14 Monoclonal Antibody Analyses of Microsomal Human Drug Metabolism and Multifunctional Cytochrome P450

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14.1 SUMMARY

Monoclonal antibody (MAb)-based methods are ideal reagents for cytochrome P450 (P450) research. The properties and molecular diversity of P450s make them extraordinarily suitable for investigation with MAbs. The P450s are present in multiple forms. Defining the role of each P450 isoform for drug metabolism in human liver microsomes (HLM) *in vitro* requires a method that is both highly specific and highly inhibitory to P450 activity and thus can identify and quantitate the contribution of single or several P450 isoforms that metabolize the drug. MAbs have been isolated that are inhibitory and specific to 13 human P450s. The MAbs may also be used to determine the role of each P450 isoform for a variety of nondrug metabolizing activities such as mutagenesis carcinogen S9 activation and binding to DNA and others described in the text.

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14.2 INTRODUCTION

Our laboratory has produced highly specific and inhibitory MAbs to human P450 1A1, 1A2, 2A6, 2B6, 2 C8, 2 C9, 2 C19, 2 C Family (8, 9, 18, 19), 2D6, 3A4/5, 2E1, and 2 J2. The MAb system identifies drugs metabolized by a single P450, multiple P450s, and alleles of polymorphic P450s. The MAbs may be used to measure the expression of specific alleles of polymorphic P450s as well as the formation of specific optically active metabolites of racemic mixtures. The *in vitro* assay system uses HLM from different individuals for the metabolism of the drug. The MAbs are added either singly or several combinatorially to determine potential interactions in the metabolism of a drug by two or more P450s. MAbs display extraordinary specificity, far greater than that of polyclonal antibodies or chemical inhibitors, which ordinarily are considerably cross reactive. We have used MAb-based analysis to examine the role of different P450 isoforms for diazepam metabolism, taxol metabolism, coumarin hydroxylation, and dextromethorphan metabolism by 2D6 and 2 C9.

14.3 MONOCLONAL ANTIBODY (MAb)

14.3.1 Monoclonal Antibodies: Production

MAbs are produced by the "hybridoma" technology [1,2]. Their discovery is a major achievement of modern biology. The hybridoma technology grew out of the clonal selection hypothesis of Burnet and colleagues [2], which states that each B-lymphocytes antibody-forming cell and its progeny is committed to the production of a single type of antibody molecule. The MAbs are chemically defined reagents that recognize a single antigenic determinant or "epitope" on a macromolecule [2]. This specificity continues for the lifetime of the cell and its progeny.

For the production of MAbs [3], myeloma tumor cells are fused with the isolated dissociated B-lymphocytes of the spleen from mice immunized with a particular immunogen. The fused cells or "hybridomas" exhibit unique and special character by combining the characteristics of the B-lymphocytes that are committed to the continuous and stable production of a specific antibody with the myeloma tumor cells imparting immortal properties to the hybridoma cells. The individual hybridomas are cloned and screened for the desired MAb. The screening is done by a variety of methods including binding assays, Western blots, and enzyme inhibition. The selected clone and its progeny produce identical antibodies directed to a single epitope. The selected clones are usually subcloned three times to assure monoclonality. These hybridomas, grown in cultures in a defined medium, produce the chemically defined MAbs free of contaminating cells and protein debris. Large amounts of MAbs can be obtained by injecting the hybridomas into the peritoneal cavity of appropriate recipient mice, where they grow as ascites tumors and produce large quantities of the specific MAb. The MAb-producing hybridomas are essentially immortal and can be frozen and stored or grown in culture indefinitely.

14.3.2 MAbAnalytic Systems: Liver Microsomes

Comprehensive reviews of the value and utility of MAbs for the study of drug metabolism have been published [4,5]. MAbs were first made to rabbit [6] and rat

TABLE 14.1 Monoclonal Antibodies (MAbs) to Human Liver Cytochrome P450s

cDNA Expressed Human P450	MAb	Ig Subtype	Enzyme Inhibition (%)	Substrate
1A1	1-7-1	Ig G_1	>90	7-Ethoxycoumarin (7-EC), phenanthrene, B[*a*]P
	1-599-16	Ig G_1	None	—
1A2	26-7-5	Ig	>90	7-EC, phenanthrene, B[*a*]P, phenacetin, *p*-nitroanisole
2A6	151-45-4	Ig G_1	>90	Coumarin, phenanthrene, 4-nitrophenol, 7-EC
2B6	49-10-20	Ig G_{2b}	>90	Diazepam, testosterone, 7-EC, phenanthrene
2 C8, 9, 18, 19	1-68-11	Ig M	>90	See 2 C substrates below
2 C8	2B1-1-1	Ig G_1	>90	Diazepam, phenanthrene, taxol
2 C9*1, *2, *3	763-15-5	Ig G_1	>80	Tolbutamide, diclofenac, warfarin, mephenytoin, phenanthrene, diazepam
	763-15-20	Ig G_1	None	—
2 C9*2	292-2-3	Ig G_1	>90	Diclofexiac, tolbutamide
2 C19	1-7-4-8	Ig G_1	>90	Mephenytoin, tolbutamide
2D6	512-1-8	Ig G_1	>90	Bufuralol, dextromethorphan, debrisoquine
	50-1-3	Ig G_1	>90	—
2D6	2D6-50	Ig G_{2b}	>90	Dextromethorphan, testosterone, alfatoxin, bufuralol
	2D6-50	Ig G_{2b}	>90	—
2E1	1-73-18	Ig M	>90	Phenanthrene, toluene, *p*-nitroanisole, chlorzoxazone
	2-106-12	Ig G_1	—	—
3A4/5	3-29-9	Ig M	>90	Diazepam, testosterone, taxol, phenanathrene, alfatoxin B1 (cyclosporim 70–80%)
	275-1-2	Ig M	None	—
2 J2	6-5-20-8	Ig G_1	>90	Arachidonic acid, terfenadine, ebastine, phenathrene

P450s [7]. Table 14.1 shows the MAbs that are P450 isoform specific and are >90% inhibitory to the expressed target P450 activity. Some, but not all the MAbs also immunoblot the target P450 (Table 14.2). The amount of inhibition of drug metabolism *in vitro* by HLM with the addition of the specific MAb quantitates the contribution of the target P450 to the metabolism of the drug. The MAb method is in stark contrast to other complex analytic systems that are relatively nonspecific and currently being

TABLE 14.2 Immunoblotting MAbs

cDNA Expressed Human P450	MAb	Ig Subtype
1A1	1-599-16	Ig G_1
1A2	26-7-5[a]	Ig G_1
2A6	151-45-4[a]	Ig G_1
2B6	49-10-20[a]	Ig G_{2b}
2 C8	281-1-1[a]	Ig G_1
2 C9*1, *2, *3	763-15-20[b]	Ig G_1
2D6	512-1-8[a]	Ig G_1
2E1	2-106-12	Ig G_1
3A4/5	275-1-2	Ig M

[a] Also inhibitory.
[b] Also immunoblots 2 C8.

used to study drug metabolism. Drugs metabolized by a single P450 isoform or metabolites formed from a single metabolic pathway may be useful as markers for identifying the target P450 *in vivo*. The MAb methodology is precise, simple, and highly reproducible. The MAbs, which are highly specific and highly inhibitory, may also be used to measure the specificity and the potency of chemical inhibitors.

14.3.3 Monoclonal Antibody and Human Liver Microsomes Analytic System

Figure 14.1 shows production and analytic system of MAbs. Specific inhibitory MAbs identify individual P450s responsible for drug metabolism in HLM *in vitro*. The MAb system analyzing HLM identifies the P450 isoforms that catalyze the metabolism of the drug substrate and quantitates the maximum contribution of each P450 for the metabolism of the drug. The MAb system uses saturating levels of both the MAb and the drug substrate using zero-order kinetics for the reaction, and thereby defining the maximum contribution of each P450 isoform to the metabolism of the drug. The system also identifies drugs that are metabolized by a single P450, two or more P450s, and polymorphic P450s. The drugs metabolized by a single P450 have potential for use as markers identifying the presence and the activity of the target P450 *in vivo*. The MAbs also identify the P450s catalyzing metabolism in alternative pathways, yielding metabolites that may be used as *in vivo* markers for specific P450s. The system can study polymorphic P450s, examine the metabolic consequences of an absent or deficient polymorphic P450 in individual microsomes, and identify other active nonpolymorphic P450s that can reduce the deleterious effect of the missing or deficient isoforms. The methodology of the MAb system is simple. Basal control levels of metabolism of the drug are measured, and the amount of inhibition of metabolism on the addition of the isoform-specific inhibitory MAb determines the quantitative role of each P450 for the metabolism of the drug. The MAb system of analysis of HLM metabolism is entirely self-contained, and the values obtained are the results of diverse elements such as interindividual differences in the amount and activity of the target P450 as well as factors such as lipid and cofactor content, P450 protein structural configuration, membrane–enzyme interactions, and other currently unknown factors. Table 14.1 is a list of the human cytochrome P450 enzymes for which MAbs have been made in the Laboratory of Metabolism, National Cancer Institute. These are MAb 1-7-1,

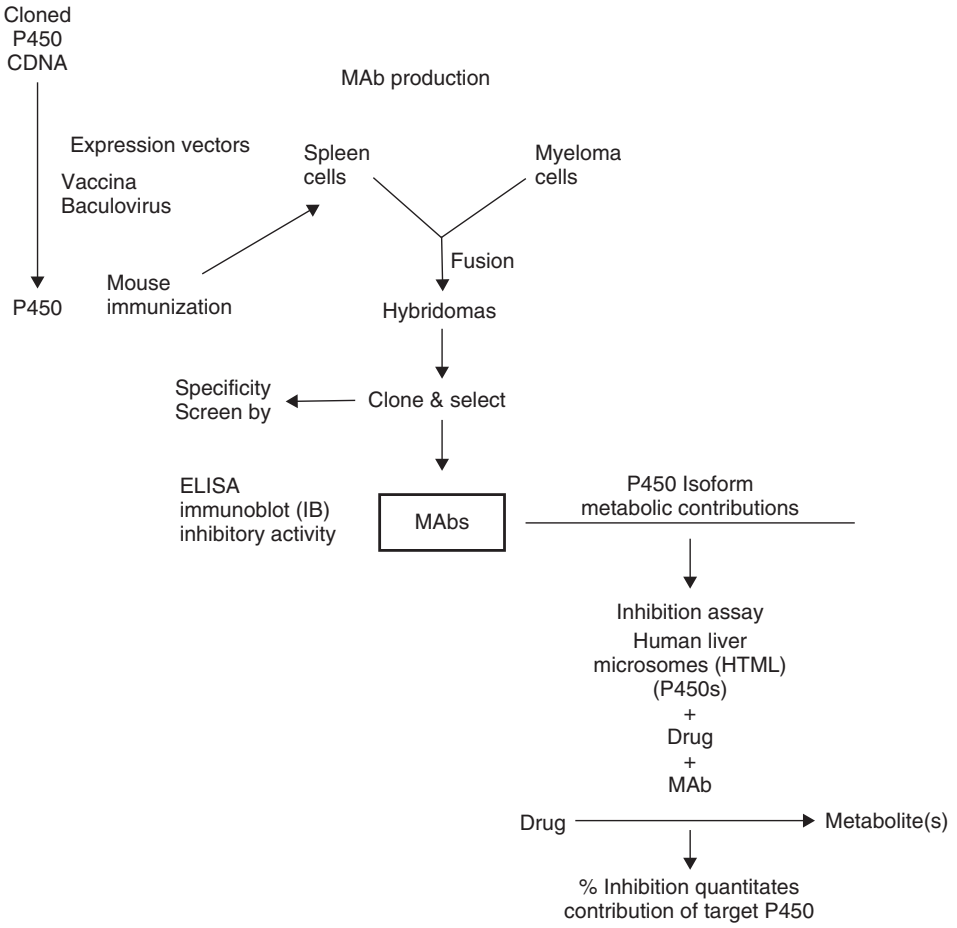

Figure 14.1 Monoclonal antibodies to human cytochrome P450s (preparation and assay).

which inhabits both 1A1 and 1A2 in rat livers but is specific only to 1A1 in human tissues [8]. The MAb 1-7-1 has also been used to phenotype 1A1 in different human tissues [9] and phenotype differences in single and twin human placenta [10]. Anti-P450 MAbs have been made to 1A2 [11], 2A6 [12], 2B6 [13], 2C8, 2C9, 2C19, 2C [14,15], 2D6 [16,17], 2E1 [18], 2J2 [19], and 3A4/5 [20]. One of the MAbs, 1-68-11, binds and inhibits each of the 2C8, 2C9, and 2C19 isoforms of the P450 2C family, indicating that they share a common epitope. Table 14.1 also shows the Ig type of each of the MAbs and their inhibitory activity toward the expressed P450 isoform, which was >80–90%. The last column of Table 14.1 lists the substrates that were used to measure the inhibitory activity of each MAb to the expressed P450.

14.4 IMMUNOBLOTTING MAbs

Table 14.2 lists nine MAbs that immunoblot the target P450. There are large interindividual variations in the protein content as well as enzyme-inducible activity of the

different P450s. The immunoblotting MAbs can be used to determine the protein amount of a specific P450 in human tissues [13] and measure interindividual differences of different populations. The immunoblotting MAbs have also been used in immunohistochemical studies. A noninhibitory MAb, anti-P450 1B1, has been used for the immunohistochemical localization of 1B1 in breast cancer [21]. The MAb anti-rat P450 2E1 has been used for immunohistochemical studies of murine lung [22] and for studies on the effects of acetone and ethanol in murine liver [23]. Among other functions, P450 2J2 metabolizes arachidonic acid and is involved in calcium regulation in cardiomyocytes [19].

14.5 MAb PROPERTIES

14.5.1 Monoclonal Antibodies and Epitope Specificity

The specificity of the MAb resides in its precise ability to recognize and bind a specific epitope on the surface of a P450. Thus, an MAb may recognize only a single species of P450 if it contains a unique epitope. In this case, the MAb would be specific for only a single isozymic form of P450. AnMAb that recognizes an epitope that is present in more than one form of P450 will bind all of those forms of cytochrome P450 containing the common epitope. On theoretical grounds, a comprehensive MAb library will contain MAbs that recognize single unique forms of P450.

14.5.2 Monoclonal Antibody Allele Specificity

Table 14.3 demonstrates the allele specificity potential of a MAb. MAb 292-2-3 specifically binds to a single allele, P450 2C9*2(Arg_{144}Cys), of three expressed P450 2C9 allozymes (*1,*2,*3). This MAb does not bind the 2 C9*1(Arg_{144}) wild type or the third allele P450 2C9*3($Ileu_{359}$Leu) [24]. Of the three alleles of P450 2C9, the wild type and two polymorphic alleles have only a single amino acid substitution relative to the wild type and differ from the wild type by a single amino acid, and thus MAb

TABLE 14.3 MAb Allele Specificity

Allele		Tolbutamide	Diclofenac
2 C9*1 Arg_{144}	Control	1.0[a]	0.30
	+MAb	0.98	0.30
	% Inhibition	1.3	0.1
2 C9*2 Arg_{144}Cys	Control	0.85	0.42
	+MAb	0.03	0.02
	% Inhibition	96.4	95.1
2 C9*3 $Ileq_{359}$Leu	Control	0.22	0.35
	+MAb	0.20	0.30
	% Inhibition	9.3	14.2

MAb 292-2-3 inhibits only the single allele 2 C9*2 arg144cys catalyzed tolbutamide and diclofenac metabolism.

[a] Activity (mmol product/min/nmole P450).

292-2-3 detects a single amino acid substitution. MAb 292-2-3 inhibits the P450 2C9*2 allozyme-catalyzed metabolism by 96% and shows no inhibition of the wild type P450 2C9*1 metabolism. The P450 2C9*2 allozyme-specific MAb does not bind to any of the other P450 2C9 isoforms, 2 C8, 2 C18, or 2 C19, or any of the other human P450s, 1A1, 1A2, 2A6, 2B6, 2D6, 2E1, or 3A4/5. The MAb 292-2-3 inhibits the metabolism of tolbutamide, diclofenac, and phenanthrene by the P450 2C9*2 by >90% and does not inhibit the enzyme activity by the other two alleles or any of the other human P450 isoforms listed above. The MAb 292-2-3 can be viewed as a prototype of an ideal and extraordinary specific reagent for the detection and measurement of the metabolic role of highly related isoforms and polymorphic alleles of human cytochrome P450 [24].

14.5.3 Monoclonal Antibody Enantiomer Specificity

Phenprocoumarin is a potent anticoagulant in common usage and is hydroxylated by the P450s of the 2 C family. One route of metabolism converts the drug to both the *S*-4-OH and to its enantiomeric *R*-4-OH (Table 14.4). The MAb specific to P450 2C8 inhibits the formation of the R-enantiomer and does not inhibit the formation of the S-enantiomer. The MAb to P450 2C9 also shows enantiomer specificity, inhibiting the *R*-4-OH formation and not inhibiting the *S*-4-OH formation [25]. The MAb anti-P450 2C9 also exhibited enantiomer selectivity. P450 2C9 catalyzes the demethylation of the endocrine disrupter pesticide methoxychlor, forming a chiral product [26]. The HLM generates primarily the *S*-mono OH form. The MAb anti-2 C9 selectively reduces the formation of the S-form from 80% to 55%, thereby enantiomer selectively changing the ratio of the S- and R-enantiomers formed. This study also found that expressed P450 2C19 exhibited the highest level of activity for demethylation but was not inhibited by MAb anti-2 C19 in HLM, thus indicating that P450 2C19 is not active in HLM. These unusual results suggest that with certain substrates (e.g., methoxychlor), there is no correlation between the expressed enzyme activity (in this case, P450 2C19) and its presence in HLM. MAbs have also been used to study the mechanism of toxicity of the chemotherapeutic agent ifosfamide, which is believed to be due to its conversion to nephrotoxic chloroacetaldehyde [27]. The study showed that metabolism of ifosfamide was catalyzed by P450 3A4 and 2B6, both of which are present in fetal, pediatric, and adult tissues. The P450 3A4 and 2B6 were found in the fetus as early as eight weeks of gestation. Thus, the MAb system can easily map the issue distribution of P450 isoforms in fetal as well as adult tissue and help to clarify the mechanism of toxicity of chemical agents.

TABLE 14.4 MAb Enantiomer Specific Hydroxylation of Phenprocoumarin

	Product Formation/% Inhibition			
Inhibitor	[*S*] 4′OH	[*R*] 4′OH	[*S*] 6′OH	[*R*] 6′OH
MAb anti-2 C8	32	<5	15	<5
MAb anti-2 C9	31	<5	52	40
Triacetyloleandomycin	52	80	40	51

TABLE 14.5 Inhibition of 7-ECO Metabolism in HLM by the Addition of a Single Purified MAb Compared to its Inhibitory Effects when in Combination with Other Mabs[a]

HLM (Activity[b])	Single MAb		Combinatorial Addition of MAbs	
	MAbs to P450	% Inhibition	MAbs to P450	% Inhibition
HL 80	Control	0	Control	0
(0.52)	2E1	26.1	2E1	33.5
	2A6	2B.9	2E1 + 2A6	30.1
	2B6	31.5	2E1 + 2A6 + 2B6	23.9
	1A2	9.5	2E1 + 2A6 + 2B6 + 1A2	4.4

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.
[a]Purified MAbs from ascites.
[b]Activity is in nanomole 7-OH coumarin formed/min/nmol total P450.

14.6 SINGLE AND COMBINATORIAL ANALYSES

Table 14.5 shows the MAb-based analysis of 7-ethoxycoumarin deethylase activity using purified MAbs added singly to the reaction mixture compared to analysis with a combinatorial system. The values obtained using the single MAb addition for measuring the contribution of P450 2E1, 2A6, 2B6, and 1A2 corresponded well with values obtained for the same four P450s using the combinatorial analysis with MAbs against each of the four P450s. The combinatorial system permits the concurrent analysis of the contribution of up to four different P450s. Table 14.6 is a summary of the interindividual differences in the activity of four different HLM comparing the single and combinatorial systems. There was wide variability in the basal activity of the different HLM samples. The largest approximate amount of activity was due to P450 2E1 and 2B6. *P*4502B6 activity ranged from 11% to 50%, P450 2E1 from 16% to 57%, P450 1A2 from 7% to 22%, and P450 2A6 from 8% to 32%. Table 14.6 uses ascites fluid as the MAb source, which, in comparison, is similar to the data in Table 14.5 using the purified MAbs. These values could not be determined by simply using the expressed P450 system for analyses. The enzyme activities exhibited by expressed P450 isoforms do not accurately reflect their activities in the HLM and obviously cannot be used to examine interindividual differences. However, the expression system is valuable for identifying P450s that exhibit enzymatic activity toward a given drug substrate. The presence of high activity of an expressed isoform may be a presumption but not a certainty indicating the presence of the isoform activity in HLM. Figure 14.2 shows a combinatorial analysis of diazepam hydroxylation to temazepam (TMZ). For this figure, the height of each column is compared to the height of the previous column. No difference in the height of the adjacent column indicated that the P450 isoform of the second column does not contribute to the reaction. The MAbs anti-2B6 and anti-3A4 were combined for convenience with the previous knowledge that P450 2B6 is inactive for TMZ formation. P450 3A4 is the only isoform that exhibits metabolic activity for TMZ formation. The amount of activity exhibited by 3A4 ranges from 90% in HLM 80 to 70% in HLM 79. P450s 2 C8, 2 C9, and 2 C19 exhibit no activity. In contrast, Fig. 14.3 shows the variable contribution in eight HLM of four P450 isoforms for the N-demethylation of diazepam to nordiazepam (NDZ). The large interindividual variation is reflected by the contribution of P450 3A4/2B6 activity, which ranges from

TABLE 14.6 MAb[a] Single and Combinatorial Analysis of Ethoxycoumarin Deethylation (ECOD)

	Single MAb		Combinatorial Addition of MAbs			
HLM	MAb to P450	Inhibition (%)	MAb to P450	Total Inhibition	% Contribution P450	
HLM75	Control		Control		Control	
	2E1	26	2E1	26	2E1	26
	2A6	28	2E1 + 2A6	58	2A6	32
	2B6	21	2E1 + 2A6 + 2B6	80	2B6	22
	1A2	22	2E1 + 2A6 + 2B6 + 1A2	98	1A2	18
HLM 76	Control		Control		Control	
	2E1	16	2E1	16	2E1	16
	2A6	22	2E1 + 2A6	42	2A6	26
	2B6	50	2E1 + 2A6 + 2B6	85	2B6	43
	1A2	14	2E1 + 2A6 + 2B6 + 1A2	96	1A2	11
HLM 77	Control		Control			
	2E1	57	2E1	57	2E1	57
	2A6	7	2E1 + 2A6	68	2A6	11
	2B6	20	2E1 + 2A6 + 2B6	79	2B6	11
	1A2	10	2E1 + 2A6 2B6 1A2	89	1A2	8
HLM 80	Control		Control		Control	
	2E1	22	2E1	22	2E1	22
	2A6	15	2E1 2A6	30	2A6	8
	2B6	43	2E1 2A6 2B6	78	2B6	48
	1A2	10	2E1 2A6 2B6 1A2	85	1A2	7

Abbreviations: 7-ECOD, 7-ethoxycoumarin deethylation; HLM, human liver microsome; MAb, monoclonal antibody.
Control Activity: HLM 75 (1.6), HLM 76 (4.6), HLM 77 (3.1), HLM 80 (1.4).
[a]MAb in ascites fluid.

20% to 80%, and the large variable contribution from P450 2C9 ranges from 10% to 46% and 3–30% from P450 2C8. There is little or no contribution from P450 2C19. It is important that a significant number of samples of HLM be examined in these studies. These data are also summarized in Table 14.7.

14.7 DRUGS METABOLIZED BY A SINGLE P450

Taxol 3′-hydroxylation is catalyzed solely by P450 3A4/5, and there is relatively constant inhibition of 6-hydroxy formation by MAb anti-2 C8 (Table 14.7), which indicated that there is relatively little interindividual variation in the nine HLM samples for taxol 6-hydroxylation catalyzed by P450 2C8. Table 14.7 shows that coumarin hydroxylation is catalyzed entirely by P450 2A6 and thus would be an excellent drug substrate marker for the *in vivo* presence of P450 2A6. The MAb system also determines the role of each P450 isoform for the metabolism of a drug through alternate metabolic pathways, such as is the case with diazepam and taxol. When a metabolite of a drug is the result of the activity or affinity of a single P450,

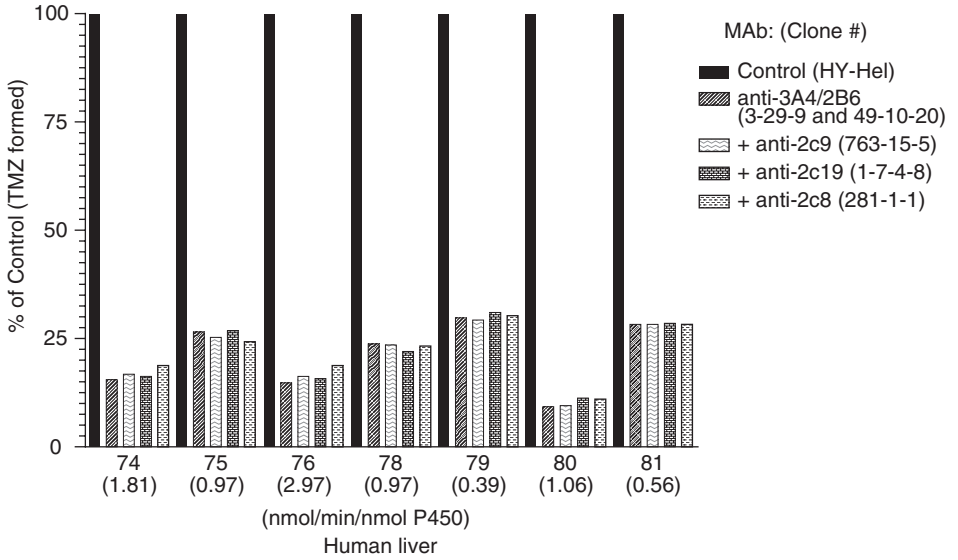

Figure 14.2 MAb combinatorial analysis of diazepam hydroxylation in human liver microsomes. The difference between each bar represents the amount of P450 activity contributed by the subsequent addition of the indicated MAb. Results are the average of duplicate incubations. Values in parenthesis are control activity.

the metabolite identifies the P450 isoform as a potential marker for the *in vivo* presence of the P450 isoform. Metabolite analysis for taxol 6-hydroxy and taxol 3-hydroxy formation catalyzed solely by P450 2C8 and 3A4 would be excellent markers for the *in vivo* presence of P450 2C8 and 3A4, respectively. Likewise, coumarin hydroxylation that is metabolized solely by 2A6 would be an excellent marker for 2A6 activity *in vivo*. There are two extensive reviews [28,29] on the use of probe drug substrates for the *in vivo* targeting of a P450 isoform for its role in the metabolism of a drug. A primary concern for this method is the specificity of the probe drug and the potential contribution of low activity isoforms to the reaction *in vivo*. In many cases, the specificity of the probe, substrate has not been determined for either the metabolism of the drug or the formation of marker metabolites arising from alternate markers must be responsible for essentially the entire metabolism of the drug, and minor P450s should not contribute to the reaction. Minor isoforms exhibiting activity for drug metabolism may cause a misreading of the identification of the *in vivo* target P450. Combinatorial analysis of diclofenac 4-OH formation indicates that the P450 2C9 is the primary and major catalyst for diclofenac hydroxylation. Minimal or very slight activity is shown by other members of the P450 2C family. Table 14.8 indicated that in 10 HLM, 83–88% of activity was due to P450 2C9, and 7–8% was contributed by P450 2C19. The activity of the expressed P450s for hydroxyl tolbutamide formation shows that two alleles of P450 2C9, 2C9*1, and 2C9*2, show high activity, with P450 2C9*2 exhibiting half the activity of the wild type. P450 2C19 exhibits moderate activity, and little or no activity is shown by the other expressed P450s—2C8, 2C18, and 2D6. Table 14.8 shows that in eight HLM, P450 2C9 contributes 77–82% of tolbutamide 6-hydroxylation activity with variable low contributions of about 5–10% from 2C8 and 2C19. Table 14.8

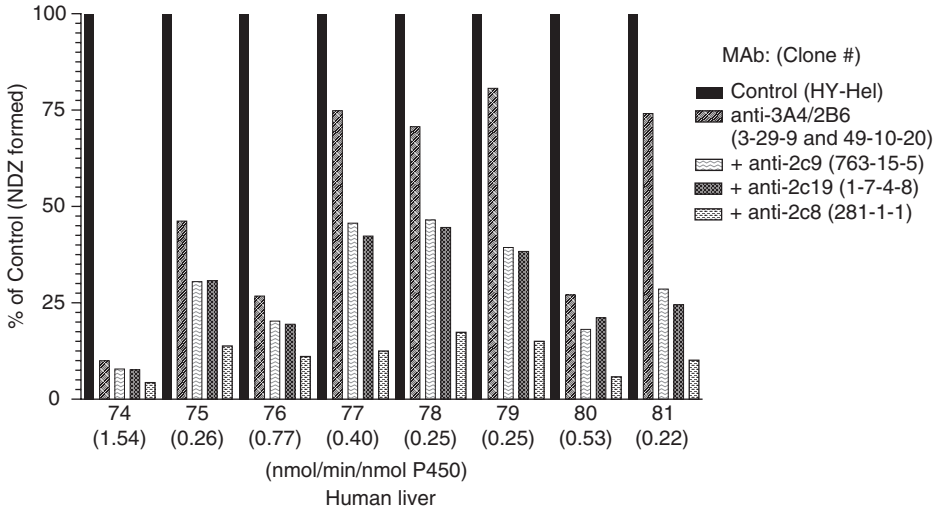

Figure 14.3 MAb combinatorial analysis of diazepam N-demethylation in human liver microsomes. The difference between each bar represents the amount of P450 activity contributed by the subsequent addition of the indicated MAb. Results are the average of duplicate incubations. Values in parenthesis are control activity.

TABLE 14.7 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)				
	Diazepam ↓ Temazepam (OH)	Diazepam ↓ Nordiazepam ($N\text{-}CH_3$)	Taxol ↓ 6-OH Taxol	Taxol ↓ 3-OH Taxol	Coumarin ↓ 7-OH Coumarin
MAb anti-1A2	0	0	0	0	0
2A6	0	0	0	0	$>90_{(8)}$
2B6	$1–5^{a}{}_{(6)}$	$6–23_{(6)}$	0	0	0
2 C8	0	$3–30_{(8)}$	$83–91_{(8)}$	0	0
2 C9	0	$2–46_{(8)}$	0	0	0
2 C19	0	$0–4_{(8)}$	0	0	0
2D6	0	0	0	0	0
2E1	0	0	0	0	0
3A4/5	$75–84_{(6)}$	$14–45_{(6)}$	0	$73–90_{(6)}$	0

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.
(), Number of HLM samples; 0, No activity of expressed enzyme.
[a]Values <5% are not reliable.

also shows the MAb-based analysis of HLM for the P450-catalyzed metabolism of 7-ethoxycourmarin, phenacetin, diclofenac, *S*-mephenytoin, dextromethorphan, and bufaralol. *S*-mephenytoin metabolism was strongly and primarily catalyzed by the expressed P450 2C19 and only slightly by P450 2C9. Table 14.8 also shows that in seven HLM, P450 2C19 was the major catalyst. Significant but very low activity was

TABLE 14.8 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)						
	7-Ethoxycoumarin ↓ 7-OH coumarin	Phenacetin ↓ Acetaminophen	Diclofenac ↓ 4-OH diclofenac	S ± Mephenytoin ↓ 4-OH mephenytoin	Tolbutamide ↓ 6-OH tolbutamide	Dextromethorphan ↓ Dextrophan	Bufuralol ↓ 4-OH bufuralol
MAb anti-1A2	$7–16_{(10)}$	$64–84_{(16)}$	0	0	0	0	$5–20_{(3)}$
2A6	$7–28_{(10)}$	$0–9_{(16)}$	0	0	0	0	0
2B6	$18–30_{(10)}$	0	0	0	0	0	0
2 C8	0	0	$0_{(2)}$	0	$3–10_{(8)}$	0	0
2 C9	0	$1–17_{(8)}$	$83–88_{(10)}$	$10–22_{(7)}$	$77–82_{(8)}$	$38–45_{(2)}$	$10–18_{(2)}$
2 C19	0	$0–9_{(8)}$	$7–8_{(2)}$	$72–87_{(2)}$	$4–8_{(8)}$	$1–4^{a}_{(2)}$	$8–27_{(2)}$
2D6	0	0	0	0	0	$35–91_{(17)}$	$9–70_{(21)}$
2E1	$16–60_{(10)}$	0	0	0	0	0	0
3A4/5	0	0	0	0	0	0	0

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.
(), Number of HLM samples; 0, No activity of expressed enzyme.
[a]Values >5% are not reliable.

contributed by 2 C9. However, when the MAb anti-2 C19 was added before the addition of the MAb anti-2 C19, the 2 C9 showed significant activity (20–25%). These results suggest that there may be competition for certain substrates by two or more P450 isoforms. Minor or moderate contributions of activity form P450 isoforms other than those responsible for the major amount of metabolism may seriously limit interpretation and cause a misreading of the role of different P450s *in vivo*. This is especially true when there is large interindividual variability in microsomal P450-catalyzed metabolism.

14.8 ADDITIONAL MAb FUNCTIONS

14.8.1 Polymorphisms

The first P450 polymorphism found to have clinical significance was the discovery of the 2D6 polymorphism. Dextromethorphan metabolism has been used as primary marker of P450 2D6 activity. Figure 14.4 is a combinatorial analysis of dextromethorphan metabolism in eight HLM. The MAb-based analysis indicated that in individuals polymorphically deficient in 2D6 activity, the P450 2C9 partially replaces the absent 2D6-catalyzed metabolism and catalyzes up to 50% of the total dextromethorphan metabolism. A parallel analysis of bufuralol metabolism with the same eight HLM in Fig. 14.4 showed that in the MAb anti-2D6-based analysis of the same eight HLM, in six of the eight HLM, 2D6 metabolized 80–90% of bufuralol metabolism. The two HLM deficient in 2D6 metabolism of dextromethorphan were also deficient in bufuralol hydroxylation. In these two HLM, 1A2 showed no activity, 2 C9 contributed 15–25% activity, and 2 C8 showed 10–40% activity. Specific and inhibitory MAbs have been used in a variety of studies. To cite several examples, MAbs have been used to identify the human P450s responsible for the *in vitro* formation of *R*- and *S*-norfluoxtine [30]. MAbs have also identified P450 2C8 and 3A4 as the principal enzymes involved in the metabolism of the insulin secretogue repaglinide [31]. P450 2D6 and 3A4 were found responsible for hydrocodone oxidation [32]. A variety of environmental agents, including acylether, nicotine, *N*-nitrosoethylamine, and *N*-nitrosobenzyl-methlyamine, are metabolized by P450 2A6 [33]. Proprofol is metabolized by P450 2B6 [34]. P450s 3A4 and 2 C9 are responsible for the metabolism of 17α-ethinylestradiol [35]. It would be useful to have a comprehensive array of MAbs to all the enzymes of phase II drug metabolism. However, that would be difficult to attain because the conjugating enzymes UDPGT, GST transferase, and sulfotransferase all have numerous different isoforms, and phenotyping an individual with a large array of MAbs to each isoform would be very difficult.

14.8.2 Endobiotic Metabolism

Table 14.9 shows the metabolism of endobiotics and nondrug xenobiotics responsible for chemical toxigenesis. Table 14.9 shows that in 18 HLM samples, P450 3A4/5 was responsible for 72–86% of testosterone 6-β-hydroxy formation. Table 14.9 also shows an analysis of the metabolism of three xenobiotics. In 18 HLM samples, 32–60% of chlorzoxazone metabolism was catalyzed by P450 2E1 and 16–46% of nitroanisole metabolism was metabolized by P450 2E1. In 18 HLM samples, 74–86% of the

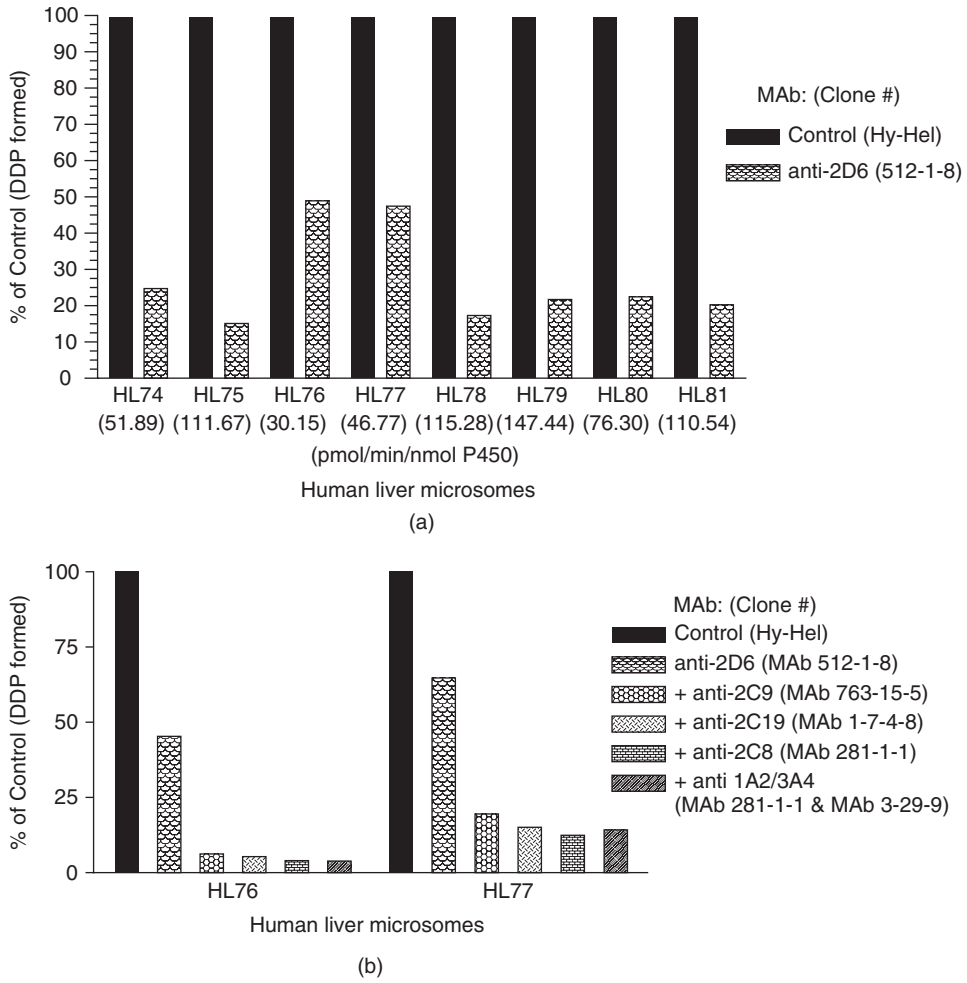

Figure 14.4 MAb analysis of dextromephorphan metabolism in human liver microsomes. Each bar represents the P450 activity relative to control after the subsequent addition of the indicated MAb. MAbs were added singly (a) or in combinatorial succession (b). Results are the average of duplicate incubations. Values in parenthesis are control activity.

metabolism of the potent carcinogen aflatoxin was metabolized by P450 3A4. The analysis of the metabolism of chlorzoxazone and nitroanisole was incomplete because the role of other P450s was not defined.

14.9 OTHER METHODS OF MICROSOMAL ANALYSIS

14.9.1 Specificity and Potency of Chemical Inhibitors

Chemical inhibitors have been used extensively to identify the role of different P450 isoforms in the metabolism of a drug. The chemical inhibitors are generally selective and do not display the specificity of the MAbs that is required to target and

TABLE 14.9 Monoclonal Antibody (MAb)-Determined Contribution of Individual P450 Isoforms to Metabolism in Human Liver Microsomes (HLM)

	% Contribution of P450 Isoforms (Amount of MAb Inhibition)			
	Testosterone ↓ 6β-OH Testosterone	Chlorzoxazone ↓ 6-OH Chlorzoxazone	4-nitroanisole ↓ 4-nitrophenol	Aflatoxin-B1 ↓ 3-hydroxylation
MAb anti-				
1A2	0	?	?	—
2A6	0	0	$22–65_{(7)}$	—
2B6	0	0	?	—
2 C8	0	—	0	—
2 C9	0	?	0	—
2 C19	0	—	?	—
2D6	0	?	?	—
2E1	0	$32–60_{(18)}$	$16–46_{(8)}$	—
3A4/5	$72–86_{(18)}$	?	0	$74–86_{(18)}$

Abbreviations: HLM, human liver microsomes; MAb, monoclonal antibody.
(), of HLM samples; 0, activity of expressed enzyme;—, Not determined; ?, with expressed P450; not yet assayed with Mab/HLM.

quantitate the role of a P450 isoform responsible for a drug's metabolism. Chemical inhibitors have been used rather extravagantly, with little attention to their lack of isoform specificity. The MAb system can be used to assess both the potency and the specificity of a chemical inhibitor. The MAb-based analyses were used to evaluate the chemical inhibitors, ketaconazole, orphenadrene, and quinidine, which are commonly used as inhibitors of P450 3A4, 2B6, and 2D6, respectively. Ketoconazole at 100 μM inhibits the metabolism of phenanthrene by 80%, indicating the participation of P450s other than 3A4. The ketoconazole inhibitor does not identify all the other P450s responsible for phenanthrene metabolism. Quinidine is thought to be a P450 2D6-specific inhibitor, and debrisoquine metabolism is generally used as a P450 2D6-specific marker. However, a report found that debrisoquine is also metabolized by P450 1A1, and the metabolism is inhibited by quinidine [36]. Thus, neither ketoconazole nor quinidine is a specific inhibitor of P450 3A4 or 2D6, respectively. Orphenadrine is commonly used as a P450 2B6 inhibitor. The MAb system was used to measure the relative potency of orphenadine compared to the specific inhibitory MAb anti-2B6. Phenanthrene metabolism was inhibited by 60% by the MAb anti-2B6, whereas 100 μM orphenadrine inhibited the reaction by only 25%, indicating its relatively poor inhibitory potency of orphenadrine compared to the inhibitory anti-2B6 MAb. Thus, the specific MAbs can be used to analyze both the specificity and potency of a chemical inhibitor. A different study examined the specificity of inhibition of expressed P450 with eight commonly used chemical inhibitors [37]. Ketaconazole and quinidine exhibited similar high inhibitory activity toward P450 3A4 and 1A1, sulphenazole showed selectivity for P450 2C9, and α-naphthoflavone (ANF) inhibited both P450 1A1 and 1A2. The remaining inhibitor selectivities were at best moderate. The major advantage of the MAbs compared to chemical inhibitors is the extraordinary specificity of the MAbs. Some of the chemical inhibitors, such as ketaconazole, can be highly selective

but not specific for a target P450. However, a major advantage of a highly selective chemical inhibitor, such as ketaconazole, is the ability to administer the inhibitor to the patient. That is not possible with the MAbs because they cannot be administered *in vivo*. Thus, chemical inhibitors can be used to alter routes of metabolism and metabolic pathways that would be therapeutically advantageous.

14.9.2 Monoclonal and Polyclonal Antibodies

MAbs derive their single-epitope specificity by being formed from a single MAb produced by a parent spleen cell. The MAb-producing spleen cells are immortalized by fusion with myeloma cells, producing hybridomas that maintain their single-epitope specificity. The hybridomas are stable and continuously produce identical MAbs that can be used universally and are transferable to any laboratory as pure reagents. In contrast, polyclonal antibodies commonly display cross-reactivity because they are heterogenous mixtures of monoclonals that, by definition, vary with each immunization, even when using pure immunogens. Undefined polyclonal antibodies resulting from independent immunizations require extensive purification and characterization to eliminate potential and undesirable cross-reactivity. The major advantage of polyclonal antibodies is the relative simplicity of their production compared to the isolation of highly specific and highly inhibitory MAbs to target specific P450 isoforms. The hybridomas producing MAbs are essentially immortal and produce identical MAbs with an identical epitope-binding character. Experimental results with polyclonal antibodies from different laboratories are not comparable because the polyclonal antibodies are epitopically different and may have different cross-reactivity. Thus, the MAbs are more reliable reagents than the polyclonal antibodies.

14.10 MULTIFUNCTIONAL CYTOCHROME P450

Cytochrome P450 is multifunctional and the MAb technology can be successfully applied to the study of a variety of diverse functions of P450 and within each system identify the P450 isoform responsible for the observed biological activity (Table 14.10). The MAb system can be used to map the protein amount and subcellular content and the activity of individual P450s in different human tissues. For each tissue or cell, the inhibitory MAbs may define the amount of P450-specific enzyme activity present and the immunoblotting MAbs measure the P450 isoform protein amount. The MAb system demystifies the "S9" used commonly for activation in the Ames mutagen detection system [38], an activation system previously described for the covalent binding of benzopyrene to DNA [39]. The activity of the "S9" is microsomal and a function of the heterogeneous mixture of P450 isoforms in the microsome-derived "S9" [39]. The MAbs can identify those isoforms responsible for the "S9" activity. The MAb system identifies the individual P450s that catalyze the conversion of compounds to electrophiles that are mutagentic [40,41] or to active metabolites that covalently bind to DNA and protein. The MAb system identifies the P450 isoforms that convert environmental agents to mutagenic, carcinogenic, toxic, and immunotoxic metabolites. The MAb system may identify the function of each P450 in the metabolism, induction, and

TABLE 14.10 Multifunctional Cytochromes P450 and Monoclonal Antibodies

Monoclonal Antibodies Identify P450 Isoform Content, Function and Expression Proteomics
Allyl(Allozyme)expression
S-9 Dymystification-mutagen activation
DNA covalent binding
Protein covalent binding
Tissue and developmental mapping
Cell culture expression
P450 in metabolism, induction, and inhibition
Conversion of environmental agents, natural products, and neutraceuticals to carcinogenic, mutagenic, toxic, and immunotoxic agents
Potency and specificity of chemical inhibitors
Monoclonal Antibodies and Drug Metabolism
Identification and quantification of drug metabolism by single, several, or polymorphic P450s
Identification of drugs and metabolites useful for *in vivo* phenotyping
Purification of proteins

inhibition of P450 activity by neutraceuticals, natural products, and other environmental chemical agents. The MAb system may also identify the expression of different P450 isoforms in cells grown in a variety of cell culture systems.

14.11 SYNOPSIS AND CRITIQUE (DRUG METABOLISM)

Different methods have been used to identify and quantitate the role of each P450 for the metabolism of a drug [42,43]. One method is the measurement of the enzyme activity of the expressed pure P450 toward specific substrates. The amount of substrate metabolized by the pure expressed enzyme determines its capability to metabolize the substrate, but it is often not a good indicator of a role of the P450 isoform for *in vitro* microsomal metabolism. The P450 expression method does not take into account different amounts of the P450 isoform protein present in HLM and its usual large interindividual variability. The HLM analysis of a P450 isoform activity depends on the P450 protein content and other factors, such as cofactor and lipid content, membrane and structural positioning, and potential for competition with P450 isoforms that may also metabolize the subject drug. Drug therapeutics and drug discovery would be promoted if drugs or combination of drugs were available or developed that would be designed specifically for highly reliable phenotyping of the patient. Knowledge of the patient's phenotype would greatly enhance the ability of the clinician to reduce adverse drug reactions and administer effective drugs or combination of drugs at the right dosage by having a precise knowledge of the patient's phenotype for the metabolism of the drugs. The MAb method is in stark contrast to other complex analytic systems that are relatively nonspecific and currently being used to study drug metabolism. The MAb system identifies and quantitates drugs metabolized by a single P450, multiple P450s, and polymorphic P450s. Drugs metabolized by a single P450 isoform or metabolites formed from a single metabolic pathway may be useful as markers for identifying the target P450 *in vivo*. The MAb methodology is precise, simple,

and highly reproducible. The MAbs, which are highly specific and highly inhibitory, may also be used to measure the specificity and the potency of chemical inhibitors.

The MAbs will prove useful for identifying and quantifying P450 proteins that vary under different conditions, which include nutritional and hormonal states, developmental stages, age, sex, and inducer exposure. A full library of MAbs to P450 could develop a P450 taxonomy and would greatly enhance the progress of P450 research.

14.12 CONCLUSION

This chapter evaluated the efficacy of the various methods used to examine the role of P450 isoforms for the metabolism of a drug. The conclusion of this analysis was that specific and potent inhibitory MAbs against the different P450 isoforms (such as those listed in Table 14.1), which are characterized for specificity and inhibitory potency and verified with HLM and the expressed P450, represents the most valuable approach and basically the only method necessary for a phenotyping study.

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