in CYP enzymes have been reported as a result of single nucleotide polymorphisms, gene deletions, and gene duplications (Rodrigues and Rushmore, 2002). CYP2D6 and CYP2C19 are polymorphically expressed in different populations such as Asian and Caucasian (Nakamura, et al., 1985; Kimura, et al., 1998). If the *in vivo* clearance of a drug is largely mediated by a polymorphically expressed allelic variant of a CYP, it is anticipated that poor metabolizers will produce elevated plasma area under the curve (AUC) and/or an increased half-life, adversely affecting the safety and efficacy of drugs. In addition, the catalytic activity of CYP3A4-dependent midazolam hydroxylation has been reported to range from 95 to 651 pmol min\(^{-1}\)mg\(^{-1}\) among pooled human liver microsomes (HLMs) from 20 individual donors (Huang, et al., 2004). This wide range of the activity might cause inter-individual variability for drug exposure. Therefore, it is important to determine the CYP contribution to the metabolism of a drug considering the attrition of drug candidates in the discovery process. A growing number of drugs have labels with recommended dose adjustments based on reaction-phenotyping and/or genotype information pertaining to drug-metabolizing enzymes (Nagar and Blanchard, 2006; Sanderson, et al., 2005).

With the advent of polypharmacy, a competitive marketplace, and greater attention paid to public safety (i.e., risk versus benefit), pharmaceutical companies have increasingly focused on new chemical entities (NCEs) with optimal PK/ADME properties that preclude costly therapeutic monitoring and genotyping of patients (Lin and Lu, 1997; Rodrigues and Rushmore, 2002). It is also advantageous to develop NCEs with minimal DDI liabilities, so that package insert (“black box”) warnings can be avoided and patient safety maximized as much as possible. The highly publicized market withdrawals of two DDI “victim” drugs, terfenadine and cerivastatin, were precipitated partially because of a drug interaction involving the inhibition of CYP3A4 (CYP3A4).
and CYP2C8, respectively (Honig, et al., 1993; Backman, et al., 2002; Emoto, et al., 2006; Emoto, et al., 2006; Greenblatt, et al., 1998; Azie, et al., 1998; Gorski, et al., 1998; Gomez, et al., 1995; Floren, et al., 1997; Backman, et al., 1994; Varhe, et al., 1994). More recently, drug interactions involving the inhibition of tizanidine (CYP1A2-dependent), repaglinide (CYP2C8/CYP3A4-dependent) and fluticasone (CYP3A4-dependent) clearance have also received some attention (Niemi, et al., 2003; Ringtong-Sanders, et al., 2006; Backman, et al., 2006). In addition, there are examples of successfully marketed “back up” drugs that have improved DDI profiles and less dependency on polymorphic CYPs for clearance (e.g., fexofenadine versus terfenadine). 

In general the consequences of higher metabolic clearance are tied to the potential effect of co-administered inhibitors on the total exposure of the new drug. Drugs that are not cleared significantly by metabolism (e.g. fm <0.3) will not suffer much when co-administered with an inhibitor of that enzyme, regardless of how potent the inhibitor is or the concentration at which it is given or attains. In contrast a drug that is cleared entirely by metabolism (fm = 1) will be markedly affected by inhibition of its clearance, with a several-fold increase in AUC possible. Consequently, a drug with a narrow therapeutic index that is metabolically cleared by several CYPs is considerably safer than a drug cleared principally by only one CYP. It is possible to determine reaction phenotyping (i.e. fm) after the completion of an antibody inhibition study or any of other approaches. By knowing the enzymes involved in metabolic clearance and their relative contribution, one can simulate the potential effect of inhibition on exposure and safety.

Table 1 summarized applications of CYP-raised antibodies in P450 research. Because CYPs are responsible for the clearance of approximately three quarters of approved drugs (Williams, et al., 2004), identification and characterization of the enzyme(s) responsible for the metabolism of NCEs (reaction-phenotyping) is an important task during the drug discovery and development processes (Rodrigues and Rushmore, 2002; Rodrigues, 1999; Lu, et al., 2003; Emoto, et al., 2006; Williams, et al., 2003). The antibodies that bind and inhibit CYPs are uniquely suited for reaction phenotyping and measure the
contribution of specific CYPs to the metabolism based clearance. The use of antibodies for CYP research is a powerful tool in drug discovery and development.
Reference List


Hong JY and Yang CS (1997) Genetic polymorphism of cytochrome P450 as a biomarker of susceptibility to environmental toxicity. [Review] [32 refs]. *Environmental Health Perspectives* 105:Suppl-62.


Kassahun K, McIntosh IS, Shou M, Walsh DJ, Rodeheffer C, Slaughter DE, Geer LA, Halpin RA, Agrawal N and Rodrigues AD (2001) Role of human liver cytochrome


Nagar S and Blanchard RL (2006) Pharmacogenetics of uridine diphosphoglucuronosyltransferase (UGT) 1A family members and its role in patient response to irinotecan. [Review] [74 refs]. *Drug Metabolism Reviews* 38:393-409.


Tang C, Shou M, Mei Q, Rushmore TH and Rodrigues AD (2000) Major role of human liver microsomal cytochrome P450 2C9 (CYP2C9) in the oxidative metabolism of


Varhe A, Olkkola KT and Neuvonen PJ (1994) Oral triazolam is potentially hazardous to patients receiving systemic antimycotics ketoconazole or itraconazole. *Clinical Pharmacology & Therapeutics* **56**:t-7.


Table 1. Applications of CYP-directed Antibodies.

| Immunoassay (immunohistochemistry, RIA, ELISA and immunoblot) | • Distribution, identification and quantification in tissue and cellular preparations  
• Enzyme induction  
• Enzyme mechanism  
• Toxicity (enzyme level induction)  
• Risk assessment (cancer susceptibility)  
• Membrane topography |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoaffinity</td>
<td>• Purification, precipitation and production</td>
</tr>
</tbody>
</table>
| Immunoinhibition | • Reaction phenotyping: quantitative contribution of a single or multiple CYPs to the metabolism of a drug or toxin (product formation or substrate disappearance)  
• DDI prediction (\( f_{mCYP} \))  
• Pharmacogenetics (phenotypic polymorphism)  
• Toxicity (toxicant formation, tissue and cell toxicity)  
• Bioactivation (mutagenesis, carcinogenesis, DNA and protein adduct formation) |