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COMMENTARY

Integrated Cytochrome P450 Reaction Phenotyping

ATTEMPTING TO BRIDGE THE GAP BETWEEN cDNA-EXPRESSED CYTOCHROMES P450 AND NATIVE HUMAN LIVER MICROSOMES

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ABSTRACT. With the increased availability of human liver tissue, recombinant (cDNA-expressed) cytochrome P450 proteins (rCYPs), and knowledge of the human CYP pool (e.g. immunoquantitated levels of each CYP form in native liver microsomes), it is now possible to carry out *in vitro* "CYP reaction phenotyping" in an integrated manner. Reaction phenotyping allows one to identify which CYP form(s) is (are) involved in the metabolism of a given drug, using a combination of data obtained with native human liver microsomes and rCYP proteins. The following describes how one can attempt to integrate such data. A total of ten drugs are included in the analysis, represented by twelve reactions (six hydroxylations, two *O*-demethylations, one *N*-demethylation, one *O*-deethylation, and two sulfoxidations) that are largely catalyzed (≥20%) by various combinations of CYPs (CYP3A4, CYP2C9, CYP1A2, and CYP2D6), and characterized by a wide range of apparent K_m values (12–820 μM). Briefly, reaction rates measured with individual rCYPs are normalized with respect to the nominal specific content of the corresponding CYP in native human liver microsomes. In turn, the normalized rates for each rCYP are summed, yielding a "total normalized rate" (TNR), and the normalized rate for each rCYP is expressed as a percent of the TNR (% TNR). Finally, % TNR is related to inhibition (percent inhibition in the presence of CYP form selective chemical inhibitors; % I) and univariate regression analysis ($r \geq 0.63$; $P \leq 0.05$; $N \geq 10$ different livers) data obtained with native human liver microsomes. Therefore, the reaction phenotype of a drug is assigned by integrating all three data sets (r, % TNR, and % I). BIOCHEM PHARMACOL **57**;5:465–480, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cDNA-expressed cytochrome P450; human liver microsomes; mixed-function monooxygenase activity; enzyme inhibition; reaction phenotyping

The human liver microsomal cytochrome P450 pool (CYP†; EC 1.14.14.1) has been shown to play a major role in the metabolism of many drugs, some of which exhibit a narrow therapeutic index or a steep dose–pharmacological response profile [1–4]. Therefore, if multiple drugs interact with a single CYP form, then the likelihood of a potentially hazardous drug–drug interaction is increased. Likewise, if the *in vivo* clearance of a drug is largely ($f_{m,CYP} \geq 40\%$) mediated by a single polymorphically expressed or allelic variant form of CYP (e.g. CYP2D6, CYP2C19, or CYP2C9), it is anticipated that "poor metabolizer" subjects will be characterized by disparate pharmacokinetics (e.g. elevated plasma AUCs and/or increased $T_{1/2}$) [2–4]. In addition, drugs are often metabolized to pharmacologically active metabolites via CYP-mediated oxidations, which implies that the pharmacodynamics of many drugs can be modulated by induction and/or inhibition of certain CYPs. Some well-documented examples include the CYP3A4-catalyzed oxidation of terfenadine to carboxyterfenadine [5], the CYP2D6-catalyzed *O*-demethylation of codeine to morphine [6], CYP2C19-catalyzed oxidation of proguanil to cycloguanil [7, 8], and the CYP2C9-catalyzed oxidation of losartan to carboxylosartan [9].

In recent years, *in vitro* methods for determining which form(s) of CYP is (are) involved in the metabolism of a given drug, so-called "CYP reaction phenotyping," have become firmly established, and the list of phenotyped compounds continues to grow [1–4, 10–14]. For instance, the *in vitro* CYP reaction phenotypes of over one hundred compounds were published between January 1995 and June

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† *Abbreviations:* CYP, cytochrome P450; K_m, apparent Michaelis constant; V_{max}, apparent maximal initial reaction velocity; CL_{int}, intrinsic clearance (V_{max}/K_m); K_i, inhibition constant or dissociation constant of enzyme–inhibitor complex; % I, percent inhibition; v_i, initial reaction velocity in the presence of inhibitor; v_o, initial reaction velocity in the presence of solvent only; [I], inhibitor concentration; [S], substrate concentration; TNR, total normalized rate; NR, normalized rate; r, correlation coefficient; rCYP, recombinant or cDNA-expressed CYP; mCYP, native human liver microsomal CYP; K_{ies}, dissociation constant of enzyme–substrate–inhibitor complex; α, K_{ies}/K_i; TAO, troleandomycin; DDC, diethyldithiocarbamate; ANF, α-naphthoflavone; f_m, fraction or percent of dose cleared via metabolism; $f_{m,CYP}$, fraction or percent of metabolism catalyzed by CYP; $T_{1/2}$, half-life of drug in plasma; AUC, area under the plasma drug concentration vs time curve; ADME, absorption–distribution–metabolism–excretion; f_u, fraction unbound; $Q_{h,b}$, hepatic blood flow; K_I, apparent concentration of mechanism-based inhibitor required to reach half-maximal rate of inactivation of the enzyme; K_{inact}, maximum inactivation constant; k, initial rate constant for inactivation of enzyme at each concentration of inhibitor; and PK/ADME, pharmacokinetics/absorption–distribution–metabolism–excretion.

1998 (*Xenobiotica*, *Drug Metabolism and Disposition*, *Journal of Pharmacology and Experimental Therapeutics*, *Biochemical Pharmacology*, *and The British Journal of Clinical Pharmacology*).

In general, if *in vitro* CYP reaction phenotype data are obtained in a timely manner, the information can be indicative of which *in vivo* drug interaction studies are required, in addition to those involving often-evaluated drugs (e.g. cimetidine, theophylline, warfarin, oral contraceptives, and digoxin). In the case of polymorphically expressed enzymes, *in vitro* CYP reaction phenotype data can alert clinicians of the need for genotyped subjects [1–4, 10–14]. As a result, many pharmaceutical companies now regularly include such data in their submissions to various regulatory agencies. In turn, these agencies have acknowledged the usefulness of this information, as evidenced by the recent issuance of a guidance package entitled "Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies *In Vitro*" (published by the Food and Drug Administration).

CYP REACTION PHENOTYPING

The reaction phenotyping process involves the integration of data obtained with native human liver microsomes, intact cell models, and rCYPs [1, 4, 10–14]. Intact cell systems (e.g. hepatocyte suspensions and liver tissue slices) allow one to acquire an overall metabolic profile and estimates of $f_{m,CYP}$ (Fig. 1). In the absence of *in vivo* human ADME data, the information obtained with validated intact cell systems is of paramount importance, so that some perspective can be given to data obtained with subcellular fractions [1, 4, 14]. However, many drugs are cleared unchanged via renal and/or biliary routes, which may be difficult to evaluate with *in vitro* models ostensibly geared towards metabolism. Furthermore, it should also be recognized that the pharmacokinetics of intravenously administered, high extraction drugs (e.g. $f_u \cdot CL_{int}/Q_{h,b} \geq 10$) are refractory to the effects of CYP inducers and inhibitors [15]. On the other hand, as a result of the effects of potent inhibitors (e.g. ketoconazole and itraconazole) on gut and liver CYP3A, the AUC of orally administered CYP3A substrates (e.g. $f_{m,CYP3A} \geq 70\%$) is elevated markedly (e.g. $\geq$ 10-fold). *In toto*, the *in vitro* CYP reaction phenotype data have to be evaluated in light of the therapeutic index of a drug, $f_{m,CYP}$, CL_{int} (vs $Q_{h,b}$) in the absence of inhibitor (intravenous route only), sites of metabolism (e.g. gut vs liver), and the $[I]/K_i$ ratios of potentially co-administered inhibitors [1, 4, 11, 14].

CYP reaction phenotyping entails an initial assessment of Michaelis–Menten kinetics, using native human liver microsomes (Fig. 1). Solubility permitting, initial reaction rates are measured over a wide range of [S], under incubation conditions that minimize substrate depletion ($\leq$ 15%) and that are linear with respect to microsomal protein concentration and time of incubation ($N \geq 3$ different livers). Increasingly, various investigators are performing incubations at lower concentrations of microsomal protein (e.g. $\leq$0.5 mg/mL) in order to minimize the non-specific or "futile" binding of substrate to proteins and thus obtain accurate estimates of K_m. Once generated, the data are analyzed by linear (e.g. Eadie–Hofstee plot) and non-linear (e.g. PCNonlin® and WinNonlin®) transformations.

Accurate CYP reaction phenotyping is predicated on the availability of adequate enzyme kinetic data because the results obtained with CYP form selective chemical inhibitors are governed by the ratio of $[S]/K_m$ (see later). In addition, if the kinetic data are described by a two-enzyme model, then the low (typically, $K_{m1} \leq 20$ μM) and high (typically, $K_{m2} \geq 50$ μM) apparent K_m values may reflect the involvement of more than one CYP form [1, 2, 8, 14]. In the majority of cases, the low apparent K_m component is most informative because this indicates which CYPs are saturable, or play a major role, at clinically relevant drug concentrations. Well-documented examples include the CYP2D6-catalyzed *O*-demethylation of dextromethorphan, the CYP2C19-catalyzed hydroxylation of omeprazole, and the CYP2C9-catalyzed 7-hydroxylation of (*S*)-warfarin [2, 14]. However, multiple forms of CYP may contribute to the same reaction, characterized by similar K_m values (e.g. CYP1A2- and CYP2C9-catalyzed *O*-demethylation of naproxen), and yield monophasic Eadie–Hofstee plots [14].

Enzyme kinetic studies are followed by regression analysis using a bank of human liver microsomes (typically $N \geq 10$ different organ donor subjects) at one or more substrate concentrations (e.g. $[S]/K_m \leq 0.1$ and/or $[S]/K_m \geq 2$). In this instance, the rates of metabolism of the drug in question are correlated with the levels of CYP form selective monooxygenase activities or immunoquantitated CYP apoprotein (Fig. 1). In general, for a correlation to be statistically significant using Student's *t*-test ($P \leq 0.05$; $N \geq 10$ livers), the correlation coefficient (r) has to be $\geq$0.63 [1, 13, 14]. Then complementary studies are carried out with reversible and mechanism-based (quasi-irreversible) chemical inhibitors (Table 1) that are selective for different forms of CYP [16–24], albeit some researchers are increasingly making use of CYP form selective inhibitory (anti-CYP peptide) antibodies [25, 26]. Finally, the data obtained with native human liver microsomes are confirmed with cDNA-expressed CYP proteins (Fig. 1). Use of rCYPs has become widespread [1, 2, 4, 14, 19, 21, 22, 24, 27], as a result of the increased availability of materials provided by commercial vendors (e.g. Gentest Corp., Panvera Corp., and Oxford Biomedical Research, Inc.). In addition, because the various CYPs in native human liver microsomes have been immunoquantitated (Table 2), it is now possible to normalize data obtained with rCYPs and to fully integrate *in vitro* CYP reaction phenotyping data [28–30].

At the present time, many pharmaceutical companies are including *in vitro* CYP reaction phenotyping in their preclinical drug-screening paradigms. Therefore, due to the relatively large numbers of compounds, it is recognized that reaction phenotyping procedures have to be standardized.

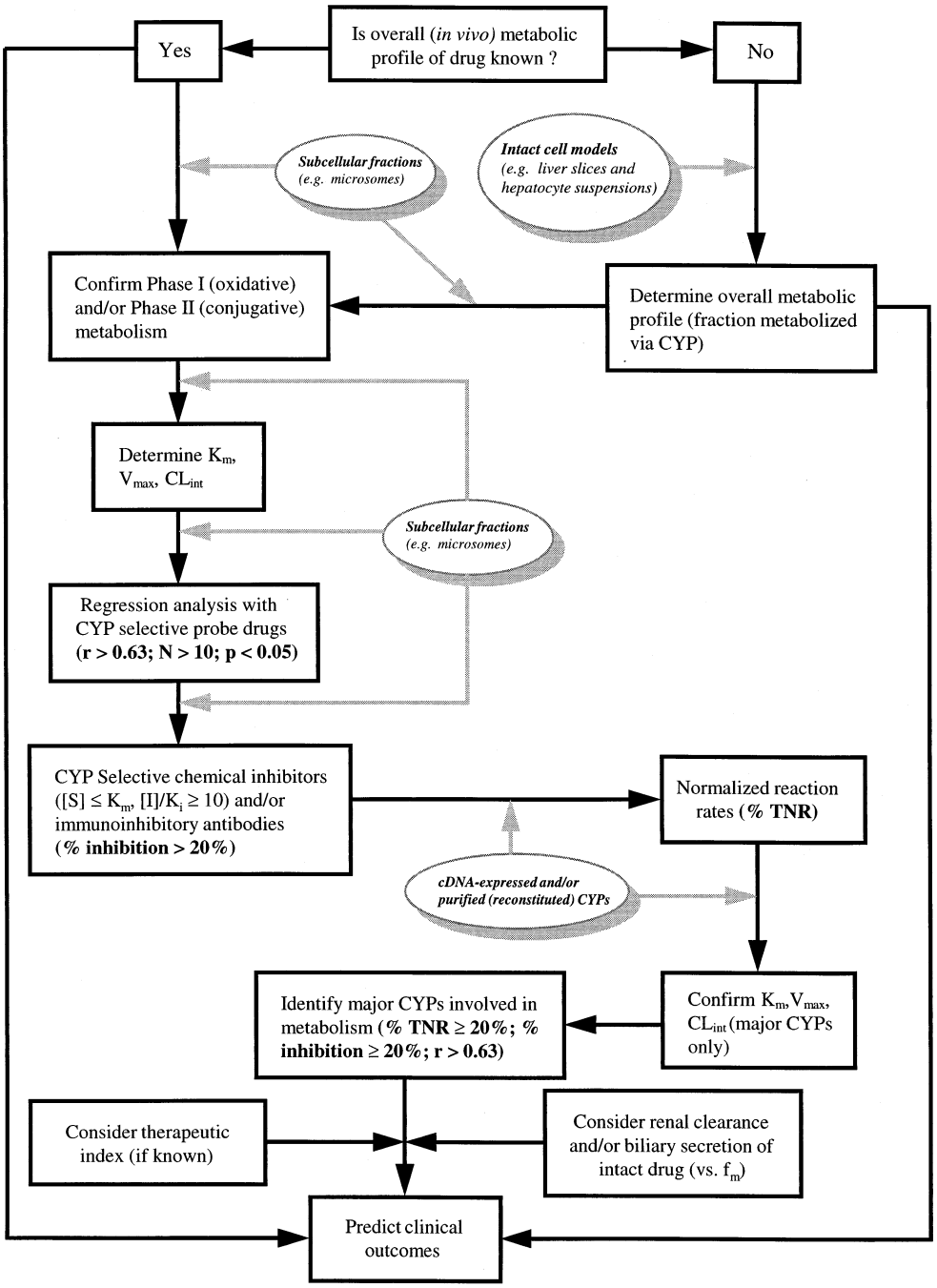

FIG. 1. Integrated *in vitro* CYP reaction phenotyping.

Overall, the number of publications describing methods for normalizing data obtained with rCYPs are limited in number [31, 32], and no consensus has been reached concerning the large-scale applicability of these approaches. Towards this end, the following describes how one can attempt to carry out integrated CYP reaction phenotyping using a relatively simple strategy.

A total of ten drugs were included in the analysis (Table 3), encompassing twelve reactions (six hydroxylations, two *O*-demethylations, one *N*-demethylation, one *O*-deethylation, and two sulfoxidations) that were characterized by a wide range of K_m values (12–820 μM) [33–37]. At the time of study, six different CYPs were included in the analysis (CYP2E1, CYP2A6, CYP3A4, CYP2C9, CYP2D6, and CYP1A2), although metabolism was primarily catalyzed by different combinations of CYP3A4, CYP2C9, CYP1A2, and CYP2D6. In agreement, it has been estimated that the majority (> 90%) of drugs are metabolized by these CYPs [38]. The majority of the data were obtained with commercially available rCYPs (Gentest Corp.) and were normalized with respect to the nominal specific content of the corresponding CYP form in native human liver microsomes

TABLE 1. Putative CYP form selective chemical inhibitors

CYP form	Inhibitor	K_i^* (μM)	[I]†	Ref.
CYP3A4	Ketoconazole	0.02	1.0	[17]
	TAO‡	(17)	25–100	[18]
CYP2C9	Sulfaphenazole	0.3	10	[17, 19]
CYP1A2	Furafylline‡	(0.6)	20	[17]
	ANF	0.01	5.0	[17]
CYP2E1	4-Methylpyrazole§	4.0	50	[20]
	DDC‡‖	4.0	50	[20, 21]
CYP2D6	Quinidine	0.07	5.0	[22]
CYP2A6	Coumarin	0.3¶	20	[23]
	8-Methoxypsoralen‡**	(1.0)	10	[24]

*K_i data obtained with native human liver microsomes or the appropriate cDNA-expressed CYPs (references indicated). Except for 8-methoxypsoralen (K_I shown), data in parentheses represent *apparent* K_i.

†Concentration of inhibitor (μM) required to ensure that $[I]/K_i \geq 10$ ($[S] \leq K_m$), and that the corresponding CYP form is selectively inhibited (% I ≥ 83%) in native human liver microsomes.

‡Mechanism-based inhibitor of CYP. Mechanism-based inhibition is characterized by K_I, k, k_{inact}, and the partition ratio.

§Also known to inhibit CYP2D6. Inhibition of CYP2E1 in native human liver microsomes is characterized by an IC_{50} of ~10 μM.

‖Also known to inhibit CYP2A6.

¶Apparent K_m for the 7-hydroxylation of coumarin by CYP2A6, competitive inhibition of CYP2A6 ($K_m \approx K_i$) assumed. Short incubation times (≤5 min) are required in order to minimize the depletion of coumarin.

**Known to inhibit multiple CYP forms (≥30% inhibition) at higher concentrations (≥ 10μM).

(Gentest Corp. data, Table 2). In all cases (rCYP and native human liver microsomes), the rate of reaction was linear with respect to the time of incubation and enzyme concentration. Assays were performed in buffer (50–100 mM potassium phosphate, pH 7.4) at 37°, in the presence of $MgCl_2$ (5–15 mM) and a NADPH-generating system [33–37]. Because of the lack of CYP2C19, CYP2B6, and CYP2C8 selective chemical inhibitors and immunoinhibitory antibodies, these three CYPs were omitted from the analysis. Their omission did not greatly affect the normalized data obtained with the rCYPs.

TABLE 2. Nominal specific content of individual CYP proteins in native human liver microsomes

CYP form	pmol CYP/mg (% Total)*		
	(1)	(2)	(3)
CYP2B6	39 (7.0)	1.0 (0.2)	3.0
CYP3A4	108 (20)	98 (29)	40
CYP3A5	1.0 (0.2)		
CYP2C8	64 (12)		
CYP2C9	96 (18)		
CYP2C19	19 (4.0)		
CYP2E1	49 (9.0)	22 (7.0)	
CYP2A6	68 (13)	42 (13)	12
CYP1A2	45 (8.0)	42 (13)	15
CYP2D6	10 (2.0)	5.0 (2.0)	15
CYP2C18	< 2.5†		
Total‡	534	344	

*Levels of each CYP (pmol/mg microsomal protein) were determined by immunoblotting procedures. Data in parentheses represent percent of total CYP: (1) Mean data obtained from the Gentest Corp. (October 1997), using a pool of liver microsomes from different organ donor subjects (N = 12); (2) mean data from sixty different livers [28]; (3) mean data from sixteen different human livers [29].

†Data for CYP2C18 were obtained from Jung *et al.* [30].

‡Total CYP was measured spectroscopically as a ferrous–carbon monoxide complex.

INHIBITION STUDIES WITH NATIVE HUMAN LIVER MICROSOMES

Once the apparent K_m of the substrate has been determined with native human liver microsomes, inhibition studies with CYP form selective chemical inhibitors (Table 1) are relatively straightforward. However, one has to consider that: (1) the volume of solvent (e.g. methanol, acetonitrile, and DMSO) in the incubations has to be kept to a minimum (≤0.5%, v/v), although data are expressed relative to solvent alone controls (equation 1); and (2) microsomes have to be characterized with respect to the levels of the various CYP forms, either by measurement of CYP selective monooxygenase activities or immunoquantitation of CYP apoprotein [28–30].

$$\% \text{ I} = \frac{v_o - v_i}{v_o} \cdot 100 \quad (1)$$

where % I represents percent inhibition; v_0 represents initial reaction velocity in the presence of solvent only; and v_i represents initial reaction velocity in the presence of inhibitor.

When using chemical inhibitors, it is important to realize that the degree of inhibition (Fig. 2) is dependent on both [S] and the mechanism of inhibition [1, 4, 39]. However, the simulations presented in Fig. 3 demonstrate that inhibition (mixed, competitive, or uncompetitive) can be independent of [S], as [S] approaches K_m ($[S]/K_m \approx 1$). In this regard, immunoinhibitory antibodies are superior to chemical inhibitors, as they can serve as noncompetitive inhibitors, and the degree of inhibition is largely independent of [S]. For the more common types of inhibition (e.g. mixed and competitive), when the ratio $[S]/K_m$ is low (i.e. when $[S]/K_m < 1.0$), the extent of inhibition is also independent of [S] (Fig. 3). This is significant because most drugs obey first order (linear) kinetics *in vivo*. At the same time, many investigators are performing metabolic stability screening *in vitro*, thus predicting CL_{int} *in vivo*, using low substrate concentrations (≤1 μM) [40]. For chemical inhibitor studies to be successful, one utilizes inhibitors that have been characterized with respect to their inhibitory potency and CYP form selectivity (Table 1). However, although potent inhibitors (e.g. $K_i < 1$ μM) are desirable, many compounds, such as TAO ($K_i \geq 20$ μM), manifest CYP form selectivity by virtue of mechanism-based inhibition (Table 1).

In general, for all types of inhibition ($[S] \leq K_m$), relatively high concentrations of inhibitor ($[I]/K_i \geq 10$) are used in order to ensure that inhibition is extensive (% I ≥ 83%), albeit higher $[I]/K_i$ ratios ($[I]/K_i \geq 30$) are needed to achieve almost complete inhibition (% I ≥ 93%) of a given

TABLE 3. Metabolism of various drugs by native human liver microsomes

Drug	Reaction	CYP(s)*	$K_m^{\dagger}$ (μM) HLM	$K_m^{\dagger}$ (μM) rCYP	Ref.
A	Sulfoxidation	3A4 2C9	230		Unpublished
	Hydroxylation	1A2 2C9	390		Unpublished
B	Hydroxylation	3A4 2C9	>300‡		Unpublished
C	Hydroxylation	3A4 2C9	>300‡		Unpublished
D	Hydroxylation	3A4	>100‡	>100‡	Unpublished
E	Hydroxylation	3A4	186	202	Unpublished
Zileuton	Sulfoxidation	3A4 2C9	820		[33]
	Hydroxylation	1A2 2C9	340		[33]
Phenacetin	*O*-Deethylation	1A2	54	46	[34]
Naproxen	*O*-Demethylation	1A2	160	250	[35]
		2C9		430	
Clarithromycin	*N*-Demethylation	3A4	59	20	[36]
Dextromethorphan	*O*-Demethylation	2D6	12	20	[37]

*Native human liver microsomal CYP form(s) shown to be involved in the metabolism of drug. CYP3A4 is considered to be most abundant CYP3A subfamily member. For each drug, CYP reaction phenotyping was carried out using chemical inhibitors and native human liver microsomes (N ≥ 3 different livers), cDNA-expressed CYPs, and univariate linear regression analysis (liver microsome bank; N ≥ 10 different livers).

†Metabolism conformed to monophasic kinetics (PCNonlin®). Apparent K_m characterizing rCYP- or native liver microsome-catalyzed oxidation. Data with native liver microsomes are reported as means of multiple livers (N ≥ 3). HLM = human liver microsomes.

‡Apparent K_m exceeds upper limit of solubility.

CYP (Fig. 3). In fact, when the [I]/K_i ratio is high (≥30), one can study inhibition at substrate concentrations that approach V_{max} or 2K_m (Fig. 3). Maximization of inhibition is critical when the metabolism of a drug is catalyzed by multiple CYPs. Fortunately, the most commonly used inhibitors (e.g. ketoconazole, ANF, sulfaphenazole, and quinidine) have been shown to be CYP selective at [I]/K_i ratios approaching 30 (K_i < 0.5 μM; [S] ≈ K_m) [17]. When the involvement of multiple CYPs is suspected, various CYP form selective inhibitors can be co-incubated [35]. This is possible because the effect of independently acting CYP-selective (competitive) inhibitors is additive. Therefore, CYP reaction phenotyping with native human liver microsomes can be carried out with CYP form selective chemical inhibitors under well-defined experimental conditions ([S] ≤ K_m; [I]/K_i ≥ 10).

NORMALIZING DATA OBTAINED WITH rCYPs

The normalization of data obtained with rCYPs involves a number of steps, which can be described as follows.

Step 1

The drug is incubated in the presence of each rCYP (rCYPn), and the choice of [S] is based on the apparent K_m obtained with native human liver microsomes. If a reaction exhibits biphasic kinetics in native liver microsomes, then incubations with rCYPs are carried out at two different substrate concentrations defined by the low (K_{m1}) and high (K_{m2}) K_m values. This approach assumes that the apparent K_m values obtained with rCYP and native liver microsomes are similar (≤3-fold difference) (Table 3).

Upon termination of the reaction, the rate of product formation is expressed on a "per nmol rCYPn" or "per pmol rCYPn" basis, after accounting for background (non-CYP) activity. This assumes that the reaction is linear with time of incubation and that the specific content of the rCYP is known (>0.1 nmol/mg) and has been measured by UV/visible spectral methods (e.g. ferrous–carbon monoxide complexation). Furthermore, the molar ratio of rCYP to NADPH–CYP reductase and cytochrome b_5 has to be considered. For the most part, rCYP needs to be limiting, and optimal reaction rates are usually achieved at relatively high NADPH–CYP reductase to rCYP ratios (≥2.0) [33–36]. By comparison, the requirement for cytochrome b_5 often depends on the rCYP and/or substrate in question [2, 35].

Interestingly, the specific content of NADPH–CYP reductase in native human liver microsomes (typically, 150–350 nmol cytochrome *c*/min/mg) has been estimated by immunoquantitation to be about 100 pmol/mg, while the specific content of cytochrome b_5 is similar to that of total (spectrally detectable) CYP (~300 pmol/mg) [41–43]. Nominally, these findings imply that the majority of CYPs (specific content < 100 pmol/mg) are limiting (vs cytochrome b_5 and NADPH–CYP reductase) in human liver

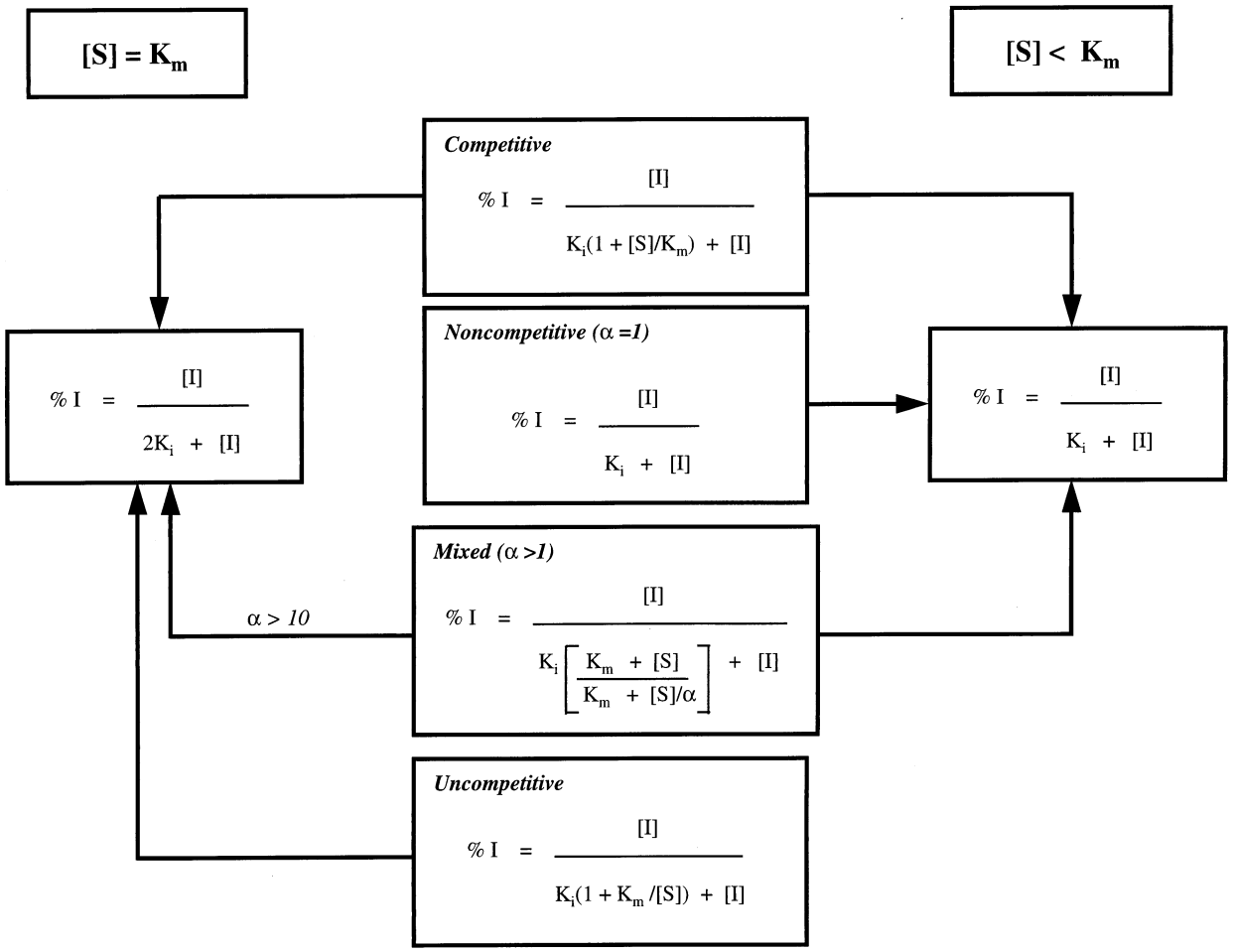

FIG. 2. Relationship between percent inhibition (% I) and substrate concentration [S] for a competitive, noncompetitive, mixed (competitive/noncompetitive), and uncompetitive inhibitor. The relationship between % I, [I], and K_i can be simplified when [S] = K_m or [S] < K_m.

microsomes (Table 2), although it is often assumed that CYP content is rate-limiting for product formation *in vivo*.

Step 2

For each rCYP, the reaction rate is multiplied by the *mean* specific content of the corresponding CYP (e.g. pmol mCYPn/mg) in native human liver microsomes (Table 2). This yields the "normalized rate" (NR), which is expressed as pmol/min per mg microsomal protein (equation 2). Although the mean specific content of each CYP is used in the analysis, one should be aware that the levels of many of the CYPs (e.g. CYP3A4 and CYP2A6) vary considerably (≥10-fold) between different livers [13, 28–30].

Step 3

The NR values for each CYP are summed to obtained the "total normalized rate" (TNR). Finally, the NR for each CYP is expressed as a percentage of the TNR (equation 2).

$$\% \text{ TNR} = \frac{\text{NR}}{\text{TNR}} \cdot 100$$

$$= \frac{pmol/min/pmol\ rCYPn \cdot pmol\ mCYPn/mg}{\Sigma\ (pmol/min/pmol\ rCYPn \cdot pmol\ mCYPn/mg)} \cdot 100 \quad (2)$$

Therefore, no attempt is made to estimate the *absolute* rate of reaction in native human liver microsomes, although % TNR can be directly related to inhibition (% I) and regression analysis data. Moreover, relatively costly and time-consuming enzyme kinetic studies can be avoided, because confirmatory K_m and V_{max} estimates are only performed with those rCYPs that are characterized by relatively high (≥20%) % TNR values (Table 3). This is important during the preclinical screening of large numbers of compounds. Nevertheless, there may be instances when one needs to determine V_{max}/K_m ratios for a number of rCYPs and simulate the contribution of each CYP over a

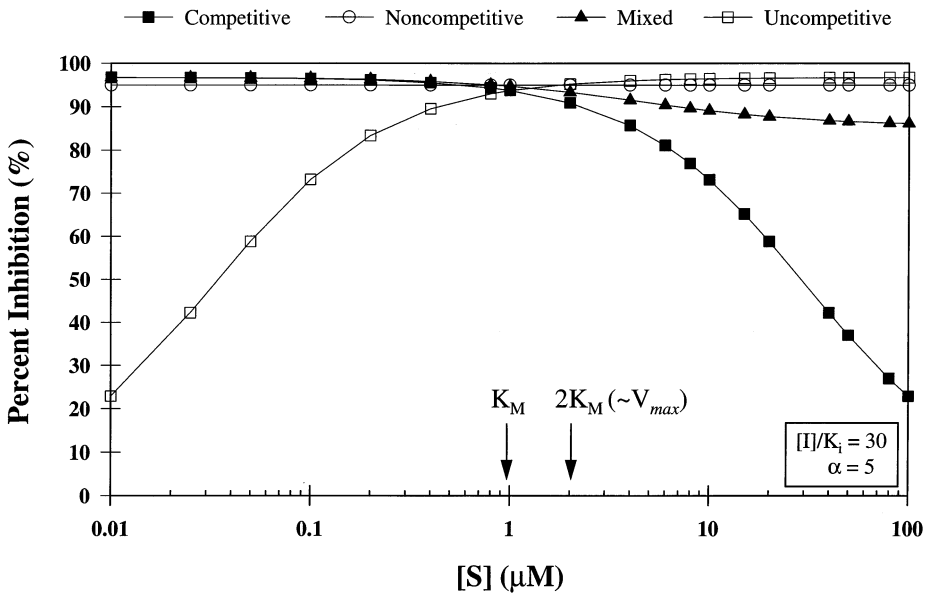

FIG. 3. Simulating the relationship between percent inhibition (% I) and substrate concentration [S] for a competitive, noncompetitive, mixed (competitive/noncompetitive), and uncompetitive inhibitor (equations presented in Fig. 2). A simple case (apparent K_m = 1 μM; [I] = 30 μM; K_i = 1 μM; [I]/K_i = 30) is modeled. In the case of mixed inhibition, α = 5. Use of low substrate concentrations ([S] < K_m) ensures that % I is maximal (≥93%), for a competitive, mixed, or noncompetitive inhibitor. When [S] ≈ K_m, inhibition is independent of the mechanism of inhibition.

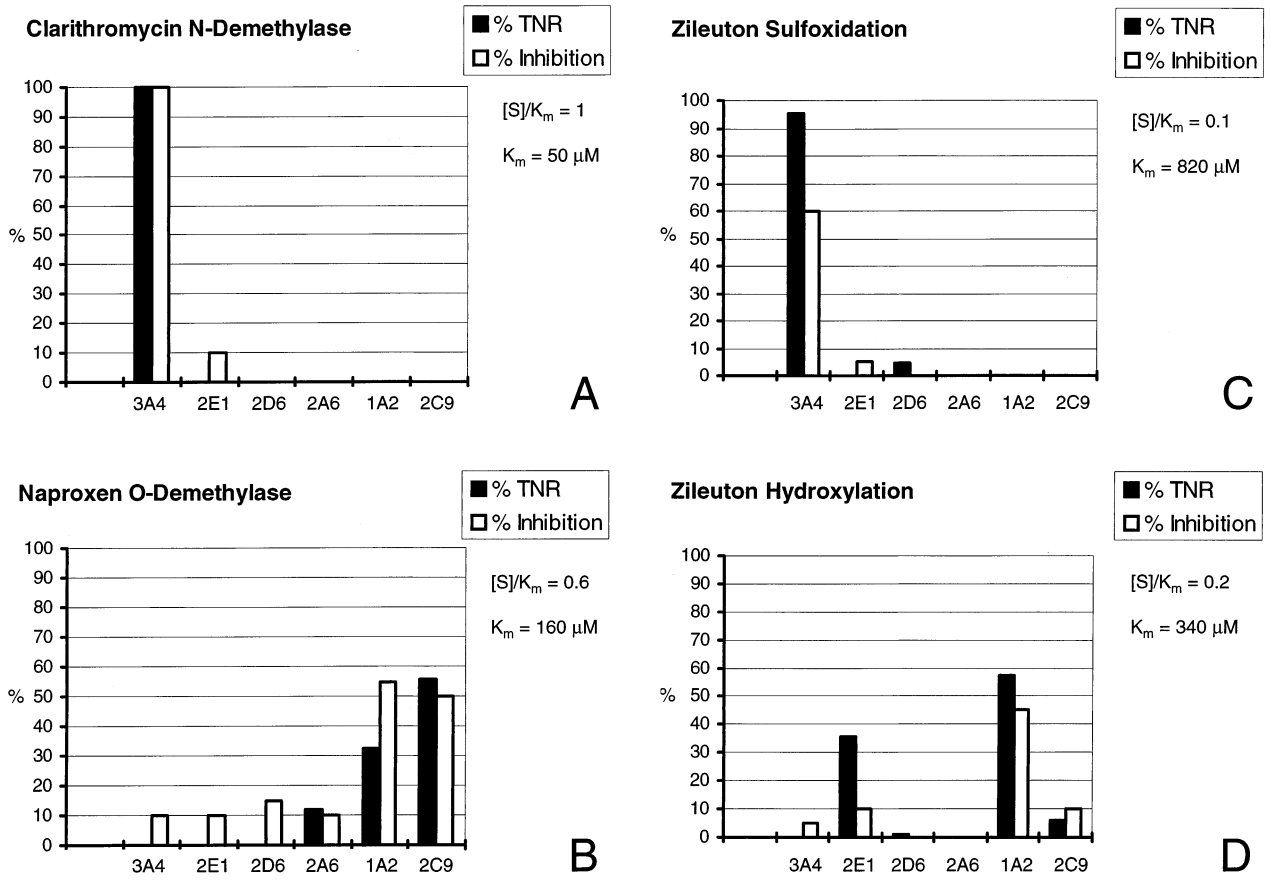

FIG. 4. Relating chemical inhibitor data (human liver microsomes) to normalized reaction rate data obtained with rCYPs (% TNR): (A) clarithromycin (*N*-demethylase); (B) naproxen (*O*-demethylase); (C) zileuton (sulfoxidase); and (D) zileuton (hydroxylase). In each case, the K_m (human liver microsomes) is indicated and is related to the [S] used in the analysis. Data represent percent inhibition (mean of N ≥ 3 different livers) in the presence of the CYP form selective inhibitors (Table 1; open bars) and % TNR (filled bars).

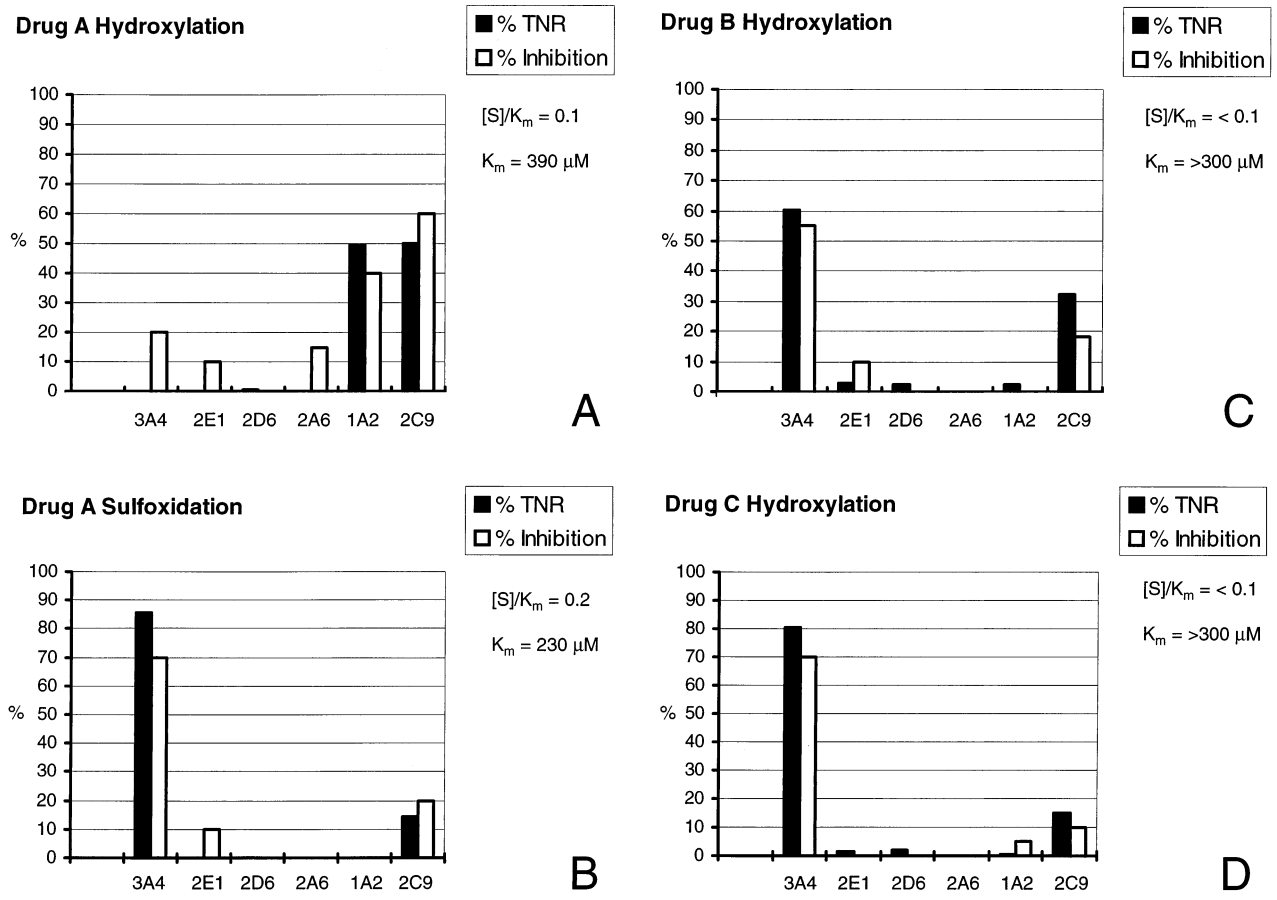

FIG. 5. Relating chemical inhibitor data (human liver microsomes) to normalized reaction rate data obtained with rCYPs (% TNR): (A) drug A (hydroxylase); (B) drug A (sulfoxidase); (C) drug B (hydroxylase); and (D) drug C (hydroxylase). The data are presented as described in the legend to Fig. 4.

wide range of substrate concentrations. The simplest case, when only two rCYPs catalyze a reaction, is described (equations 3–6). For each CYP, data (v_1 or v_2 vs [S]) are analyzed by non-linear transformation to yield estimates (± standard error) of apparent K_m and V_{max}, where V_{max} (pmol/min/pmol rCYPn) is multiplied by the specific content of the enzyme in native human liver microsomes (pmol mCYPn/mg):

$$v_{tot} = \frac{(V_{max1} \cdot \text{pmol mCYP}_1\text{/mg}) \cdot [S]}{K_{m1} + [S]} + \frac{(V_{max2} \cdot \text{pmol mCYP}_2\text{/mg}) \cdot [S]}{K_{m2} + [S]} \quad (3)$$

$$v_{tot} = v_1 + v_2 \quad (4)$$

At any given [S], % contribution of CYP1

$$= v_1/v_{tot} \cdot 100 \quad (5)$$

At any given [S], % contribution of CYP 2

$$= v_2/v_{tot} \cdot 100 \quad (6)$$

If kinetic data with human liver microsomes are described by a two-enzyme model, then the contribution of the low K_m and high K_m CYPs towards overall metabolism can also be simulated over a wide range of substrate concentrations (equations 7–10). As described above, data (v_{tot} vs [S]) are analyzed by non-linear transformation in order to obtain estimates of apparent K_{m1}, K_{m2}, V_{max1} and V_{max2}:

$$v_{tot} = \frac{V_{max1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{max2} \cdot [S]}{K_{m2} + [S]} \quad (7)$$

$$v_{tot} = v_1 + v_2 \quad (8)$$

At any given [S], % contribution of low $K_m(K_{m1})$

$$\text{CYP(s)} = v_1/v_{tot} \cdot 100 \quad (9)$$

At any given [S], contribution of high $K_m(K_{m2})$

$$\text{CYP(s)} = v_2/v_{tot} \cdot 100 \quad (10)$$

In general, however, it is advisable to relate this type of simulated data to inhibition data (% I) obtained with native human liver microsomes. In the simplest case,

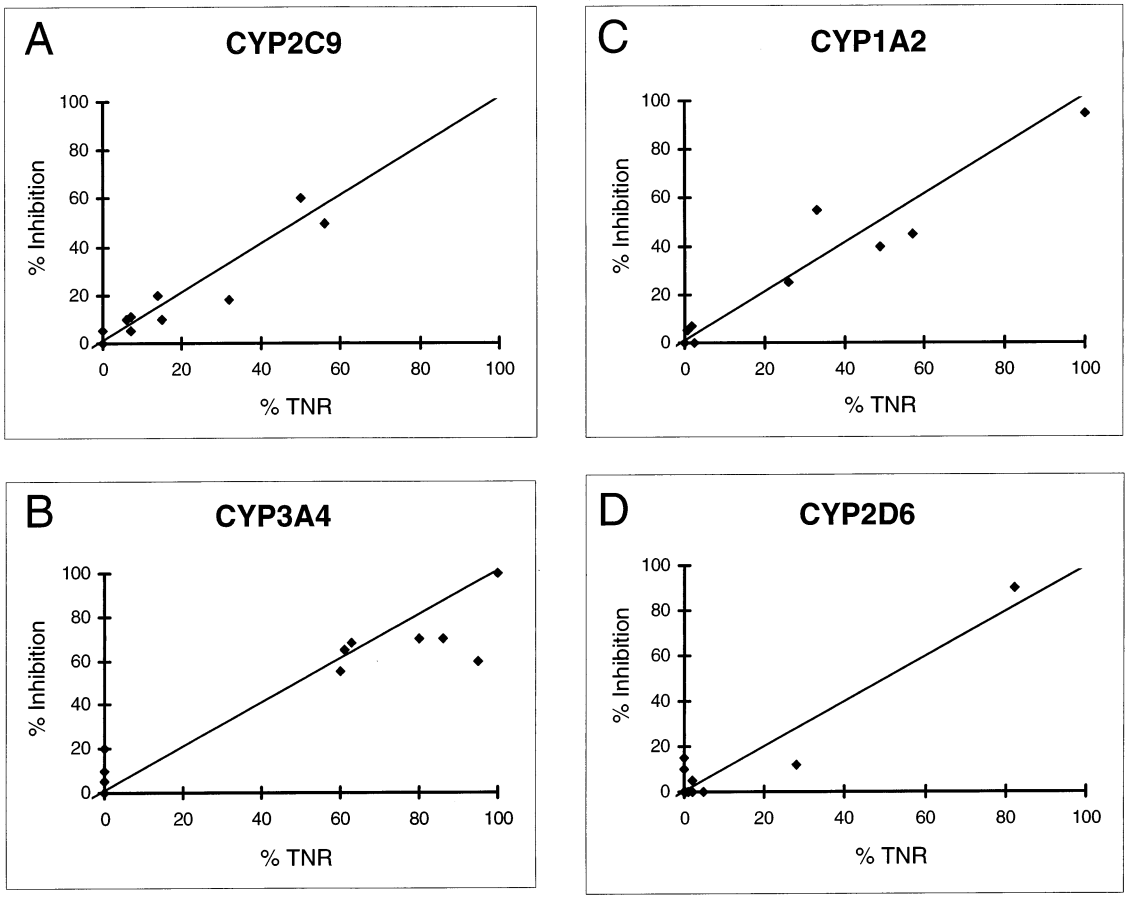

FIG. 6. Relationship between percent inhibition (CYP form selective inhibitors/native liver microsomes) and % TNR (rCYPs) for: (A) CYP2C9; (B) CYP3A4; (C) CYP1A2; and (D) CYP2D6 substrates (N = 12 different reactions; Table 3). In each case, the line of identity (1:1 proportionality) is shown. Inhibition studies were carried out with furafylline (CYP1A2), sulfaphenazole (CYP2C9), ketoconazole (CYP3A4), and quinidine (CYP2D6); see Table 1.

apparent K_{m1} and K_{m2} are reflective of only two different CYPs (e.g. CYP2D6 and CYP3A4), and the contribution of each enzyme towards overall metabolism equates with % I ($[I]/K_i \geq 30$) in the presence of the corresponding CYP form selective chemical inhibitor (equations 11 and 12). Noncompetitive inhibitors are particularly useful in this instance, because % I is independent of [S], and one can consider % I over the entire range of substrate concentrations used in the simulation. At any given [S],

$$\% \text{ Contribution of low } K_m(K_{m1}) \text{ CYP} = v_1/v_{tot} \cdot 100 \approx \% \, I_1 \quad (11)$$

$$\% \text{ Contribution of high } K_m(K_{m2}) \text{ CYP} = v_2/v_{tot} \cdot 100 \approx \% \, I_2 \quad (12)$$

Perspective

In the final analysis, the usefulness of a normalization procedure, such as the one described herein, will be dependent upon a number of factors:

(1) The accuracy of the immunoassays used to quantitate the various CYPs in native human liver microsomes. In turn, accuracy will depend on factors such as assay linearity, the selectivity of the anti-CYP antibodies, and the quality of the cDNA-expressed CYP proteins used in the construction of standard curves. Ideally, one employs cDNA-expressed CYP proteins that have been purified to electrophoretic homogeneity and exhibit high (>17 nmol/mg) specific contents. If heme incorporation is not quantitative (<90%), excess apoprotein may lead to erroneous over-estimates of CYP levels in native human liver microsomes. This can be problematic when using microsomes (insect cell or lymphoblast) containing cDNA-expressed CYP protein as a standard, because it is difficult to accurately evaluate heme incorporation into microsomal (vs electrophoretically homogenous/purified) CYP.

(2) The availability of a complete range of fully characterized rCYPs (e.g. CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2A6, CYP1A2, and CYP2B6) and the appropriate CYP form selective chemical inhibitors or immunoinhibitory antibodies.

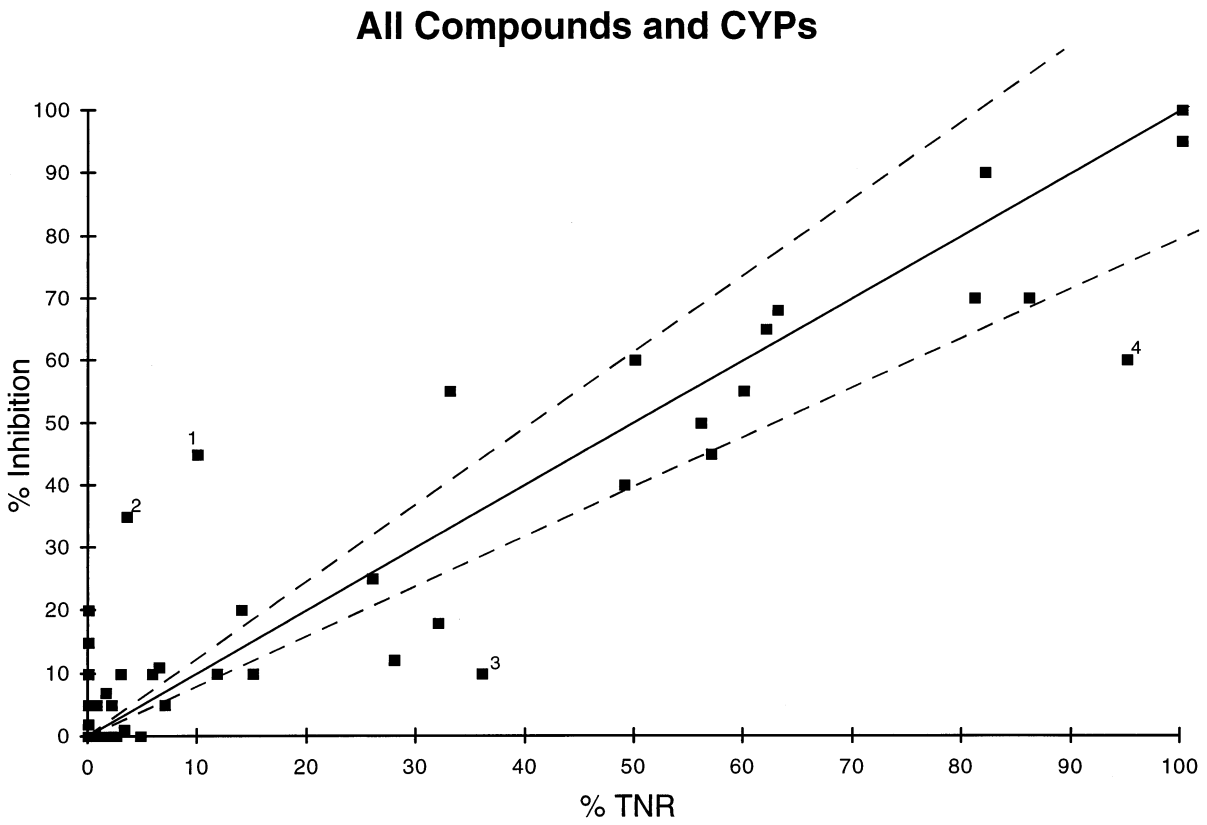

FIG. 7. Relationship between percent inhibition (CYP form selective inhibitors/native human liver microsomes) and % TNR (rCYPs). Data were obtained with ten different compounds (N = 12 different reactions; Table 3), and the analysis encompassed the major CYPs (e.g. CYP3A4, CYP2C9, CYP2D6, and/or CYP1A2) and minor CYPs (e.g. CYP2A6 and CYP2E1) involved in each reaction. The line of identity (±20%) is shown. Outlier data are indicated: 4-methylpyrazole inhibition of (1) dextromethorphan O-demethylase and (2) "drug D" hydroxylase activity. Additional outliers represent CYP2E1-mediated hydroxylation (3) and CYP3A-mediated sulfoxidation (4) of zileuton.

Fortunately, progress is being made in all of these areas, although selective inhibitors for some CYPs (e.g. CYP2C19 and CYP2C8) are not available at the present time.

(3) Recognition that microsomes containing heterologously expressed CYPs are "artificial" systems and can differ considerably from native microsomal systems. For instance, the phospholipid environment, lipid to protein ratio, and NADPH–CYP reductase to CYP ratio can be markedly different. However, these concerns can be addressed if rCYPs are used to confirm data obtained with native human liver microsomes (Fig. 1).

TABLE 4. Naproxen O-demethylation catalyzed by various human liver microsomal CYP2C subfamily members

	O-Demethylase rate				% I‖	
CYP	pmol/min/pmol rCYP*	pmol/min/mg†	% TNR‡	K_i§ (μM)	$f_{m,CYP2C} = 1.0$	$f_{m,CYP2C} = 0.5$
2C8	<0.1	<1.0	<0.1	63	5.0	3.0
2C9	8.0	770	90	0.3	91	46
2C18	0.5	1.0	0.1	29	10	5.0
2C19	4.7	80	10	NI	<1.0	<1.0
TNR		860				

*Rate of reaction obtained with purified and fully reconstituted rCYP2Cs [35].

†Rate normalized with respect to the nominal specific content of each CYP2C protein in native human liver microsomes (Table 2, Gentest data).

‡Data expressed as percent of TNR, where TNR = 860 pmol/min/mg.

§K_i of sulfaphenazole with various rCYP2Cs [19], NI: no inhibition detected.

‖Predicted inhibition (% I) of each CYP2C, assuming that the final sulfaphenazole concentration is 5.0 μM ($[S]/K_m = 0.6$), that the mechanism of inhibition is competitive (Fig. 2), and that each CYP2C protein contributes to 100% ($f_{m,CYP2C} = 1.0$) or 50% ($f_{m,CYP2C} = 0.5$) of the total metabolism in native human liver microsomes. Only 50% of naproxen O-demethylase activity in native human liver microsomes is mediated by CYP2C (non-CYP1A2) proteins [35].

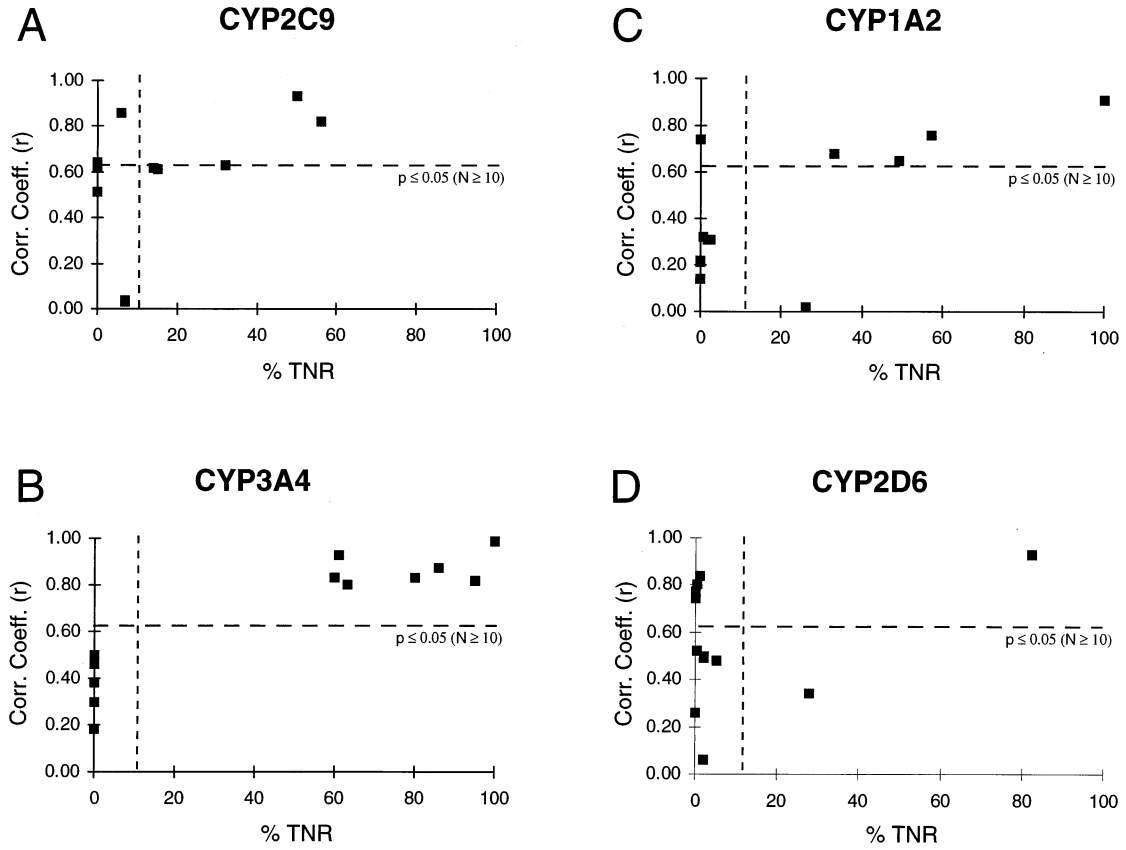

FIG. 8. Relationship between correlation coefficient (r) and % TNR (rCYPs) for: (A) CYP2C9, (B) CYP3A4, (C) CYP1A2, and (D) CYP2D6 substrates. Data were obtained with drugs presented in Table 3. In each case, univariate linear regression analysis was carried out with a bank of liver microsomes (N ≥ 10 different livers) and CYP form selective probe activities (tolbutamide hydroxylase, CYP2C9; 7-ethoxyresorufin O-deethylase, CYP1A2; erythromycin N-demethylase, CYP3A4), or immunoquantitated CYP protein (CYP2D6). Using Student's *t*-test, where $t = [r \cdot \sqrt{(N-2)}]/[\sqrt{(1-r^2)}]$, the correlation is considered statistically significant ($P \leq 0.05$; $t \geq 2.31$) when $r \geq 0.63$. Statistically significant correlations when % TNR is low (≤10%) are considered spurious.

RELATING NORMALIZED rCYP DATA TO NATIVE HUMAN LIVER MICROSOMES

% TNR vs % I

As illustrative examples, the partial *in vitro* CYP reaction phenotype profiles (% I vs % TNR) of clarithromycin, naproxen, and zileuton are presented in Fig. 4 [33, 35, 36], while the profiles of three additional compounds are shown in Fig. 5. Two of the compounds (zileuton and drug A) are metabolized by two distinct pathways (hydroxylation and sulfoxidation), each catalyzed by a different combination of CYPs. The data presented in Fig. 6 illustrate how % TNR is related to % I for four clinically relevant CYPs (CYP3A4, CYP2D6, CYP2C9, and CYP1A2). All twelve reactions are included in the analysis (Table 3). In all cases, the data are distributed about the line of identity and indicate that the % TNR obtained with rCYPs is proportional to % I under defined experimental conditions. Data for all CYPs included in the analysis (CYP3A4, CYP2C9, CYP1A2, CYP2E1, CYP2D6, and CYP2A6) are shown in Fig. 7. In this instance, the majority of the data are also distributed (±20%) about the line of identity, with the greatest deviations occurring at low background values of % I (≤20%) and % TNR (≤10%). However, erroneous estimates of % I in liver microsomes can give rise to marked deviation at higher values of % TNR and % I (≥30%) (Fig. 7). Inhibition, and thus the contribution of a given CYP, can be overestimated when non-CYP selective inhibitors are used. For example, 4-methylpyrazole is only selective for CYP2E1 over a relatively narrow concentration range and has been shown to inhibit CYP2D6 (point 1, Fig. 7) [16, 37]. Problems can also arise when the metabolism of a low turnover substrate is catalyzed by multiple CYPs. In this instance, a low signal to noise ratio leads to poor estimates of % I in human liver microsomes and inaccurate measurement of rCYP-catalyzed activity. Zileuton (points 3 and 4, Fig. 7) is an example of such a drug, because low rates of hydroxylation (CYP1A2 and CYP2C9) and sulfoxidation (CYP2C9 and CYP3A4) are catalyzed by at least two different CYPs [33].

The utility of integrated CYP reaction phenotyping is further illustrated with naproxen, which has been shown to undergo CYP1A2-($f_{m,CYP1A2} \sim 50\%$) and CYP2C9-($f_{m,CYP2C9} \sim 50\%$) catalyzed O-demethylation in the presence of native human liver microsomes [35]. Neverthe-

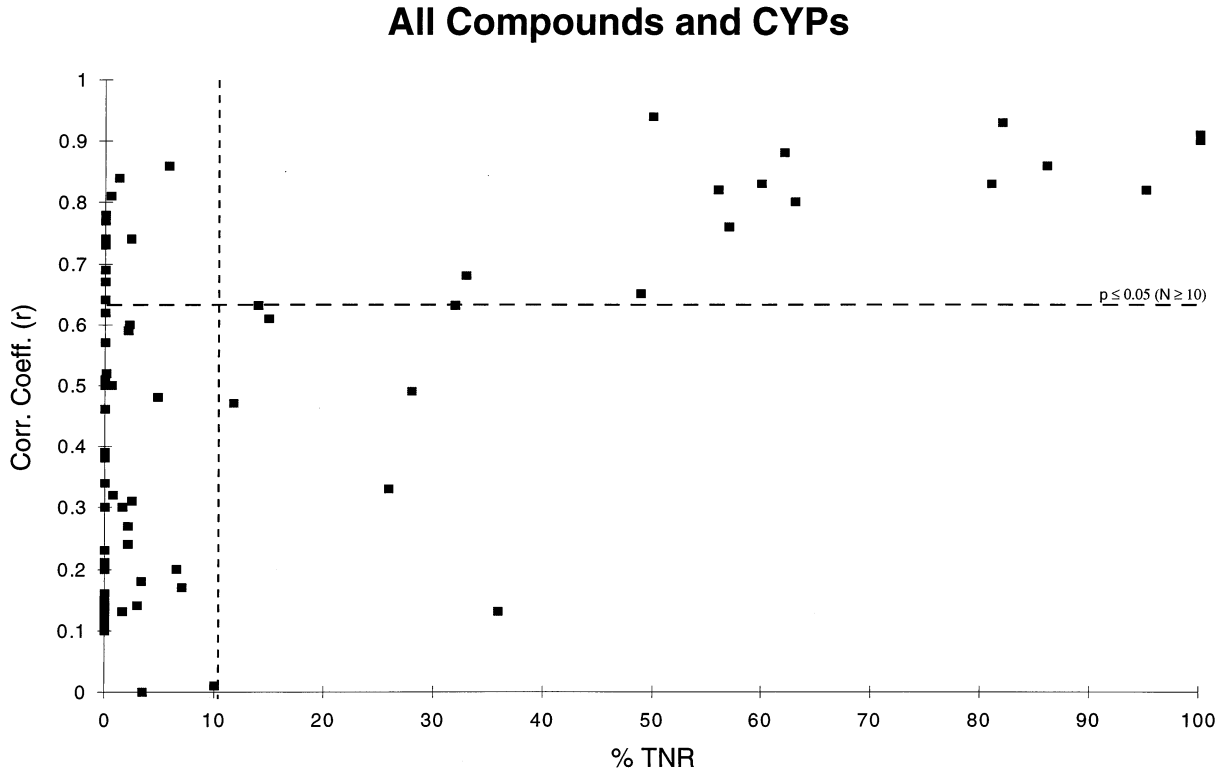

FIG. 9. Relationship between correlation coefficient (*r*) and % TNR (rCYPs). In each case, univariate linear regression analysis was carried out with a bank of liver microsomes (N ≥ 10 different livers). Statistically significant correlations ($P \leq 0.05$; $r \geq 0.63$) where % TNR is low (≤10%) are considered spurious. Data were obtained with drugs presented in Table 3, and regression analysis was performed with CYP form selective probe activities (tolbutamide hydroxylase, CYP2C9; 7-ethoxyresorufin *O*-deethylase, CYP1A2; erythromycin *N*-demethylase, CYP3A4; coumarin 7-hydroxylase, CYP2A6; *p*-nitrophenol hydroxylase or chlorzoxazone hydroxylase, CYP2E1) or immunoquantitated protein (CYP2D6).

less, appreciable rates of *O*-demethylation (>0.5 pmol/min/pmol rCYP) are observed with at least three rCYP2Cs (Table 4). Upon normalization of the data, it is apparent that CYP2C9 will be the major CYP2C catalyzing the reaction (% TNR ~ 90%) at substrate concentrations approaching K_m in native liver microsomes. However, because only about 50% of the activity is attributed to CYP2C proteins, CYP2C9 would be expected to contribute to about 45% of the overall metabolism. A similar conclusion is reached upon consideration of the inhibitory potential of sulfaphenazole, a known competitive inhibitor of rCYP2C9 (K_i = 0.3 μM), rCYP2C8 (K_i = 63 μM), and rCYP2C18 (K_i = 29 μM) [19]. Under well-defined experimental conditions ([S]/K_m = 0.6), sulfaphenazole (5 μM) would be expected to inhibit CYP2C9 markedly (% I > 90%; [I]/K_i ~ 17) in native human liver microsomes, while exerting only a minimal effect on CYP2C8, CYP2C19, and CYP2C18 (% I < 10%; [I]/K_i < 0.2) (Table 4). In agreement, about half of the naproxen *O*-demethylase activity ([S]/K_m = 0.6) in native human liver microsomes is inhibited by sulfaphenazole (5 μM) [35], indicating that CYP2C9 is maximally inhibited. Therefore, integrated CYP reaction phenotyping can differentiate between various CYP subfamily members. It is hoped that this type of analysis will be extended to include other CYP2C substrates such as tolbutamide, diazepam, phenytoin, warfarin, ibuprofen, taxol, and *(S)*-mephenytoin [2].

% *TNR Versus* r

The data presented in Fig. 8 illustrate how the correlation coefficient, the product of univariate regression analysis, can be related to % TNR. For CYP3A4, CYP2D6, CYP1A2, and CYP2C9, the data indicate that the correlation is statistically significant ($P \leq 0.05$; N ≥ 10 livers; $r > 0.63$) only when % TNR is greater than 30%. In the majority of cases, a low % TNR (<30%) is characterized by a relatively weak correlation ($r < 0.60$). These findings are further illustrated in Fig. 9, where data for all CYPs (CYP1A2, CYP2C9, CYP3A4, CYP2D6, CYP2E1, and CYP2A6) are combined. However, one has to be aware of spurious correlations (% TNR < 10%; % I < 10%; $r > 0.63$, $P < 0.05$) as a result of co-regression between markers of different CYPs. This can be avoided by carrying out multivariate regression analysis, or simply performing univariate regression analysis of the CYP marker activities. If the latter is performed ahead of time (N ≥ 15 livers), then selected liver samples can be omitted from the analysis, and one can attempt to ensure that correlations between CYP markers are weak ($r < 0.4$; N ≥ 10 livers).

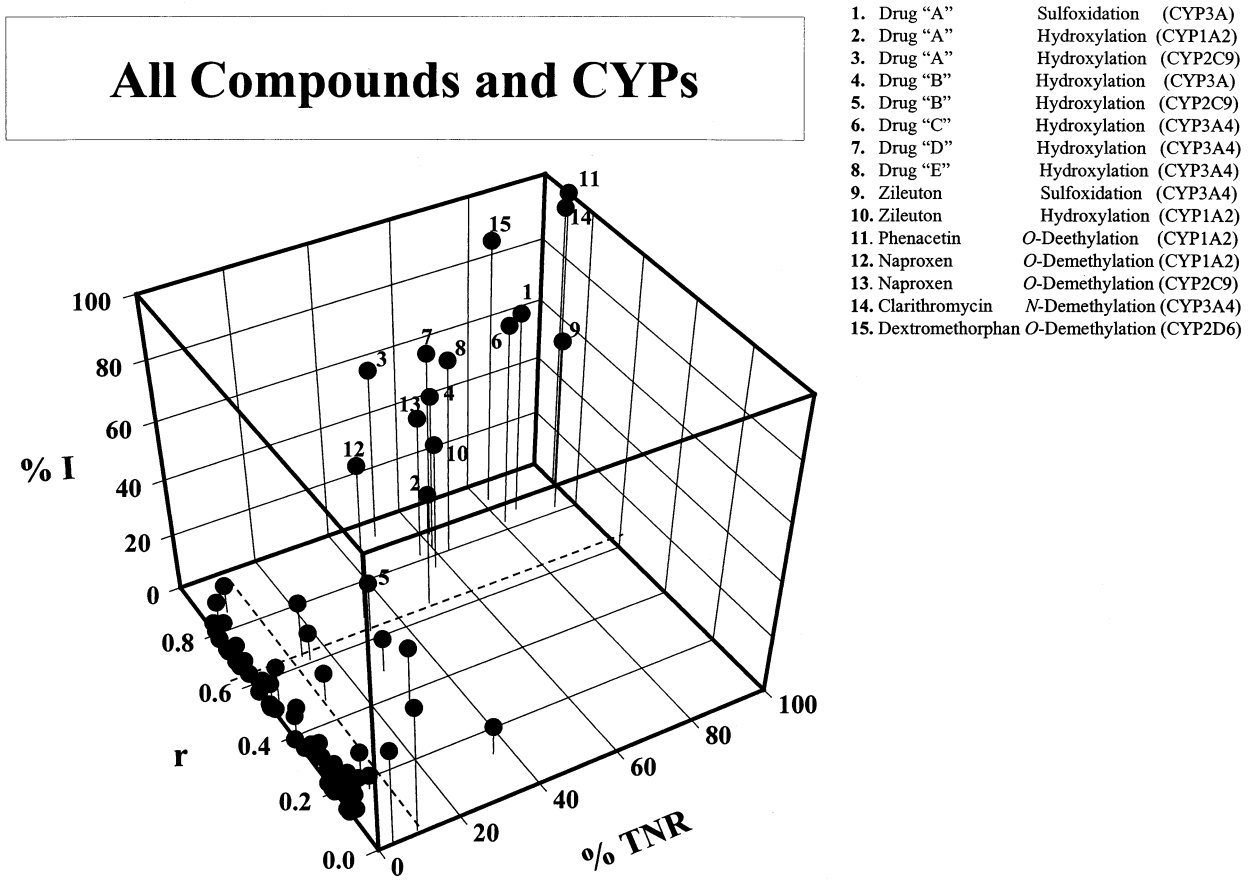

FIG. 10. Relationship between correlation coefficient (*r*), % TNR, and % I. Data presented in Figs. 7 and 9 are shown. Numbers refer to reactions listed in Table 5.

By comparing all three data sets (*r*, % TNR, and % I), the CYP reaction phenotype of a drug can be assigned using three criteria (Fig. 10):

(1) Reaction(s) is (are) catalyzed primarily by a single form of CYP (% TNR $\geq$ 60%; % I $\geq$ 60%; $r \geq 0.63$, $P \leq 0.05$, N $\geq$ 10 livers), and the individual contribution of each of the other CYPs is minor (% TNR < 20%; % I $\leq$ 10%; $r < 0.63$). Examples include clarithromycin *N*-demethylation and 14-(*R*)-hydroxylation (CYP3A4), low K_m phenacetin O-deethylation (CYP1A2) and dextromethorphan O-demethylation (CYP2D6), and drug C and D hydroxylation (CYP3A4) (Table 5). In the case of drug E, hydroxylation is primarily (≥60%) catalyzed by CYP3A4, although CYP2D6 (~10%), CYP2C19 (<10%), CYP1A2 (~10%), and CYP2C9 (~10%) also contribute (unpublished observations).

(2) Reaction(s) is (are) catalyzed by two or three different CYPs, and the contribution of each CYP varies (% TNR = 20–60%; % I = 10–60%; $r \geq 0.63$, $p \leq 0.05$, N $\geq$ 10 livers). This type of profile is exemplified by the CYP1A2/CYP2C9-catalyzed O-demethylation of naproxen and hydroxylation of zileuton, and the CYP3A4/CYP2C9-catalyzed hydroxylation of drug B (Table 5).

(3) Multiple (more than three) CYPs contribute equally to each reaction, and no one CYP form dominates (% TNR < 30%; % I < 30%; $r < 0.63$). In this instance, inhibitory drug–drug interactions are minimized because no single CYP enzyme has the potential to contribute to the overall clearance of the drug (e.g. $f_{m,CYPn} \leq 30\%$). However, it is possible that significant induction (>3-fold) of a "minor" form contributing only 30% to total product formation could result in an appreciable interaction.

CONCLUSIONS

As a result of the increased availability of rCYPs, native human liver microsomes, and CYP form selective chemical inhibitors and marker substrates, it is now possible to perform *in vitro* CYP reaction phenotyping in an integrated manner. The results described herein, albeit with a relatively small data set of ten compounds, show that one can attempt to normalize data obtained with rCYPs. In turn, the normalized data (% TNR) can be related to inhibition (% I) and regression analysis data obtained with native human liver microsomes. The normalization process is relatively straightforward (equation 2) and, after input of

TABLE 5. ***In vitro*** **CYP reaction phenotype (major CYPs) of various drugs**

Drug	f_m (%)*	Reaction†	CYP(s)‡	% TNR§	Human liver microsomes	
					% I‖	*r*¶
A		**1.** Sulfoxidation	3A4	86	70	0.87
		2. Hydroxylation	1A2	49	40	0.65
		3. Hydroxylation	2C9	50	60	0.93
B		**4.** Hydroxylation	3A4	60	55	0.83
		5. Hydroxylation	2C9	32	18	0.63
C		**6.** Hydroxylation	3A4	80	70	0.83
D	<10	**7.** Hydroxylation	3A4	61	65	0.93
E		**8.** Hydroxylation	3A4	63	68	0.80
Zileuton	<10	**9.** Sulfoxidation	3A4	95	60	0.82
		10. Hydroxylation	1A2	57	45	0.76
Phenacetin	≥57	**11.** *O*-Deethylation	1A2	100	95	0.91
Naproxen	≤30	**12.** *O*-Demethylation	1A2	33	55	0.68
		13. *O*-Demethylation	2C9	56	50	0.82
Clarithromycin	~35	**14.** *N*-Demethylation	3A4	100	100	0.90
Dextromethorphan	≥40**	**15.** *O*-Demethylation	2D6	82	90	0.93

*Percent of dose metabolized via indicated pathway(s) *in vivo*.

†Bolded numerals refer to data presented in Fig. 10.

‡Only those CYPs that play a major role in the metabolism of each drug are shown.

§Percent of TNR obtained with rCYPs.

‖Inhibition observed with CYP form selective inhibitors ($[I]/K_i \geq 10$; $[S]/K_m \leq 1.0$), upon incubation with native human liver microsomes.

¶Correlation coefficient (univariate regression analysis) obtained with CYP form selective activities (CYP3A4, erythromycin *N*-demethylase; CYP2C9, tolbutamide hydroxylase; CYP1A2, 7-ethoxyresorufin *O*-deethylase) or immunoquantitated protein (CYP2D6) in a bank of native human liver microsomes. Only statistically significant correlations ($P \leq 0.05$; $N \geq 10$ different livers) are shown (see legend to Fig. 8).

**Extensive metabolizer subjects.

initial reaction rate data and CYP immunoquantitation data, can be carried out using commercially available software (e.g. MSExcel®). Because inhibition data (equation 1) can also be analyzed using spreadsheets, one can integrate both data sets (% TNR vs % I) very rapidly (Figs. 4–6). In the near future, as the use of immunoquantitation data and CYP form selective inhibitory (anti-CYP peptide) antibodies becomes widespread [25, 26, 28–30], it is envisioned that data obtained with rCYPs will be normalized, and numerous integrated CYP reaction phenotyping strategies will be developed. Standardization of these strategies will be important, so that the generation of databases and accurate forecasting of drug–drug interactions and polymorphic oxidations can be expedited [44, 45]. Moreover, given the trend towards high-throughput screening, the eventual goal will be to carry out *in vitro* CYP reaction phenotyping using currently available multi-well microplate technology [44]. For a given series of compounds, because decreases in CL_{int} can be reflective of changes in the CYP reaction phenotype, the data need to be analyzed in parallel with *in vitro* metabolic stability data (e.g. parent drug consumption half-life in microsomes at a substrate concentration of ~1 μM) [40]. This type of analysis requires rapid integration of both data sets. However, while *in vitro* CYP reaction phenotyping data *per se* can be used to screen out numerous compounds preclinically, it is worth reemphasizing that the clinical significance of the data can only be fully evaluated in light of information garnered from clinical trials and human PK/ADME studies:

(1) Therapeutic index.
(2) The fraction of the dose cleared via metabolism (f_m vs renal and/or biliary clearance of parent drug).
(3) The fraction of metabolism catalyzed by CYP ($f_{m,CYP}$ vs other routes of metabolism).
(4) Intrinsic clearance (vs $Q_{h,b}$) in the absence of inhibitor (intravenous route only).
(5) The sites of metabolism (e.g. hepatic vs gut).
(6) The route of administration (e.g. oral vs intravenous).
(7) The *in vivo* potency of co-administered inhibitors ($[I]/K_i$) and inducers [1, 4, 15, 45, 46].
(8) The incidence of polymorphism, or allele frequencies (e.g. CYP2D6, CYP2C9, and CYP2C19), within different populations [47–49].

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Note added to proof:

Lasker *et al.* [*Arch Biochem Biophys* **353:** 16–28, 1998] have reported recently that CYP2C9 (vs CYP2C8 and CYP2C19) is quantitatively the most predominant CYP2C subfamily member in native human liver microsomes.

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