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Received 8 December 2000

Predicting drug pharmacokinetics in humans from *in vitro* metabolism studies

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Abstract

The pharmaceutical industry is committed to market safer drugs with fewer side effects, predictable pharmacokinetic properties and quantifiable drug–drug interactions. There is an increasing need to develop robust, enhanced-throughput *in vitro* assays, which accurately extrapolate to humans. The major drug metabolizing human hepatic cytochrome P450s (CYPs; CYP1A2, 2C9, 2C19, 2D6 and 3A4) have been co-expressed functionally in *Escherichia coli* with human NADPH-cytochrome P450 reductase and validated as surrogates to their counterparts in human liver microsomes (HLM) with respect to their kinetic and inhibition properties. Using these recombinant enzymes, fully automated *in vitro* assays to assess CYP inhibition and determine the enzymology of drug oxidation have been developed and validated. IC₅₀ values determined for a series of test compounds in HLM and recombinant CYPs were similar ($r^2 = 0.9$, $P < 0.001$). There was a good correlation between the sum of individual CYP intrinsic clearance (Cl_{int}) and HLM Cl_{int} ($r^2 = 0.8$, $P < 0.001$) for ten prototypic substrates for which clearance was CYP-dependent. Several *in vitro* incubation milieu (e.g. CYPs, HLM, human hepatocytes) are routinely used and the level of non-specific binding was investigated with respect to effects on K_m and K_i determinations. There were clear correlations between binding and lipophilicity (logD_{7.4}) for a selection of bases ($r^2 = 0.98$, $P < 0.001$) and acids ($r^2 = 0.79$, $P < 0.001$) that may allow prediction of this property. Our laboratory has shown that recombinant enzymes are suitable for ‘frontline’ predictive human metabolism studies in early drug discovery.

Key words: cytochrome P450, drug metabolism.

Abbreviations used: HLM, human liver microsomes; CYP, cytochrome P450; Cl_{int}, intrinsic clearance.

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Introduction

The ability to predict accurately pharmacokinetic parameters in humans from relatively straightforward *in vitro* screens is an important paradigm currently facing drug-metabolism scientists in the pharmaceutical industry. Clinical experiences in the last decade have highlighted the importance of assessing the potential for inhibition [1] and induction of drug metabolism [2]. In addition, identification of the drug-metabolizing enzymes responsible for the biotransformations commonly encountered in drug development may predict interindividual variability in pharmacokinetics and prioritize drug–drug interaction studies. As $\approx 60\%$ of all marketed drugs are primarily cleared by cytochrome P450 (CYP)-dependent metabolism [3] the initial efforts of the industry have been centred on this enzyme family.

Currently the use of both human hepatic tissue fractions as well as heterologous-expressed CYPs is considered the most thorough approach to evaluating both the inhibitory potential of new chemical entities and to determining the enzymology of metabolism. For example, a common approach to identify the CYP enzyme(s) involved in the metabolism of a new chemical entity requires correlation analyses within a bank of human liver microsomes (HLM), chemical and antibody inhibition, and subsequent confirmation with recombinant enzymes.

As part of an extensive collaboration between academia and the major pharmaceutical companies, our laboratory has characterized extensively the major drug-metabolizing human CYP isoforms, CYP1A2, 2C9, 2C19, 2D6 and 3A4, expressed in *Escherichia coli* [4]. Using these recombinant enzymes, assays have been developed and validated to determine both the potential for CYP inhibition [5] and the enzymology of drug oxidation by the individual CYPs [6]. In addition, an accurate determination of values such as K_i and K_m is essential to the success of *in vitro*–*in vivo*

extrapolations. The extent of non-specific binding of compounds to the protein/phospholipid milieu of *in vitro* incubations is currently being evaluated.

The now routine use of combinatorial chemistry and high-throughput screens in drug-discovery programmes generates much larger numbers of chemically diverse compounds. Therefore there is increasing expectation for the analysis of CYP–drug interactions to be ‘enhanced’ throughput and ensure rapid result turnaround. The fully automated assays developed at AstraZeneca R&D Charnwood are used as a ‘front-line’ approach to new-chemical-entity screening in early drug discovery.

Kinetic properties of recombinant human CYPs

The five major drug-metabolizing human hepatic CYPs were functionally co-expressed with human NADPH-cytochrome P450 reductase in *E. coli* and characterized with respect to their kinetic and inhibition properties. Using automation technology the kinetic parameters V_{\max} and K_m of CYP1A2, 2C9, 2C19, 2D6 and 3A4 were determined and compared with results obtained for HLM, recombinant CYPs from the major commercial sources and the wider literature. Probe assays were the fluorometric-based ethoxyresorufin O-dethylation for CYP1A2 and radiometric analysis of naproxen O-demethylation for CYP2C9, diazepam N-demethylation for CYP2C19, dextromethorphan O-demethylation for CYP2D6 and erythromycin N-demethylation for CYP3A4. The dealkylation of ethoxyresorufin, dextromethorphan and erythromycin were all shown to be specific reactions for CYP1A2, CYP2D6 and CYP3A4 respectively, which allowed direct comparison with kinetic data for HLM. The complex kinetics for the discrete oxidation of naproxen and diazepam in HLM necessitated a comparison of kinetic data for CYP2C9 and CYP2C19 with data obtained using established, commercial CYP preparations. Turnover numbers of CYPs expressed in *E. coli* towards these substrates were generally equal to or even greater than those of the major commercial suppliers. The mean turnover number \pm S.D. for ethoxyresorufin O-dethylation by CYP1A2 in *E. coli* was $0.6 \pm 0.2 \text{ min}^{-1}$ and in B-lymphoblasts $0.4 \pm 0.1 \text{ min}^{-1}$; CYP2C9 (naproxen O-demethylation), $6.7 \pm 0.9 \text{ min}^{-1}$ in *E. coli* and 4.9 min^{-1} in B-lymphoblasts; CYP2C19 (diazepam N-demethylation), $3.7 \pm 0.3 \text{ min}^{-1}$ and $0.2 \pm 0.1 \text{ min}^{-1}$;

CYP2D6 (dextromethorphan O-demethylation), $4.7 \pm 0.1 \text{ min}^{-1}$ and $4.4 \pm 0.1 \text{ min}^{-1}$; CYP3A4 (erythromycin N-demethylation), $3 \pm 1.2 \text{ min}^{-1}$ and 1.6 min^{-1} . The apparent K_m values for the specific reactions were also similar (K_m ranges for expressed CYPs and HLM were: ethoxyresorufin, 0.5–1.0 μM ; dextromethorphan, 1.3–5.9 μM and erythromycin, 18–57 μM), indicating little if any effect of N-terminal modification on the *E. coli*-expressed CYPs. The data generated for all the probe substrates by HLM and recombinant CYPs also agreed well with literature values.

The molar ratio of NADPH-cytochrome P450 reductase to recombinant CYP has been manipulated for the *E. coli* expression constructs to produce the appropriate, optimal reaction kinetics for probe substrates [4]. For example, optimal CYP2C19-mediated diazepam N-demethylation can be achieved, in the absence of cytochrome b_5 , by increasing the molar ratio of NADPH-cytochrome P450 reductase/CYP2C19 to $\approx 20:1$ [4]. Indeed, in order to optimize CYP expression systems, further elucidation of the role and importance of ancillary electron transporters such as cytochrome b_5 in the metabolism of xenobiotics is required [7].

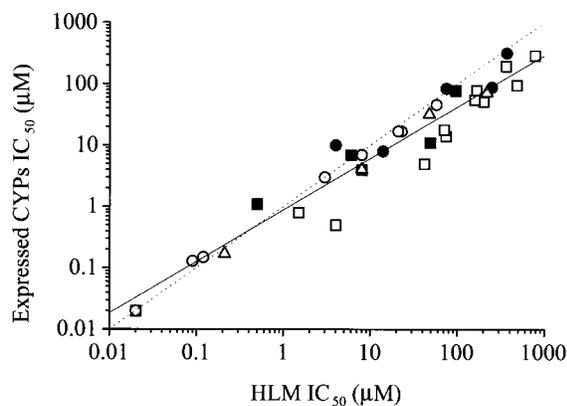
Inhibition properties of recombinant human CYPs

Fully automated inhibition screens for the major human hepatic CYPs have been developed and

Figure 1

Comparison of IC_{50} values determined in recombinant CYPs and HLM for a series of compounds

The data represent IC_{50} values for the five different isoforms CYP1A2(■), CYP2C9(□), CYP2C19(●), CYP2D6(O) and CYP3A4(△). For CYP2C9 and CYP2D6, where more than one cDNA-expression system was used, the figure reflects only IC_{50} values determined from *E. coli* membranes expressing the respective isoform. The solid line depicts linear regression analysis of the data ($r^2 = 0.9$, $P < 0.001$). The dashed line depicts unity.



validated. Furafylline, sulfaphenazole, omeprazole, quinidine and ketoconazole were identified as specific markers for the respective CYP1A2 ($IC_{50} = 6 \mu M$), CYP2C9 ($0.7 \mu M$), CYP2C19 ($6 \mu M$), CYP2D6 ($0.02 \mu M$) and CYP3A4 ($0.1 \mu M$) inhibition screens.

Figure 1 shows the correlation between IC_{50} values determined for a series of test compounds in human liver microsomes and recombinant CYPs ($r^2 = 0.9$, $P < 0.001$). In particular, the CYP1A2, CYP2D6 and CYP3A4 screens demonstrated the flexibility to accept either enzyme source. As a result of incomplete selectivity, recombinant CYPs are required for analysis of CYP2C9 and CYP2C19 inhibition using the substrates chosen. Good agreement was demonstrated between IC_{50} values determined in these assays and values published by other laboratories using a wide range of analytical techniques and similar incubation conditions, which provided confidence in the universality of these inhibition screens.

Enzymology of drug oxidation

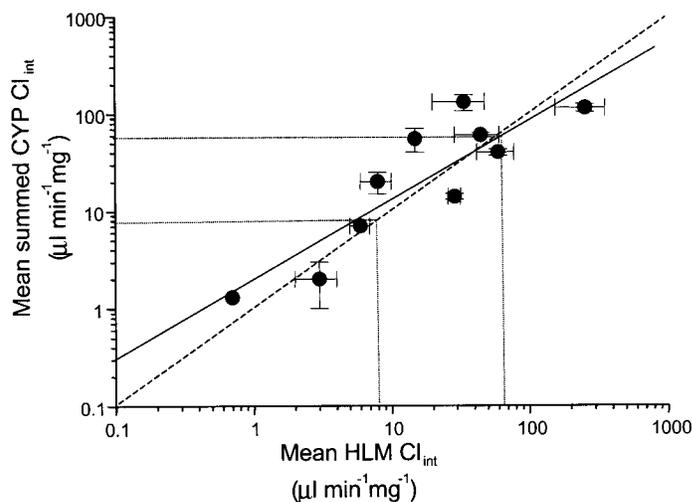
Much interest is currently focused on the identification of the drug-metabolizing enzymes responsible for the biotransformations commonly encountered in drug development [8]. Such information, determined early in the drug-discovery process, is useful for several purposes, including;

understanding ligand–enzyme structure–activity relationships; expanding the database for substrates of the polymorphic isoforms; assessment of potential inter-subject variability; prediction of drug–drug interactions and ultimately direction of clinical trials. Using recombinant enzymes a fully automated assay to determine the enzymology of drug oxidation by the major human hepatic CYPs has been developed and validated [6]. Ten prototypic substrates were chosen for which clearance was primarily CYP-dependent oxidation and the activities of these five major CYPs were represented. A range of intrinsic clearance (Cl_{int}) values were obtained for substrates in both pooled HLM ($1\text{--}380 \mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) and recombinant CYPs ($0.03\text{--}7 \mu l \cdot \text{min}^{-1} \cdot \text{pmol}^{-1}$) and thus the percentage contribution of individual CYPs towards their oxidative metabolism could be estimated. All the assignments were consistent with the available literature data. Tolbutamide was metabolized by CYP2C9 (70 %) and CYP2C19 (30 %), diazepam by CYP2C19, ibuprofen by CYP2C9 (90 %) and CYP2C19 (10 %) and omeprazole by CYP2C19 (68 %) and CYP3A4 (32 %). Metoprolol and dextromethorphan were primarily CYP2D6 substrates and propranolol was metabolized by CYP2D6 (59 %), CYP1A2 (26 %) and CYP2C19 (15 %). Diltiazem, testosterone and verapamil were metabolized predominantly by CYP3A4. In addition, the metabolite profile for the CYP-

Figure 2

Comparison of summed CYP Cl_{int} with HLM Cl_{int} for several probe substrates

The summed CYP and HLM Cl_{int} data points represent the mean Cl_{int} determinations and the error bars reflect the S.D. from the mean. The dotted boxes illustrate HLM Cl_{int} values of $< 8 \mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (low clearance) and $> 65 \mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (high clearance). The solid line depicts linear regression analysis of the data ($r^2 = 0.8$, $P < 0.001$) with the equation $\log CYP Cl_{int} = 0.91 \times \log HLM Cl_{int} + 0.3$. The dashed line indicates the line of unity.



dependent clearance of several markers determined by MS was as predicted from the literature.

There was a good correlation between the sum of individual CYP Cl_{int} and HLM Cl_{int} ($r^2 = 0.8$, $P < 0.001$) for the substrates, indicating that recombinant CYPs may be used to predict HLM Cl_{int} data (Figure 2). The summed CYP Cl_{int} correctly predicts a low HLM Cl_{int} ($< 8 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) for tolbutamide, diazepam and metoprolol; an intermediate HLM Cl_{int} ($8\text{--}65 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) for ibuprofen, propranolol, dextromethorphan, diltiazem and testosterone; and a high HLM Cl_{int} ($> 65 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) for verapamil. However, the summed CYP Cl_{int} of omeprazole and propranolol does over-predict somewhat HLM Cl_{int} . One possible explanation for this is an increase in 'futile' binding with increased protein concentration for some compounds. Typical assay conditions used $0.2\text{--}0.4 \text{ mg}$ of protein $\cdot \text{ml}^{-1}$ CYPs (exact amount depended on the CYP expression level as all incubations contained 100 pmol of CYP $\cdot \text{ml}^{-1}$) and $1 \text{ mg} \cdot \text{ml}^{-1}$ HLM. The HLM Cl_{int} of propranolol at $0.4 \text{ mg} \cdot \text{ml}^{-1}$ was determined to be $22 \pm 4 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ which compares more favourably with the summed CYP Cl_{int} at the same protein level ($55 \pm 15 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

Non-specific binding

It is generally assumed that unbound drug concentration more closely reflects the activity of a drug than does total plasma concentration. It follows that only unbound drug interacts with drug-metabolizing enzymes, either as an inhibitor

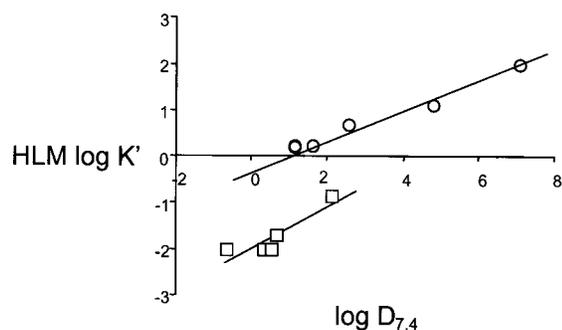
or substrate. The accurate measurement of kinetic parameters such as K_i and K_m underpins successful *in vitro*–*in vivo* extrapolations and the effect of non-specific binding on these determinations is currently of great interest to scientists in this field. Despite this, the total concentration added to *in vitro* incubation milieu is traditionally used in estimates of apparent K_m or K_i values. Indeed, several recent reports have confirmed that drugs can bind substantially to microsomal fractions [9–11]. The relationship between physicochemical descriptors and binding of compounds to a typical microsomal incubation has been explored. Several literature sources were interrogated for microsomal binding data and $\log D_{7.4}$ measurements. Figure 3 shows the relationship between binding of acids and bases to HLM with lipophilicity. Two distinct trends, one for bases and the other for acids, were observed which allow a physicochemical description of this phenomenon that predicts microsomal binding. Moreover, it is clear that for a given $\log D_{7.4}$, the microsomal binding of bases is at least an order of magnitude greater than that of acids. Further efforts to examine differential non-specific binding between separate *in vitro* models and the effect on extrapolation to humans (e.g. for the prediction of Cl_{met} and the potential for drug–drug interactions) are underway.

In conclusion, these data indicate that *E. coli*-expressed CYPs appear faithful surrogates for the native (HLM) enzymes and the data suggest that such recombinant enzymes may be suitable for predictive human metabolism studies in early drug discovery. Therefore, although this technology is very much in its infancy, the data in this report demonstrate the potential for the optimization and prediction of various human pharmacokinetic parameters from enhanced-throughput *in vitro* assays.

Figure 3

Relationship of the lipophilicity of bases, neutrals and acids to microsomal binding

Binding to HLM is expressed as $\log K'$, where $K' = [\text{Bound}]/[\text{Free}]$. Circles represent basic compounds, the solid line through these points is a linear regression ($r^2 = 0.98$, $P < 0.001$). Squares represent acidic compounds, the solid line depicts a linear regression analysis of these data ($r^2 = 0.79$, $P < 0.001$).



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Received 1 November 2000

Evolution of bioinorganic motifs in P450-containing systems

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Abstract

The choice of bioinorganic motifs by Nature results in a spectacular variety of active-site structures even within the same protein family. Here, we use the concept of the bioinorganic motif to discuss the function and evolution of P450-containing and other related systems. Apart from P450, these systems include a FAD flavoprotein or domain, a FMN domain, ferredoxins and cytochrome b_5 . Analysis of available complete genomes can shed light on what an ancestral P450-containing system could be.

Introduction

The P450 enzymes constitute a large superfamily of haem-thiolate proteins involved in the metabolism of diverse chemical compounds. Usually, they act as terminal oxidase in multicomponent electron-transfer (ET) chains, called here P450-containing mono-oxygenase systems, although self-sufficient non-mono-oxygenase P450s are also described. In the previous review [1], we postulated that all P450-containing mono-oxygenase systems share common structural and functional domain architecture. Apart from P450 itself, these

systems can comprise several fundamental redox domains: FAD flavoprotein or domain, FMN domain, ferredoxin (Fd) and cytochrome b_5 . These ubiquitous redox domains, in various combinations, are widely distributed in biological systems. FMN domain, Fd or cytochrome b_5 transfer electrons between the FAD domain and P450. While P450-containing systems are found throughout all kingdoms of life, some organisms lack one or more of these redox domains completely. Here, we review the current state of the affairs in the light of data that became available as a result of the genomic revolution and the progress in structural biology.

In 1995, the first complete genome sequence of a free-living organism, the bacterium *Haemophilus influenzae*, was published [2]. Since that, complete genomes of 36 species, including three eukaryotes, have been sequenced (see <http://www.ebi.ac.uk/genomes/>; when this paper is published, the number is likely to have increased). The analyses of the complete genome data provide unprecedented insight into molecular evolution of biochemical systems.

In the same time, we have witnessed an extraordinary increase in quantity and quality of protein structural information. In the last 5 years, the structures of key components of P450-containing systems were determined, viz. NADPH:cytochrome P450 reductase (CPR), NADPH:adrenodoxin reductase (AdR) and adrenodoxin (Adx), together with structures of new P450s from different kingdoms of life and other haem-thiolate proteins.

On the other hand, our knowledge of function of P450 and P450-containing systems does not catch up with the relevant sequence and structural information. For example, of 83 *Drosophila melanogaster* P450s, CYP6A2 is the only functionally

Key words: cytochrome b_5 , electron transfer, ferredoxin, flavoprotein, genome.

Abbreviations used: 3D, three-dimensional; CPR, NADPH:cytochrome P450 reductase; AdR, NADPH:adrenodoxin reductase; Adx, adrenodoxin; b5R, NADH:cytochrome b_5 reductase; BIM, bioinorganic motif; ET, electron transfer; FADPNR, FAD-dependent pyridine nucleotide reductase; Fe3, Fe₃S₄ bacterial-type ferredoxin; Fd, ferredoxin; Fld, flavodoxin; FNR, ferredoxin:NADP⁺ reductase; NOS, nitric oxide synthase; PdR, putidaredoxin reductase; Pdx, putidaredoxin; Rdx, rhodocoxin; RdR, rhodocoxin reductase; SiR, sulphite reductase; SiR-FP, SiR flavoprotein subunit; SPTR, SWISS-PROT/TrEMBL; TdR, terpredoxin reductase; Tdx, terpredoxin.

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