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PHARMACOKINETICS AND DISPOSITION

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Identification of human cytochrome P_{450}s that metabolise anti-parasitic drugs and predictions of in vivo drug hepatic clearance from in vitro data

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Abstract *Objective*: Knowledge about the metabolism of anti-parasitic drugs (APDs) will be helpful in ongoing efforts to optimise dosage recommendations in clinical practise. This study was performed to further identify the cytochrome P_{450} (CYP) enzymes that metabolise major APDs and evaluate the possibility of predicting in vivo drug clearances from in vitro data.
Methods: In vitro systems, rat and human liver microsomes (RLM, HLM) and recombinant cytochrome P_{450} (rCYP), were used to determine the intrinsic clearance (CL_{int}) and identify responsible CYPs and their relative contribution in the metabolism of 15 commonly used APDs.
Results and discussion: CL_{int} determined in RLM and HLM showed low ($r^2 = 0.50$) but significant ($P < 0.01$) correlation. The CL_{int} values were scaled to predict in vivo hepatic clearance (CL_H) using the 'venous equilibrium model'. The number of compounds with in vivo human CL data after intravenous administration was low ($n = 8$), and the range of CL values covered by these compounds was not appropriate for a reasonable quantitative in vitro–in vivo correlation analysis. Using the CL_H predicted from the in vitro data, the compounds could be classified into three different categories: high-clearance drugs (> 70 % liver blood flow; amodiaquine, praziquantel, albendazole, thiabendazole), low-clearance drugs (< 30 % liver blood flow; chloroquine, dapsone, diethylcarbamazine, pentamidine, primaquine, pyrantel, pyrimethamine, tinidazole) and intermediate clearance drugs (artemisinin, artesunate, quinine). With the exception of artemisinin, which is a high clearance drug in vivo, all other compounds were classified using in vitro data in agreement with in vivo observations. We identified hepatic CYP enzymes responsible for metabolism of some compounds (praziquantel—1A2, 2C19, 3A4; primaquine—1A2, 3A4; chloroquine—2C8, 2D6, 3A4; artesunate—2A6; pyrantel—2D6). For the other compounds, we confirmed the role of previously reported CYPs for their metabolism and identified other CYPs involved which had not been reported before.
Conclusion: Our results show that it is possible to make in vitro–in vivo predictions of high, intermediate and low CL_{int} drug categories. The identified CYPs for some of the drugs provide a basis for how these drugs are expected to behave pharmacokinetically and help in predicting drug–drug interactions in vivo.

Keywords Anti-parasitic drug · Metabolism · Microsomes

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Introduction

In efforts to reduce attrition rates of new chemical entities (NCE) in drug discovery, drug absorption, distribution, metabolism, excretion and toxicology (ADME-Tox) studies have become a major activity in the pharmaceutical industry [1]. To ensure that only drugs with minimal adverse reactions (ADR) are licensed, drug regulatory agencies like the Food and Drug Administration (FDA) are also increasingly demanding data on ADME-Tox in relation to new drug applications (NDA) [2]. This not only affects the drug discovery and development process but also the optimal use of drugs already on the market.

Most anti-parasitic drugs (APDs) in use had already been introduced in the 1940s, before bioanalytical methods were available to evaluate ADME parameters

and optimise the pharmacokinetics (PKs) of the drugs. Because chemotherapy is often the principal means to control parasitic diseases, optimisation of their use is urgently needed. In addition, use of drug combinations to increase therapeutic efficiency and to avoid the emergence of drug resistance is now being advocated in tropical medicine [3]. In recent years, the gross PKs of some APDs has been studied in humans [4], but studies are also needed on the mechanistic basis for PK properties, therapeutic or toxicological outcome observed in the use of these drugs.

There have been many methodological developments in the study of drug metabolism. The increased availability of human tissue (liver microsomes, slices and hepatocytes) and recombinantly expressed human drug metabolising enzymes are giving an insight into how humans metabolise drugs (reviewed in [1]). The identification of cytochrome P_{450} (CYP)-specific marker reactions, antibodies and chemical inhibitors selective for some CYPs have improved the capacity to qualitatively and quantitatively estimate the role of different enzymes in the metabolism of test compounds [5, 6]. There are many reports on the success and failure of making in vitro–in vivo correlations of drug clearance which could indicate limitations inherent in the models used and a need for further research [7]. The specific aim of this study was to identify the major CYPs involved in the metabolism of APDs and to predict in vivo clearance from in vitro intrinsic clearance data. This was part of our group's effort to elucidate the metabolism of APDs (Fig. 1.). We have previously evaluated the inhibitory effects [8] and inducing effects [9] of APDs on CYPs. This information will be useful in rationalising the PKs of these drugs and predicting potential drug–drug interactions.

Materials and methods

Chemicals

Substrates, metabolites and inhibitors were purchased from different companies: phenacetin, paracetamol, coumarin, 7-hydroxycoumarin, diclofenac, amodiaquine (AQ), albendazole (ABZ), dextromethorphan, debrisoquine, diethylcarbamazine, pentamidine, praziquantel (PZQ), primaquine, pyrantel, pyrimethamine,

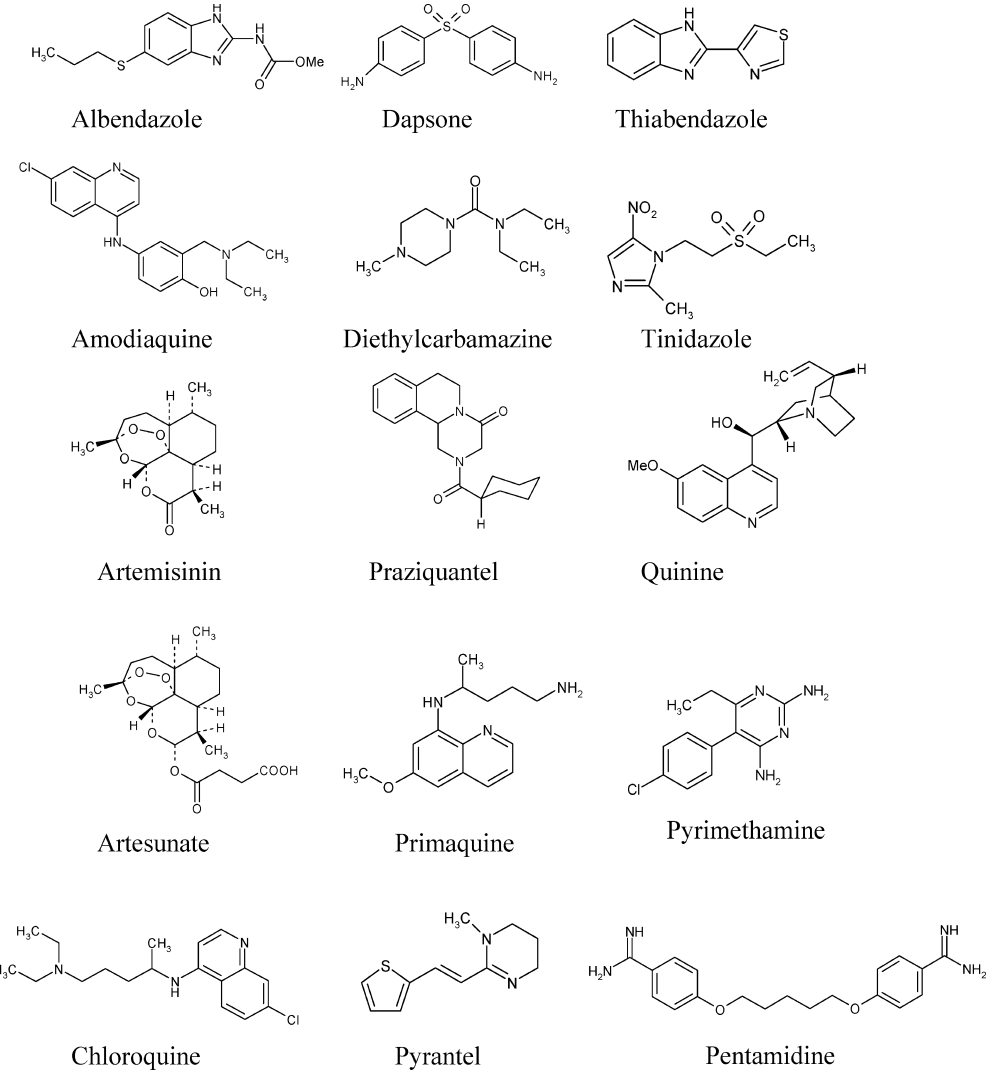

Fig. 1 Structures of the 15 antiparasitic drugs studied

quinine, thiabendazole, tinidazole, quinidine, α-naphthoflavone, reduced NADPH (Sigma Chemical Co., St. Louis, MO); bufuralol, 1′-hydroxybufuralol, *S*-mephenytoin, 4′-hydroxymephenytoin, 1′-hydroxymidazolam, 4-hydroxydebrisoquine, sulfaphenazole (Ultrafine, Manchester, UK); paclitaxel, 6α-hydroxypaclitaxel, 4′-hydroxydiclofenac, midazolam (GenTest Co., Woburn, MA); artemisinin, dapsone, quercetin (Aldrich Chemical Co., Milwaukee, WI); ketoconazole (Janssen Biotech, Flander, NJ); ticlopidine, dextrophan (ICN Biomedicals Inc., Aurora, Ohio). Metoprolol, α-hydroxymetoprolol, and demethylated metoprolol were obtained from AstraZeneca (Mölndal, Sweden). Bupropion, hydroxybupropion, chloroquine (CLQ), desethylchloroquine and *N*-desethylamodiaquine were gifts from Karolinska Institute (Stockholm, Sweden). Dr. Michael Ashton (Göteborg University, Göteborg, Sweden) kindly provided artesunate. All other reagents were of analytical or HPLC grade.

Human liver microsomes and recombinant cytochrome P_{450}s (rCYPs)

Twenty human liver microsomes (HLM) were obtained from an in-house bank of liver microsomes maintained at AstraZeneca Research and Development (Mölndal, Sweden). CYP activities (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) in individual HLM were determined using diagnostic marker substrates. The following marker reactions were used: phenacetin demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4′-hydroxylation (CYP2C9), *S*-mephenytoin 4′-hydroxylation (CYP2C19), bufuralol 1′-hydroxylation, debrisoquine 4-hydroxylation, dextromethorphan *O*-demethylation, metoprolol α-hydroxylation and demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1) and midazolam 1′-hydroxylation, testosterone 6β-hydroxylation (CYP3A4). Pooled HLM were prepared from a set of liver pieces of patients undergoing liver resections. Recombinant human CYP enzymes 1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 were from yeast (AstraZeneca, Sweden). CYP enzymes CYP2A6, 1B1, 2E1, 3A5, and 4A11 were from lymphoblastoid cell lines (GenTest Corp, Woburn, MA). The microsomal preparations were stored at −80 °C until use.

Incubation conditions with HLM, RLM and rCYP enzymes

All reactions were performed in 96-well plates. Each reaction mixture consisted of the appropriate enzyme, substrate, 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), in 0.1 M potassium phosphate buffer pH 7.4 in a final volume of 200 µl. The reactions were started by the addition of NADPH after a preincubation of 5 min at 37 °C. All reactions were stopped by the addition of 150 µl ice-cold acetonitrile. After centrifugation at $4500 \times g$ for 20 min, 50 µl supernatant was analysed by means of liquid chromatography/mass spectrometry (LC/MS).

Chromatography

The LC/MS system consisted of an HP 1100 system (Hewlett-Packard, Palo Alto, CA) and a Finnigan LCQ ion trap mass spectrometer (Finnigan Mat, San Jose, CA) employing an atmospheric pressure ionisation interface. Chromatography was performed on a Symmetry C_{18} column (3.9×150 mm i.d.; 5 µm, Waters, Milford, Massachusetts). The mobile phases consisted of acetonitrile as eluent A and 5 mM ammonium acetate buffer (pH 3) as eluent B, or 10 % of acetonitrile in 12 mM formic acid solution (v/v) as eluent C. Gradient runs of combinations of these eluents at a flow rate of 1 ml/min are shown in Table 1. The effluent was split with approximately 0.3 ml/min introduced into the mass spectrometer. Source parameters of mass spectrometer (e.g., spray voltage, temperature, gas flow rates, etc.) were individually optimised for each compound. The mass spectrometer was operated in the positive ion electrospray mode (except 7-hydroxycoumarin which was monitored in negative mode). The spray voltage was set to 4.0 kV, heated capillary temperature to 200 °C, source CID set to off. Nitrogen was used as the sheath and auxiliary gas and set to 70 and 20 (arbitrary units), respectively. The mass spectrometer was operated in the selected ion monitoring (SIM) mode. Instrument control, data acquisition and data evaluation were performed using Xcalibur software (version 1.2, Finnigan). The lower limit of quantification (LLOQ) was 0.1 µM for all the APDs investigated except artemisinin (LLOQ, 1 µM) and artesunate (LLOQ, 0.5 µM). The LLOQ was 0.05 µM for all the metabolites of CYP marker reactions.

Determination of intrinsic clearance

Intrinsic clearance (CL_{int}) studies were performed for 15 APDs with HLM, recombinant CYP enzymes (rCYPs) and RLM using the substrate disappearance approach [10, 11]. The typical incubation mixture consisted of 0.1–2 mg/ml HLM (0.5 mg/ml RLM or 20 pmol rCYPs), 1.0 µM substrates, 1 mM NADPH in 0.1 M phosphate buffer pH 7.4 in a final volume of 200 µl. After adding NADPH to initiate reaction, ice-cold acetonitrile was added at 0, 10, 20, 30, 45, 60 min to stop the reaction. The CL_{int} was determined as follows:

$$CL_{int} = (\ln 2 \times \text{incubation volume}) / \left(T_{1/2} \times \text{Protein or enzyme amount}\right)$$

$T_{1/2}$ was determined from the elimination rate constant $k = \ln 2 / T_{1/2}$. For the clearance determination of artemisinin and artesunate, the substrate concentrations used were increased to 10 µM and 5 µM, respectively, to increase their response for LC/MS detection. The use of low substrate concentrations, usually 1.0 µM, to estimate drug clearance stems from assumptions derived from the Michaelis–Menten kinetics: rate of metabolism $(v) = V_{max}[S]/(K_m + [S])$ and $CL_{int} = V_{max}/K_m$ (Michaelis–Menten equation). Under linear conditions, when [S] is 10 % or less of K_m (which most drugs are therefore assumed to be at in vivo), the equation reduces to $v = (V_{max}[S])/K_m$ which can be rearranged to:

$$CL_{int} = V_{max}/K_m = \text{rate of metabolism}/[S].$$

Detailed derivations of these equations and discussions of the assumptions are as presented previously [7, 12, 13].

In vitro–in vivo correlations

The CL_{int} in the incubation is expressed as µl/min/[mg (microsomes) or pmol (enzyme)]. This can be scaled to the apparent clearance ($CL_{int,app.}$) for the whole liver (70 kg person with a 1.4-kg liver, 20-g liver/kg body weight). In the scaling process, the currently used factor is 45 mg HLM per gram liver. Using these factors, the equation is [14]:

$$CL_{int,app} = \frac{0.693}{\ln \text{ vitro } T_{1/2}} \times \frac{\text{ml incubation}}{\text{mg microsomes}} \times \frac{45 \text{ mg microsomes}}{\text{g liver}} \times \frac{20 \text{ g liver}}{\text{kg body weight}}$$

To estimate hepatic clearance (CL_H) due to metabolism, a number of models have been proposed and include (a) the 'well stirred' or the 'venous equilibrium' model, (b) the parallel tube model, and (c) the dispersion model. Most in vitro–in vivo predictions are made using the venous equilibrium model due to its simplicity and the fact that studies have observed little difference in the values predicted by the three models [13]. The hepatic clearance, CL_H, is expressed as:

$$CL_H = \frac{Q_H \cdot f_{uB} \cdot CL_{int}}{Q_H + f_{uB} \cdot CL_{int}}$$

Table 1 Liquid chromatography (LC)/mass spectrometry (MS) chromatographic conditions for 15 anti-parasitic drugs and 14 specific cytochrome P_{450} (CYP) enzyme marker reactions analysis

Compound	HPLC conditions[a]		Retention time (min)	MS detection $[M+H]^+$ (m/z)
	Mobile phase	Linear gradient program of mobile phase		
Antiparasitic drugs				
Albendazole	A and B	0–6 min, A from 35% till 80%	4.9	266
Amodiaquine	A and B	0–6 min, A from 5% till 35%	4.6	356
Artemisinin	A and B	0–5.5 min, A from 50% till 98%	5.0	283
Artesunate	A and B	0–5.5 min, A from 50% till 98%	4.2	402 ($[M+NH_4]^+$)
Chloroquine	A and B	0–6 min, A from 5% till 35%	4.5	320
Dapsone	A and B	0–6 min, A from 10% till 95%	4.9	249
Diethylcarbamazine	A and B	0–1 min A keep at 5%, 1–5 min A from 5% till 28%	4.2	200
Pentamidine	A and B	0–6 min, A from 5% till 45%	5.0	341
Praziquantel	A and C	0–6 min, A from 30% till 75%	5.3	313
Primaquine	A and B	0–6 min, A from 5% till 65%	5.5	260
Pyrantel	A and B	0–6 min, A from 5% till 40%	4.7	207
Pyrimethamine	A and B	0–5.5 min, A from 10% till 70%	4.7	249
Quinine	A and B	0–6 min, A from 5% till 57%	5.1	325
Thiabendazole	A and B	0–6 min, A from 5% till 57%	5.2	202
Tinidazole	A and B	0–6 min, A from 5% till 70%	5.4	248
Specific CYP enzyme marker reactions				
O-Deethylphenacetin (CYP1A2)	A and C	0–1 min A keep at 5%, 1–4 min A from 5% till 45%	2.5	152
7-Hydroxylcoumarin (CYP2A6)	A and C	Constant: 0–5 min, A 20% and C 80%	3.3	161 ($[M-H]^-$)
Hydroxylbupropion (CYP2B6)	A and B	0–4 min, A from 20% till 47%	3.3	256
6α-Hydroxylpaclitaxel (CYP2C8)	A and C	0–1 min A keep at 5%, 1–8 min A from 5% till 95%	7.4	870
N-Desethylamodiaquine (CYP2C8)	A and B	0–6 min, A from 5% till 35%	4.4	328
4′-Hydroxyldiclofenac (CYP2C9)	A and C	0–2.5 min, A 20% till 95%, 2.5–5 min, A 95%	4.0	312
4′-Hydroxyl-S-mephenytoin (CYP2C19)	A and C	0–6 min, A from 5 till 70%	4.7	235
1′-Hydroxylbufuralol (CYP2D6)	A and C	0–4 min, A from 5% till 30%	2.6	278
4-Hydroxyldebrisoquine (CYP2D6)	A and B	0–3.5 min, A from 10% till 45%	2.6	192
O-Demethyldextromethorphan (CYP2D6)	A and B	0–5 min, A from 10% till 60%	4.3	258
α-Hydroxylmetoprolol (CYP2D6)	A and B	0–5 min, A from 10% till 60%	3.2	284
O-Demethylmetoprolol (CYP2D6)	A and B	0–5 min, A from 10% till 60%	3.4	254
1′-Hydroxylmidazolam (CYP3A4)	A and C	0–4 min, A from 20 till 60%	2.8	342
6β-Hydroxyltestosterone (CYP3A4)	A and B	0–4 min, A from 30% till 62%	3.5	305

[a]Chromatography was performed on a symmetry C_{18} column (3.9 × 150 mm i.d.; 5 μm). The mobile phase consisted of acetonitrile (A), 5 mM ammonium acetate buffer (pH 3) (B), or 10 % of acetonitrile in 12 mM formic acid solution (v/v) (C), run gradiently at a flow rate of 1 ml/min. The effluent was split with approximately 0.3 ml/min introduced into the mass spectrometer

where CL_{int} is the intrinsic clearance reflecting the actual metabolic capacity of the enzyme system with free access to substrate, f_{uB} is the free fraction in whole blood and Q_H is the total liver blood flow (the value of hepatic blood flow used here is 1400 ml/min). In the present study, the CL_H was calculated using the total incubation concentrations for all the drugs since the binding to HLM of the compounds was not evaluated. The CL_H of a drug cannot exceed the hepatic blood flow, 1400 ml/min. In general, drugs that have CL_H above 980 ml/min/70 kg (70 % Q_H) are classified high-clearance drugs and those below 420 ml/min/70 kg (30 % Q_H) are classified low-clearance drugs. In order to compare values across different studies, the conventional unit—ml/min/kg—is used. Using this unit, the limit of CL_H becomes 20 ml/min/kg with high-clearance drugs having values >14 ml/min/kg, and low-clearance drugs <6 ml/min/kg. In vitro–in vivo correlations of drug clearance was done for compounds for which drug clearance was measured after i.v. administration.

Enzyme kinetics

Incubation conditions used for 14 marker reactions in different recombinant human CYP enzymes and pooled HLMs were optimised for linearity with respect to time and protein concentrations. The metabolites formed were determined by LC/MS and quantified by external standardisation using authentic references. The detailed chromatographic conditions are shown in Table 1.

Contribution of CYPs to HLM APD metabolism

The percent contributions of CYPs to the metabolism of 15 APDs were estimated by applying the relative activity factor (RAF) values as proposed by Crespi [14] using the values of the activities. In brief, the RAF factor method aims to estimate the relative quantity of a specific CYP in HLM based on its metabolic activity on an enzyme-specific reaction in both the pure form of the enzyme and within the HLM. The activity can be measured as velocity (v) or CL_{int} [either as V_{max}/K_m or $\ln 2/(T_{1/2}$ x protein concentration)]. On dividing the activity of HLM for the marker reaction (pmol product/min/mg HLM) by the activity of the recombinant CYP enzyme on the marker reaction (pmol product/min/pmol rCYP), the RAF is derived as pmol rCYP/mg HLM. The RAFs of eight major CYPs (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4) were determined as the ratio of the activity of each enzyme marker reaction. The CYP enzyme-specific marker substrates for the 8 CYPs were: phenacetin (CYP1A2), coumarin (CYP2A6), bupropion (CYP2B6), paclitaxel, amodiaquine (CYP2C8), diclofenac

Table 2 Comparison of relative activity factors by different marker substrates and calculating methods. RAF_{CL} relative activity factor calculated from the clearance (V_{max}/K_m) of specific marker reaction in human liver microsomes (HLM) and rCYP enzyme, RAF_{Vmax} relative activity factor calculated from the V_{max} of specific marker reaction in HLM and rCYP enzyme, RAF_v relative activity factor calculated from the velocity of specific marker reaction at substrate concentration at K_m in HLM and rCYP enzyme

CYPs	Marker	Microsomes	V_{max} (pmol/min/mg HLM or pmol rCYP)	K_m (μM)	RAF_{CL} (V_{max}/K_m)	RAF_{Vmax} (V_{max})	RAF_v (velocity at K_m)	% of Relative content in HLM [a]
					(pmol CYP/mg HLM)			
CYP1A2	Phenacetin *O*-demethylation	HLM	718.1	30.0				
		rCYP1A2	6.5	37.8	**140.5**	198.9	241.3	**14.7**
CYP2A6	Coumarin 7-hydroxylation	HLM	431.0	2.4				
		rCYP2A6	3.5	4.6	**235.5**	127.6	184.0	**24.7**
CYP2B6	Bupropion hydroxylation	HLM	137.0	76.0				
		rCYP2B6	14.9	160.6	**9.7**	5.8	7.2	**1.0**
CYP2C8	Paclitaxel 6α-hydroxylation	HLM	107.9	10.9				
		rCYP2C8	1.1	4.9	**42.6**	38.6	50.1	**4.5**
	Amodiaquine *N*-desethylation	HLM	1695.9	3.4				
		rCYP2C8	4.7	0.8	81.8	345.3	177.1	(8.2)
CYP2C9	Diclofenac 4′-hydroxylation	HLM	1256.4	4.6				
		rCYP2C9	9.4	6.1	**175.9**	130.8	166.2	**18.5**
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	HLM	69.4	23.1				
		rCYP2C19	2.6	50.2	**57.3**	27.3	48.0	**6.0**
CYP2D6	Bufuralol 1′-hydroxylation	HLM	62.9	7.4				
		rCYP2D6	17.0	6.0	3.0	3.4	3.1	(0.3)
	Debrisoquine 4-hydroxylation	HLM	47.7	74.1				
		rCYP2D6	3.1	205.3	**43.2**	18.9	32.4	**4.5**
	Dextromethorphan *O*-demethylation	HLM	159.1	3.6				
		rCYP2D6	15.6	12.1	34.5	11.1	17.8	(3.7)
	Metoprolol α-hydroxylation	HLM	69.5	106.0				
		rCYP2D6	4.6	99.2	14.0	14.7	13.2	(1.5)
	Metoprolol *O*-demethylation	HLM	822.0	199.0				
		rCYP2D6	12.2	91.6	31.1	61.8	37.2	(3.3)
CYP3A4	Midazolam 1′-hydroxylation	HLM	1021.7	2.7				
		rCYP3A4	3.3	2.2	**248.1**	276.8	277.8	**26.0**
	Testosterone 6β-hydroxylation	HLM	5327.2	42.7				
		rCYP3A4	15.9	111.1	871.6	344.4	551.5	(56.7)
Total HLM	CYP concentration	480pmol/mg			**953**			

[a]The percentage relative content of each of the eight major CYPs in HLM were calculated from the total amount obtained using the RAF_{CL} data in bold. When there was more than one marker reaction, the one with a substrate commonly used and normally given to humans was used. Shown in bold. The relative CYP content calculated by other marker substrates are shown in parentheses

(CYP2C9), S-mephenytoin (CYP2C19), bufuralol, debrisoquine, dextromethorphan, metoprolol (CYP2D6), midazolam and testosterone (CYP3A) (Table 2).

Using RAF_{CL}, the relative contribution on the clearance of each substrate by CYPs to that in HLM was calculated using equations described previously [15]. Briefly, the relative contribution is calculated by multiplying the metabolic rate (velocity, pmol product/min/pmol rCYP or CL_{int}, μl/min/pmol rCYP) of the test compound by rCYP by the RAF (pmol rCYP/mg HLM), which gives the predicted activity in the HLM due to that specific CYP (pmol product/min/mg HLM or μl/min/mg HLM). To calculate percentage contribution, this value is then divided by the activity of HLM for the test compound and multiplied by 100. Since the metabolites are not known for most of the drugs or authentic standards not available, the drug clearances were determined by the substrate depletion approach described above.

Inhibition studies

On review of reported studies on inhibitor concentrations that would show inhibitor-CYP selectivity, an average of 10 μM α-naphthoflavone, quercetin, sulfaphenazole, ticlopidine, quinidine and 2 μM ketoconazole were considered suitable for a one-concentration screening study. The inhibitors were dissolved in methanol. Two microliters of the stock solution were added to 200-μl incubation mixtures. The inhibitory effect of each inhibitor was assessed by comparing the catalytic activity of the metabolism of three substrates (albendazole, amodiaquine or praziquantel) in HLM with and without inhibitors.

Data analysis

All data points represent the mean of duplicate determinations. Estimation of in vivo clearance was made from in vitro $T_{1/2}$ data according to the venous equilibrium model (well-stirred model) using equations described previously [10, 16]. The liver was taken as the main site of drug metabolic clearance. Predictions from in vitro data were compared to the reported in vivo clearances. K_m and V_{max} values of each marker substrate in pooled HLM and eight rCYPs were determined using non-linear least-squares regression analysis with GraFit software (version 3.0, Erithacus Software Limited, Middlesex, UK) and SigmaPlot Enzyme Kinetics Module for Windows 7.0 (SPSS, Inc., Chicago, IL). Correlations between the activities of respective CYP enzyme in individual HLMs and

the clearance of substrates, artemisinin, amodiaquine and praziquantel, or the formation of their major metabolites were determined by least-squares linear regression analysis using SigmaPlot 2001 (version 7.0, SPSS Science Software UK Ltd., UK). $P < 0.05$ was considered statistically significant.

Results

Drug clearance by RLM and HLM

In this study, the in vitro clearance of 15 APDs has been done in both HLM and RLM for species comparison only (HLM were used in all other investigations). Table 3 shows the in vitro $T_{1/2}$ values, from which CL_{int} for the 15 APDs are calculated (Table 4) for RLM and HLM. The predicted in vivo CL_H values in humans from the in vitro CL_{int} data are shown in Table 4. Linear regression analysis of predicted hepatic clearance, CL_H, between RLM and HLM yielded a low correlation coefficient (r^2) of 0.50, which was statistically significant ($P < 0.01$; Fig. 2). The clearances in RLM, however, showed much higher values than those in HLM (from 1.6-fold for pyrimethamine to 19-fold for diethylcarbamazine).

Table 3 Identification of cytochromes P_{450} (CYPs) involved in the metabolism of 15 anti-parasitic drugs. Note that all rat liver microsomes (RLM) are used with 0.5 mg/ml and recombinant CYP enzymes (rCYPs) are used at 20 pmol per incubation. *HLM* human liver microsome

Compound	$T_{1/2,RLM}$ (min)	HLM concentration (mg/ml)	$T_{1/2,HLM}$ (min)	1A1	1A2	2A6	1B1	2B6	2C8	2C9	2C19	2D6	2E1	3A4	3A5	4A11
				(% of substrate consumed in rCYPs)												
Albendazole	31.6	0.09	39.2	100	93		91		52	28	73	43	43	54	42	
Amodiaquine	22.6	0.1	15.8	100	19	20	49		94			13				
Artemisinin	28.0	0.4	87.4				17	25				33		39		
Artesunate	12.2	1	20.9			69	91	42					49			59
Chloroquine	106.8	1	454.0	27					18			12	13	5		
Dapsone	91.5	1	100.8		16				19	18	20	18		19		
Diethylcarbamazine	160.7	1	1036		12			17								
Pentamidine	201.3	2	>2000	63											16	23
Praziquantel	24.6	0.2	41.8		23						91	20		11	33	
Primaquine	18.8	1	92.6		21	13		30				45	23			
Pyrantel	121.9	1	161.3	28					19	18		39				
Pyrimethamine	>1500	1	623.3				11				13					
Quinine	36.8	0.4	190.4	97							15			22		
Thiabendazole	69.0	0.1	39.6	100	100		100									
Tinidazole	161.0	2	298.0					17						8		

Table 4 Calculated intrinsic clearance (CL_{int}[a]) of 15 anti-parasitic drugs (APDs) in rat liver microsomes (RLM), human liver microsomes (HLM), and the relative contributions of cytochromes P_{450} (CYPs) to the metabolism of APDs in HLM using the relative activity factor (RAF) method. *N.D.* not detectable due to immeasurably long elimination half-life

Compound	$CL_{int,RLM}$	$CL_{int,HLM}$	Predicted CL_H[b] (ml/min/kg)	rCYP (% contribution in HLM)								
	(µl/min/mg protein)			1A2	2A6	2B6	2C8	2C9	2C19	2D6	3A4	Sum
Albendazole	43.8	204.4	18.2	53			0.3	3.5	2.2	0.6	5.4	65
Amodiaquine	61.3	440.1	19.1				67			0.2		68
Artemisinin	49.4	19.8	9.8			10				6.5	25	42
Artesunate	113.6	33.2	12.3		120	1.3						121
Chloroquine	3.2	1.5	1.4				54			53	13	120
Dapsone	15.1	6.9	5.0				10	48	12	4.3	31	105
Diethylcarbamazine	8.6	0.7	0.6									N.D.
Pentamidine	6.9	<0.1	<0.1									N.D.
Praziquantel	56.3	82.9	16.0	39					14	0.2	30	83
Primaquine	73.9	7.5	5.3	60						23		83
Pyrantel	11.4	4.3	3.4							90		90
Pyrimethamine	<0.9	1.1	1.0									N.D.
Quinine	37.7	9.1	6.1						3.8		70	74
Thiabendazole	20.1	174.9	17.9	395								395
Tinidazole	8.6	1.2	1.1			12					77	89

[a]The CL_{int} was determined by the elimination half-life of substrate [$CL_{int} = (\ln 2 \times \text{incubation volume})/(T_{1/2} \times \text{protein or enzyme amount})$]

[b]Predicted drug in vivo hepatic clearances (CL_H) were scaled from in vitro $CL_{int,\ HLM}$ using the normal human body weight of 70 kg with a 1.4-kg liver, 20 g liver/kg body weight. In the scaling process the currently used factor is 45 mg HLM per gram liver. The value of hepatic blood flow used here is 1400 ml/min/70 kg

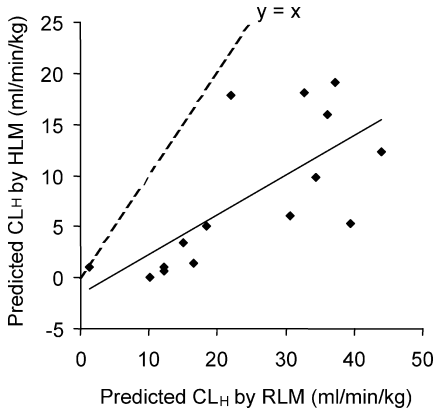

Fig. 2 A correlation analysis of hepatic clearances (CL_H) of 15 anti-parasitic drugs in rats and humans ($n = 15$, $r^2 = 0.50$, $P < 0.01$). Data were calculated from drug intrinsic clearance in vitro and employed the normal human and rat body weights of 70 kg and 0.25 kg, respectively

Comparison of clearance from in vitro and in vivo studies

Table 5 summarises the in vivo CL data obtained for some of the drugs taken from PK literature reports of intravenous administration [17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32]. Tinidazole showed the lowest mean clearance and amodiaquine the highest. The interindividual range of values for each substrate varied from twofold for tinidazole to tenfold for amodiaquine. Using the well-stirred model, the clearance of each substrate was calculated from the in vitro $T_{1/2}$ and was scaled to in vivo units of ml/min/kg body weight, to enable direct comparison with literature data. Table 4 and Table 5 show the predictions of in vivo clearances from in vitro data. In this study, we obtained a significant in vitro–in vivo correlation, $r^2 = 0.82$ ($P < 0.01$) for the eight compounds for which there was in vivo data (Table 5). This seemingly high correlation was, however, not robust in that omission of one or two compounds resulted in a significant loss in correlation to coefficients around 0.6. The data are, therefore, not of sufficient quantity and range to perform a useful correlation analysis. Whereas this study focuses on the role of hepatic CYPs, for some of the drugs studied, like artesunate, dapsone and albdendazole, other non-CYP and/or extrahepatic enzymes are involved in their metabolism and disposition (Table 5). This has important implications in efforts to estimate total in vivo clearance, CL_{tot}, from CYP metabolism-based hepatic clearance CL_H.

Relative activity factors

Table 2 shows the RAFs determined in our laboratory using recombinant enzymes expressed in yeast and a pool of HLM made from 20 livers. The CYP concentration of the pooled liver microsomes was 480 pmol/mg. The RAFs were determined using a variety of kinetic measures of activity (v = velocity at K_m, V_{max} = velocity at saturating substrate concentrations, $CL = V_{max}/K_m$) and with more than one marker substrate for some CYP enzymes. Using the RAFs of some marker substrates the total amount of CYP in the human liver according to RAFs was twice (953 pmol/mg) the actual estimated amount of CYP. This indicates that the RAFs do not give the actual amount of CYP but the relative amounts that would be associated with reported activities on specific marker reactions. When analysed this way, the RAFs obtained using $CL = V_{max}/K_m$ as an activity marker (Table 2) give relative abundances of the different CYPs in proportions that are consistent with literature data obtained from immunoquantification studies (reviewed [5]). Use of the RAF calculated from CL was also shown to be better than those derived from velocity at any concentration or at V_{max} by Nakajima et al. [15].

Table 5 Comparison of in vivo clearances of eight anti-parasitic drugs between predicted values from in vitro studies with those reported after i.v. drug administration in humans and their metabolic profiles

	Predict $CL_{in\ vivo}$ (ml/min/kg)	Clinical in vivo CL[a] (ml/min/kg)		CYP enzymes mediating biotransformation in humans	Main metabolite in humans (reported data)	Renal clearance (% dose)	Protein binding (%)
		Mean	Range				
Amodiaquine	19.1	115	27–288	CYP2C8	N-mono- and di-desethyl	0.15	>90
Artesunate	12.3	41.5	13–70	(Esterases and hepatic enzymes)	Dihydroartemisinin		
Chloroquine	1.4	12.3	6.4–18	CYP2C8, 2D6, 3A	Mono- and di-desethyl 7-chloro-4-aminoquinoline N-mono-and di-oxide	20–60	50–64
Dapsone	5.0	0.65	0.3–0.90	CYP3A4, 2E1, 2C, and N-acetyltranferase	Hydroxylamine (toxic) mono-acetyl	20	50–80
Pentamidine	<0.1	17.87	8.8–23.8	CYP2D6 and 1A1	No data available	2–11	
Primaquine	5.3	5.8	4–7.5		Carboxy and others	0.7	45–65
Quinine	6.1	3.4	1.8–4.6	CYP3A4 and 2C19	3-Hydroxy	20	80–90
Tinidazole	1.1	0.5	0.4–0.7		No data available	20	

[a]Data were obtained from healthy volunteers except for artesunate which was from patients with moderate malaria and pentamidine which was from patients with late stage *Trypanosoma gambiense* sleeping sickness

Identification of CYPs responsible for the metabolism of 15 APDs

The CYP enzymes involved in the metabolism of each APD were studied at 1 μM (except for artemisinin 10 μM and artesunate 5 μM) of the substrate concentration with a microsomal concentration of 0.1–2.0 mg/ml and 20 pmol/200 μl incubation of rCYP. Table 3 shows the catalytic activities of the 13 rCYPs towards the elimination of each APD. The table also shows the in vitro $T_{1/2}$ data in HLMs and RLMs. The mainly hepatic CYP1A2, 2D6 and 3A4 were involved in the metabolism of most of these compounds. The mainly extrahepatic CYP1A1, 1B1 also showed high catalytic activity with some substrates. The formation of metabolites of amodiaquine, chloroquine and praziquantel were also determined using LC/MS by comparing with the authentic references. The main metabolite of AQ in HLM was desethylAQ, which was mediated mainly by CYP2C8 as we previously showed [18]. Two hydroxylated metabolites were formed from PZQ after incubation with HLMs. Formation of 4-hydroxylPZQ was catalysed by CYP1A2 and 2C19, whereas that of a previously unidentified mono-hydroxylated metabolite (X-OH-PZQ) was mediated by CYP3A4 and 3A5 (Fig. 3a, b). According to its LC/MS/MS chromatographic and mass spectrometric characteristics, the hydroxy group of X-OH-PZQ was not located at the cyclohexyl ring. The desethylated chloroquine was the major metabolite of CLQ in HLM which was catalysed by CYP2C8, 2D6 and 3A4 (Fig. 3c).

Contribution of CYP enzymes to the metabolism activity of substrates in HLM

Following the results from CYP identification studies of the 15 APDs (Table 3), further investigations were done to determine their in vitro $T_{1/2}$ values in the rCYP enzymes. The CL_{int} for these compounds in HLM ranged from less than 0.1 μl/min/mg for pentamidine to 440 μl/min/mg for amodiaquine.

The RAF approach

The RAFs of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 were calculated as the ratio of the marker clearance ($CL = V_{max}/K_m$) in HLM to that in rCYPs (Table 2). The estimated relative contribution of each CYP enzyme in the clearance of these APDs by HLM is shown in Table 4. When calculating the relative contribution of CYPs in the metabolism of a compound metabolised by more than one CYP, RAFs obtained from rCYPs from the same source were used, yeast or lymphoblastoid cells. It is not recommended to mix RAFs derived from different sources when calculating relative contributions on the metabolism of a compound because the RAF values also depend on the source of recombinant CYPs [6].

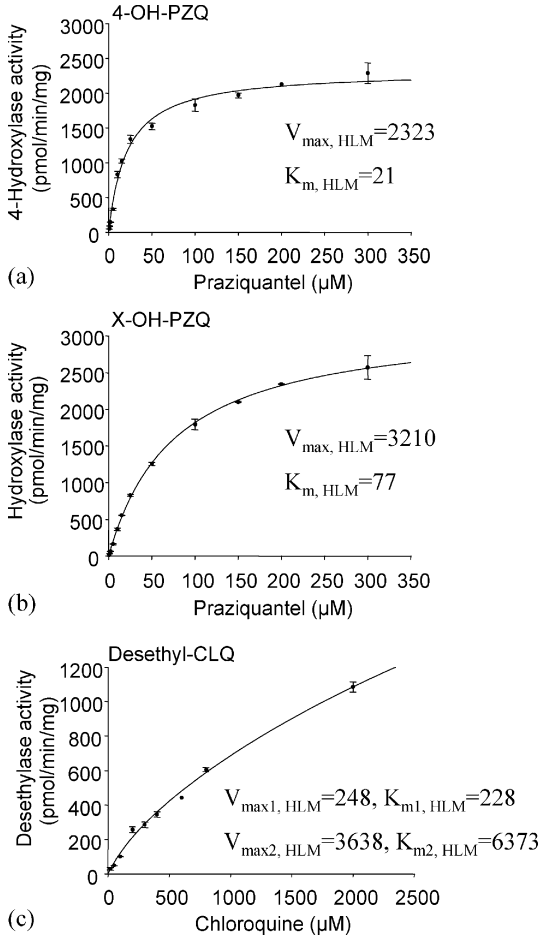

Fig. 3 Plots of enzyme metabolic activity versus substrate concentration of praziquantel (PZQ) (**a** and **b**) and chloroquine (CLQ) (**c**) in human liver microsomes (HLM). *Inset* V_{max} and K_m values for the formation of PZQ 4- and unidentified hydroxylated (X-OH) metabolites and CLQ desethylated metabolite in HLM, respectively. The V_{max} (pmol/min/pmol) and K_m (μM) values of each reaction in recombinant cytochrome P_{450} (CYP) enzymes were as follows: PZQ 4-hydroxylation: $V_{max, CYP1A2} = 33$, $K_{m, CYP1A2} = 54$; $V_{max, CYP2C19} = 4$, $K_{m, CYP2C19} = 7$; PZQ X-hydroxylation: $V_{max, CYP3A4} = 7$, $K_{m, CYP3A4} = 65$; CLQ desethylation: $V_{max, CYP2C8} = 6.9$, $K_{m,CYP2C8} = 458$; $V_{max, CYP2D6} = 1.7$, $K_{m, CYP2D6} = 62$; $V_{max, CYP3A4} = 2.3$, $K_{m, CYP3A4} = 582$. The formation of X-OH-PZQ was calculated semi-quantitatively using 4-OH PZQ as a reference because of the lack of the reference compound

With the exception of artemisinin and thiabendazole, the total relative contribution of the eight major CYP enzymes to the metabolism of each compound in HLM were between 65 % and 120 %, which means the metabolism of these compounds in HLM was mainly mediated by the CYPs. Table 4 also shows the relative significance of each CYP to the metabolism of compounds by the relative contribution data, such as CYP1A2 to the metabolism of albendazole and thiabendazole, CYP2C8 to amodiaquine, CYP2D6 to pyrantel, and CYP3A4 to quinine. For thiabendazole, an unrealistic total contribution by CYP1A2 of 394 % was observed. At 1.0 μM substrate concentration, we found a large amount of hydroxylated metabolite of thiabendazole formed with recombinant CYP1A2, while it was

Table 6 Relative contributions of cytochromes P_{450} (CYPs) to the metabolism of substrates in human liver microsomes (HLM) by inhibition studies

	Blank[a]	Control[b]	α-Naphthoflavone (10 μM) 1A2	Quercetin (10 μM) 2C8	Sulfaphenazole (10 μM) 2C9	Ticlopidine (10 μM) 2C19	Quinidine (10 μM) 2D6	Ketoconazole (2 μM) 3A4	Sum
Albendazole (μM)[c]	1.08	0.50	0.75	0.96	0.55	0.85	0.59	0.62	
Contribution			42.8 %	77.9 %	7.4 %	60.3 %	12.1 %	19.5 %	220 %
Amodiaquine (μM)[d]	0.94	0.15	0.66	0.93	0.21	0.24	0.26	0.53	
Contribution			64.5 %	98.0 %	8.0 %	11.6 %	14.5 %	47.4 %	244 %
Desehtyl-AQ (μM)	0.00	0.89	0.40	0.14	0.83	0.78	0.79	0.56	
Contribution			55.4 %	84.4 %	7.1 %	11.6 %	10.5 %	37 %	206 %
Praziquantel[e]	0.98	0.41	0.75	0.70	0.53	0.68	0.51	0.70	
Contribution			34.8 %	28.9 %	12.4 %	27.6 %	10.4 %	28.8 %	143 %
4-hydroxyl-PZQ (μM)	0.00	0.26	0.00	0.01	0.22	0.02	0.23	0.21	
Contribution			100.0 %	94.9 %	13.5 %	90.7 %	11.2 %	16 %	327 %
X-Hydroxyl-PZQ (μM)	0.00	0.29	0.31	0.30	0.26	0.37	0.25	0.00	
Contribution			−6.9 %	−3.1 %	10.8 %	−26.0 %	15.8 %	100.0 %	90.6 %

[a]Blank sample: incubation without NADPH and inhibitors
[b]Control sample: incubation without inhibitors
[c]Incubation condition for albendazole (ABZ): 1 μM of ABZ, 0.1 mg/ml HLM, 45 min
[d]Incubation condition for amodiaquine (AQ): 1 μM of AQ, 0.1 mg/ml HLM, 45 min
[e]Incubation condition for praziquantel (PZQ): 1 μM of PZQ, 0.2 mg/ml HLM, 30 min

not detectable in HLM. In this case, the erroneous enzyme contributions (exceeding 100 %) and the formation of enzyme-source specific metabolite (hydroxylated thiabendazole in rCYP1A2) may have been caused by differences between HLM and recombinant CYP1A2 in cofactor and supporting enzyme (cytochrome b5 and CYP NADPH reductase) requirements in the metabolism of index and test compounds. For diethylcarbamazine, pentamidine and pyrimethamine, it was difficult to estimate the relative contributions of CYPs to their elimination in HLM because of the immeasurably long $T_{1/2}$ in rCYPs even at high enzyme concentrations of 20 pmol per incubation.

The selective inhibition approach

α-Naphthoflavone (CYP1A2), quercetin (CYP2C8), sulfaphenazole (CYP2C9), ticlopidine (CYP2C19 and CYP2D6), quinidine (CYP2D6) and ketoconazole (CYP2C8, 3A4, 3A5) have been shown to be potent diagnostic inhibitors. Three high-clearance drugs—albendazole, amodiaquine and praziquantel—were chosen to evaluate the utility of using selective inhibitors in CYP identification studies. The inhibitory effect by these six inhibitors on either the clearance of substrates or the formation of metabolites of AQ and PZQ are shown in Table 6. When the extent of inhibition of each enzyme was added up, the total contribution calculated for most of the drugs was over 200 %. The relative contribution by the inhibition studies was also different from those obtained from the RAFs approach. Only the predicted CYP contribution for the formation of hydroxylated metabolite, X-OH-PZQ, of praziquantel was close to 100 %, which was only mediated by CYP3A and only inhibited by ketoconazole. The overestimated contribution of CYPs to the metabolism of substrates using inhibition studies could therefore be a reflection of the lack of selectivity of these inhibitors for the respective CYPs.

The activity correlation in a panel of HLM approach

Correlations between the clearances of amodiaquine, artemisinin, and praziquantel and 9 CYPs activities in a panel of 20 HLM were made (Table 7). The marker substrates for the 9 CYPs were: phenacetin (CYP1A2), coumarin (CYP2A6), bupropion (CYP2B6), paclitaxel (CYP2C8), diclofenac (CYP2C9), S-mephenytoin (CYP2C19), bufuralol (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3A). The correlation results of CYP activities derived from the 20 HLMs are summarized in Table 8. From our experience with CYP activity correlation studies, r^2 values of over 0.7 indicate metabolically significant association between enzyme activity and compound metabolism. Values lower than this, even if they are statistically significant, usually are of no biological importance. Inter-CYP correlations (r^2) in the HLM samples used should, therefore, be less than 0.7 in order to differentiate metabolically relevant correlations in the metabolism of a compound by two or more CYPs. Table 8 shows that the HLM samples used in this study did not exhibit any metabolically significant inter-CYP activity correlation. The highest correlation was between CYP2B6 and CYP2C8: $r^2 = 0.52$.

Artemisinin metabolism showed a significant correlation ($r^2 = 0.75$, $P < 0.01$) with CYP3A4 activity. A good correlation ($r^2 = 0.96$, $P < 0.0001$) was observed between amodiaquine metabolism and CYP2C8 activities. In this case, CYP 2C9 activities also showed a good correlation ($r^2 = 0.69$, $P < 0.01$, respectively) with AQ metabolism. This apparent correlation is likely

Table 7 Correlation coefficients of substrate clearance and/or formation of major metabolites with specific cytochrome P_{450} enzyme activities in a panel of 20 human liver microsomes (HLM). The incubations were performed with substrate concentrations of 10 μM artemisinin (0.4 mg/ml HLM, 30 min), 1 μM amodiaquine (0.1 mg/ml HLM, 20 min) and 10 μM praziquantel (0.2 mg/ml, 30 min)

Compound	1A2	2A6	2B6	Correlation coefficient (r^2)					
				2C8	2C9	2C19	2D6	2E1	3A4
Artemisinin	0.02	0.08	0.40**	0.12	0.40**	0.06	0.02	0.02	0.75***
Amodiaquine	0.02	0.13	0.43	0.96***	0.69**	0.01	0.04	0.00	0.01
Desethyl-AQ	0.01	0.10	0.42	0.99***	0.64**	0.03	0.04	0.01	0.00
Praziquantel	0.00	0.01	0.26*	0.09	0.48***	0.33**	0.01	0.03	0.81***
4-Hydroxyl-PZQ	0.61***	0.01	0.00	0.03	0.12	0.45**	0.02	0.02	0.02
X-Hydroxyl-PZQ	0.05	0.03	0.36**	0.09	0.37**	0.07	0.08	0.04	0.82***

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$

Table 8 Correlation coefficients (r^2) of cytochrome P_{450} (CYP) activities in a panel of 20 human liver microsomal samples. CYP activity marker reactions used were: CYP1A2 (phenacetin demethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (paclitaxel 6α-hydroxylation), CYP2C9 (diclofenac 4′-hydroxylation), CYP2C19 (S-mephenytoin 4′-hydroxylation), CYP2D6 (bufuralol 1′-hydroxylation), CYP2E1 (chlorzoxazone 6-hydroxylation), CYP3A4 (midazolam 1′-hydroxylation)

CYPs	Activity range	Correlation coefficients (r^2)								
	(pmol/min/mg)	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4
1A2	51–1334	1	0.00	0.02	0.00	0.00	0.05	0.01	0.08	0.00
2A6	94–1054		1	0.19	0.04	0.01	0.03	0.00	0.00	0.02
2B6	1.3–474			1	0.52***	0.24*	0.01	0.07	0.03	0.15
2C8	14–114				1	0.46***	0.01	0.00	0.00	0.03
2C9	289–3292					1	0.06	0.01	0.03	0.29*
2C19	1.0–240						1	0.06	0.00	0.16
2D6	45–198							1	0.12	0.05
2E1	361–1570								1	0.04
3A4	27–2100									1

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$

derived from the correlations ($r^2 = 0.46$, $P < 0.001$) between the activities of CYP2C8 and 2C9, in the liver bank. The relative enzyme contribution studies, Table 3 and Table 4, do not support the role of CYP2C9 since this rCYP did not metabolise amodiaquine.

For the formation of praziquantel hydroxylated metabolites, the activities of CYP1A2 and 2C19 showed the highest correlation ($r^2 = 0.61$ and 0.45, $P < 0.05$) with PZQ 4-hydroxylase, and CYP3A4 showed correlation ($r^2 = 0.82$, $P < 0.0001$) with X-hydroxylase. This was consistent with the CYP identification results (Table 3). Interestingly, a strong correlation ($r^2 = 0.81$, $P < 0.0001$) was observed with PZQ elimination and CYP3A4 activity in HLMs, while almost no correlation ($r^2 = 0.05$) was found with CYP1A2 activity. This was not in line with the results of CYP identification and relative contribution of CYPs to PZQ metabolism by RAFs and inhibition methods, which showed that CYP 1A2 was more important to PZQ metabolism than CYP3A4 (Table 4 and Table 6). However, it has to be noted that, in the correlation studies, 10 μM PZQ (which is equivalent to human plasma concentration after therapeutic doses) was used (Table 7); whereas, in the CYP identification (Table 3 and Table 4) and inhibition studies (Table 6), the PZQ concentration used was 1.0 μM. The relative contributions of CYP1A2, 2C19 and 3A4 to PZQ metabolism activity in HLM measured at substrate concentrations of 1 μM and 5 μM showed that the contribution of CYP3A4 increased from 36 % to 59 %, while contributions of CYP 1A2 and 2C19 decreased from 47 % to 35 % and 17 % to 5 %, respectively. These data seem to indicate that, at low concentrations of PZQ, the high affinity and low turnover CYPs 1A2 and 2C19 are important and at high plasma concentration, the low affinity and high turnover CYP3A4 takes over (Fig. 3).

Discussion

Our in vitro studies on drug clearance and the identification of important hepatic CYPs responsible for the metabolism of APDs provide insights into the pharmacokinetic properties of these drugs. Rigorous evaluation of in vitro–in vivo correlations was not possible due to the scantiness of i.v. pharmacokinetic data on most of the APDs, and that, for the few compounds it was available,

the spread of clearance values was poor. Qualitative predictions to high, intermediate and low clearance drug categories were, however, possible. A combination of various in vitro reaction phenotyping approaches led to a fairly accurate identification of major CYPs involved in the metabolism of test compounds.

Interspecies differences

In the pharmaceutical industry, preclinical predictions of human PKs of a drug are based on in silico, in vitro and animal studies (reviewed in [7, 12, 33]). In vitro systems (liver microsomes, tissues slices or hepatocytes) derived from different species can assist in selecting a suitable animal pharmacokinetic and/or toxicological model. For the APDs, there was a low but significant correlation between metabolic stability in rats and in humans but the RLM were generally associated with higher CL_{int} rates than humans. These data point to the limitations of using animal-derived tissue for metabolism studies with the intention of predicting metabolism in humans.

In vitro–in vivo correlations for drug clearance

The variation in human in vivo CL values and the tendency to under-predict are consistent with literature data for other drugs [34]. The variation in human CL values is, however, not the only major reason for failure to predict the in vivo situation. In a study of 1163 compounds from 48 chemistry programmes [35] and 48 compounds in the same chemical series (Andersson et al., personal communication) in rats showed very poor in vitro–in vivo correlations in CL. Many factors have been proposed to explain these poor correlations. They range from protein binding, role of transporters, uncertainty of actual drug concentration available for metabolism in the cells, scaling factors and complexity of models used, role of other enzymes, extrahepatic metabolism and other means of drug clearance [7, 12, 33]. Using drug elimination rate at a substrate concentration of 1.0 μM to estimate drug clearance may not always satisfy the assumptions of Michaelis–Menten kinetics, namely when $[S] << Km$ $CL_{int} = V_{max}/K_m$ = rate of metabolism/[S], especially for low-clearance drugs whose plasma concentration may be higher than 1 μM. The worrying aspect is that each compound seems to be affected differently by one or a combination of some of these factors, thus compromising the HTS dream. We, however, believe that these HTS screens for metabolic stability are still useful as a starting point from which to explore in greater detail the contribution of the likely causes of poor prediction of in vivo situations.

In screening campaigns, working classifications of drugs into high-clearance (> 70 % of hepatic blood flow) and low-clearance (< 30 % of hepatic blood flow) categories are used. What we can salvage from the predictions from our in vitro data is that albendazole, amodiaquine, praziquantel and thiabendazole are high-clearance drugs, artemisinin, artesunate and quinine, medium-clearance drugs, and chloroquine, dapsone, diethylcarbamazine, pentamidine, primaquine, pyrantel, pyrimethamine and tinidazole, low-clearance drugs. These categorisations were generally in agreement with in vivo observations (Table 4, Table 5). Considering the various sources of error and uncertainties, for now it will suffice to say that for those with in vivo clearance above hepatic blood flow (e.g. amodiaquine), there are other major non-hepatic metabolism means of drug clearance. For some drugs, such as dapsone, the contribution of the cytosolic enzyme *N*-acetyltranferase (NAT2) [36] is not included in the estimation of in vivo clearance from in vitro HLM-derived metabolism data. For some drugs, such as arteminisin whose metabolism by the screened CYPs does not account for 100 % of the metabolism, it can be suspected that other yet unidenitified microsomal enzymes are involved (Table 4).

Identification of major CYPs involved in the metabolism of a compound

CYPs are a major drug metabolising enzyme system. Studies to identify the major enzymes associated with a drug's metabolism therefore start by evaluating the role of CYPs (CYP identification). In this study we applied the three major approaches being used for reaction phenotyping: (a) RAF, (b) activity correlation analysis and (c) diagnostic inhibitors. Each of these studies has its pros and cons which, for some drugs, require one to use them in combination. We reported, for the first time to our knowledge, the CYPs involved in the metabolism of praziquantel, primaquine, tinidazole, artesunate and pyrantel (Table 4). For other drugs, artemisinin [37], amodiaquine [18], quinine [28], chloroquine [32], thiabendazole [38], albendazole [39] and dapsone [40], we confirm enzymes identified (Table 4, Table 5). For some, pentamidine [41], chloroquine [42], the reasons for some discrepancies in the role of some CYPs can either be in vitro conditions, CYP identification method and/or enzyme system used. In this study we also report estimations of the relative contribution of each enzyme. This is important if one is going to consider the clinical significance of enzyme regulation of the involved CYP(s). Our studies also demonstrated that it is difficult to apply the RAF factor on compounds that are metabolised very slowly (pentamidine, diethylcarbamazine) or metabolically very unstable (thiabendazole). This might be due to failure to achieve optimal in vitro conditions for evaluating their metabolism. Our data also shows that in addition to the documented 4-OH PZQ, X-OH-PZQ is also a major metabolite of PZQ.

Some limitations of enzyme identification approaches

The use of liver microsomes and experimental conditions optimal for CYPs automatically excludes

non-microsomal and extrahepatic enzymes. This can be important for some drugs, e.g. dapsone, which is also metabolised by the cytosolic NAT2 and amodiaquine, which is also metabolised by the extrahepatic CYP1A1 and 1B1 [18]. The role of microsomal enzymes like UGTs that require different incubation conditions for activity will also be missed. The use of correlation studies can suffer from intra CYP activity correlations in the panel of HLM used (Table 8). For example, if only the correlation studies had been used, the intra-correlation between CYP2C8 and CYP2C9 would have made it difficult to decide which enzyme metabolises amodiaquine. The use of diagnostic inhibitors suffers from the lack of selectivity for target CYPs. This is reflected by the more than 200 % calculated relative contribution from assumed selective inhibitors (Table 6). It can also lead to false CYP identification as was the case in the study by Jewell et al. [43]; these authors concluded that CYP3A4 was responsible for the metabolism of amodiaquine from inhibition studies using ketoconazole. Ketoconazole also inhibits CYP2C8, the hepatic enzyme we conclusively demonstrated to be responsible for amodiaquine metabolism [18]. The RAF factor may suffer from its assumption that the factors that affect the metabolism of enzyme marker reaction are similar to those of the test compound, which is not true for some enzymes and test compounds. This could result in unrealistic enzyme percentage contributions of over 100 % for some compounds (Table 4). The choice of compound concentration used in both intrinsic clearances and in CYP identification is important. The relative contribution of CYPs in praziquantel metabolism changed with increasing drug concentrations, the high affinity CYP1A2 and CYP2C19 being important at 1.0 μM PZQ. At 5 μM and 10 μM, the contributions of these enzymes reduced as the contribution of CYP3A4 became dominant. In principle, it means that the role of enzymes continuously changes as a function of drug concentration in vivo for a compound metabolised by enzymes with different affinities and catalytic activities.

Clinical significance of these studies

Despite the limitations discussed above, the results of our study offer potentially useful explanations of the observed PKs of APDs in humans. For example, finding that at in vivo plasma concentrations, CYP3A4 is the major enzyme involved in metabolising PZQ explains why the plasma levels reduced drastically and sometimes with loss of therapeutic effect in subjects taking carbamazepine or dexamethasone [44, 45] known inducers of CYP3A4. Knowledge of the role of CYP3A4 in PZQ metabolism also explains why Diekmann et al. [46] and Jung et al. [47] observed that cimetidine, ketoconazole and miconazole inhibited the disposition of PZQ. Co-administering PZQ with inhibitors of its metabolism in these studies increased the therapeutic efficacy of the drug and reduced treatment regiments for neurocysticercosis from 2 weeks to 1 day. This approach to optimise drug treatment has a precedent in the co-administration of cyclosporin with ketoconazole, which resulted in lower doses for the expensive immuno-suppresant and also resulted in more stable and predictable PKs [48]. That amodiaquine (CYP2C8), pyrantel (CYP2D6), chloroquine (CYP2D6 and CYP2C8), and artesunate (CYP2A6) to a major extent (>50 %) appear to be metabolised by polymorphic enzymes hints at the possibility of inter-individual as well as interethnic variations in the disposition of these drugs. Drugs which are mainly metabolised by one CYP, e.g. pyrantel, amodiaquine, tinidazole and thiabendazole, could be prone to drug–drug interactions involving inhibition, induction and or polymorphic regulation of the major route of their elimination as there are no other compensatory enzymes. Our results also imply that a slow rate of metabolism contributes to chloroquine's long half-life, besides the traditional explanation based on its large volume of distribution. The 98 % renal clearance of diethylcarbamazine [49] is consistent with the metabolic stability we observed in this study. Our results have also highlighted areas that need further work towards improving the in vitro experimental design in order to make them more predictive of in vivo situations. Results of our study will assist in predicting drug combinations that might be associated with drug–drug interactions through inhibition of particular CYPs and the potential role of CYP genetic polymorphisms in the pharmacokinetic variability of some drugs.

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