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Pharmacokinetic and pharmacodynamic assessment of a five-probe metabolic cocktail for CYPs 1A2, 3A4, 2C9, 2D6 and 2E1

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Aims
The primary objectives of the present study were to establish whether there was a pharmacokinetic or pharmacodynamic interaction between the probe drugs caffeine (CYP1A2), tolbutamide (CYP2C9), debrisoquine (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3A4), when administered in combination as a cocktail. Furthermore, the tolerability of these probe drugs, both alone and in combination as a cocktail was assessed.

Methods
Twelve healthy volunteer subjects (age range 22–48 years) were entered into an open, fixed sequence, 6-limb, single centre study. The randomization was such that all drugs were given individually followed by the full 'cocktail' as the last treatment limb. The phenotypic index used to assess the intrinsic activity of the CYP isoforms included metabolite/parent ratios in plasma and urine (CYPs 1A2, 2E1 & 2C9), parent/metabolite ratios in urine (CYP2D6) and plasma AUC_{last} (CYP3A4). Blood pressure and blood glucose measurements were used to assess pharmacodynamic interactions. Tolerability was assessed through reporting of adverse events.

Results
Overall, there was little evidence that the probe drugs interacted metabolically when co-administered as the cocktail. The ratio of the geometric mean (and 90% confidence interval) of the phenotypic index, obtained after administration of the probe as part of the cocktail and when given alone were: caffeine, 0.86 (0.67–1.10), midazolam, 0.96 (0.74–1.24), tolbutamide, 0.86 (0.72–1.03), debrisoquine 1.04 (0.97–1.12) and chlorzoxazone, 0.95 (0.86–1.05). There was no difference in blood pressure and blood glucose concentrations following the cocktail and dosing of the individual probes. There was no effect on ECG recordings at any time-point. The adverse events reported for individual drug administrations were mild, transient and expected. Overall no more adverse events were reported on the cocktail study days than on the days when the drugs were administered alone.

Conclusions
The five probe drugs when coadministered, in this dosing regimen, demonstrated no evidence of either a metabolic or pharmacodynamic interaction that might confound the conclusions drawn during a cocktail study. The present cocktail methodology has the potential to become a useful tool to aid the detection of clinically important drug–drug interactions during drug development.

Introduction

Multiple drug therapy is common in clinical practice. The resultant potential for drug–drug interactions (DDIs) can cause significant safety problems and be a bar to drug registration or lead to early withdrawal from the market, as was the case with mifebradil [1]. Therefore, there is a need to identify possible DDIs during early drug development. Furthermore, knowledge of DDIs at this stage can potentially improve the design of initial patient studies, since more subjects will be eligible for entry into a study, because a wider range of comedications can be safely taken with the development drug. Additionally, the results of such studies are less likely to be confounded by unexpected drug interactions. Many DDIs occur due to inhibition or induction of the cytochrome P450 system. *In vitro* hepatic microsomal studies can provide preliminary information about the CYP isoforms that are likely to be affected clinically. Definitive data for each CYP isoform can be obtained by using an *in vivo* 'probe' drug that is a significant and selective substrate of that enzyme in man. Therefore, when a compound with inhibitory potential is introduced, the metabolism of the probe drug is readily impaired. When two or more isoforms are involved, several DDI studies may have to be performed to understand the clinical potential for interaction [2]. In this case, a 'cocktail' approach may provide a more elegant solution than the traditional single probe, single study approach [3, 4]. Whereas this methodology has been widely studied in academia [5–8], its applicability to the drug development process has been questioned [4, 9, 10]. The 'cocktail' approach involves the simultaneous administration of two or more probe drugs to a single subject to assess the activity of several distinct CYP enzymes. By administering the cocktail, the effect of the test drug on a number of distinct CYP isoforms can be established within one clinical study.

Several studies have been published in which cocktail methodology has been employed either to phenotype or to evaluate the effect of marketed drugs on CYPs. Results from a 4-drug phenotyping cocktail for CYP1A2, CYP2C19, CYP2D6, CYP3 A, N-acetyltransferase (NAT) and xanthine oxidase indicated no pharmacokinetic or pharmacodynamic interaction [7]. A five-drug cocktail (the 'Pittsburgh' cocktail) has been pioneered by Frye *et al.* [5]. The cocktail was used to demonstrate the selective inhibition of CYP2D6 by chloroquine [6]. The Pittsburgh cocktail has subsequently been modified by Palmer *et al.* to include diclofenac as a probe for CYP2C9 and midazolam has replaced dapsone as a CYP3A probe [8]. These studies provide good evidence to support the use of a cocktail to investigate multiple CYP activity. However, before use within the regulatory environment of drug development, we feel that for the cocktail approach to become a useful tool further validation work is required.

Of prime concern is the possible occurrence of pharmacokinetic interactions between the coadministered probe drugs. Furthermore, pharmacodynamic interactions particularly affecting blood pressure would be of particular concern. Several probe drugs (e.g. debrisoquine, midazolam) can affect blood pressure, which in turn could influence hepatic blood flow and consequently the metabolic clearance of the probe compounds.

The present study was designed to examine whether pharmacokinetic and pharmacodynamic interactions occur within a five-probe drug cocktail consisting of tolbutamide (CYP2C9), chlorzoxazone (CYP2E1), caffeine (CYP1A2), debrisoquine (CYP2D6) and midazolam (CYP3A4). Tolbutamide was chosen because it is the most widely reported probe for CYP2C9 in the literature [4, 11] and has less variable absorption compared to diclofenac [12].

Methods

The study protocol was approved by the Astra Charnwood Ethics Committee and carried out in conformity with the Declaration of Helsinki (amended 48th General Assembly, Somerset West, Republic South Africa, October 1986).

Subjects

Twelve healthy Caucasian, nonsmoking volunteer subjects (five males and seven females) were included in the study, after providing written informed consent. Smoking status was confirmed by self-report. Additionally, all subjects were extensive metabolisers with respect to CYP2D6, identified through genotyping for the following alleles, CYP*3, CYP*4 and CYP*5. The respective means and ranges for the age and BMI of the subjects were 31 (22–48) years and 24.1 (19.8–28.6) kg m^{-2}.

Study design

An open, fixed sequence, 6-limb, single centre study was performed. All drugs were given individually followed by the full 'cocktail' as the last treatment limb. A protocol led interim review of the data was undertaken after 6 subjects had completed the study.

After a prestudy screening visit (Visit 1) to assess eligibility, each subject was assigned to receive each of the probe drugs alone and in combination as a cocktail (Visits 2–7). For practical reasons, the treatment order

(caffeine, midazolam, debrisoquine, tolbutamide, chlorzoxazone, cocktail) was the same in all subjects. All probes were given after a standard breakfast, and dosing occasions were separated by a washout of at least 48 h. Midazolam was given intravenously, and all other probes were given orally. The dose of midazolam was based on subject body weight (0.025 mg kg^{-1}). For the cocktail administration limb (Visit 7) the probe drugs were given in the following order: tolbutamide (250 mg), chlorzoxazone (250 mg), caffeine (100 mg), debrisoquine (5 mg) and midazolam (0.025 mg kg^{-1}). The first probe drug was taken 34 min before midazolam administration, and the remaining probe drugs (excepting midazolam) were taken at two-minute intervals thereafter. This approach was used in part for logistical reasons, but also to ensure that there was systemic exposure to the oral probes at the time of intravenous midazolam administration. An overview of the sampling regimen and the matrix collected for each probe is given in Table 1.

Blood glucose concentrations were monitored during the tolbutamide and cocktail limbs, using a handheld glucometer. Standing and supine blood pressure and heart rate were monitored at intervals over 24 h. Specific attention was paid to those measurements taken during the debrisoquine and midazolam alone limbs and the cocktail limb. Adverse events were monitored on each study day by a combination of self-reporting and direct questioning. A final poststudy follow-up visit (Visit 8) took place 7–10 days after the cocktail dosing limb.

Measurements of plasma and urine drug concentration

Caffeine The determination of caffeine and paraxanthine in plasma (100 ml) involved sample preparation by protein precipitation with acetonitrile, before reverse phase HPLC separation on a C_{18} column, followed by tandem mass spectrometry. The lower limit of quantification for the assay was 0.10 mg ml^{-1} for both caffeine and paraxanthine analysis. The precision of the caffeine assay was 5.4% and 2.8% at respective concentrations of 0.20 and 4.0 mg ml^{-1}. At the same concentrations the respective precision of the paraxanthine assay was 5.4% and 2.8%.

Midazolam The determination of midazolam in plasma (500 ml) involved solid phase extraction before reverse phase HPLC on a CN column followed by tandem mass spectrometry. Quantification was achieved by peak area to internal standard ratio. The lower limit of quantification for the assay was 0.26 ng ml^{-1}. The precision of the assay was 9.2% and 4.3% at respective concentrations of 0.50 and 16 ng ml^{-1}.

Tolbutamide The procedure for the determination of tolbutamide, hydroxytolbutamide and carboxytolbutamide in urine (500 ml) was based on sample dilution followed by injection on to a reverse-phase C_8 high performance liquid chromatography (HPLC) system. Tolbutamide, hydroxytolbutamide and carboxytolbutamide are chromatographically separated from other urine constituents and selectively detected by monitoring specific parent-daughter ion transitions using a triple quadruple mass spectrometer. The respective lower limit's of quantification for tolbutamide, hydroxytolbutamide and carboxytolbutamide were 5 ng ml^{-1}, 1.0 mg ml^{-1} and 5.0 mg ml^{-1}. For tolbutamide, the precision of the assay at 5 ng ml^{-1} was 17.8% and 6.45% at 398 ng ml^{-1}. For hydroxytolbutamide, the precision of the assay at 1.0 mg ml^{-1} was 8.25% and 3.36% at 50 mg ml^{-1}. For carboxytolbutamide, the precision of the assay at 5.0 mg ml^{-1} was 13.6% and 6.58% at 100 mg ml^{-1}.

Table 1
Details of the probe cocktail used

Cytochrome P450 Enzyme	Probe (Dose)	Route	Matrixs sampled	Sample time(s)	Phenotypic index	Reference
CYP1A2	Caffeine (50 mg)	oral	plasma	6 h 30 min	Paraxanthine/caffeine ratio	[13]
CYP2C9	Tolbutamide (250 mg)	oral	urine	6–12 h	hydroxy- and carboxy tolbutamide/tolbutamide ratio	[14]
CYP2D6	Debrisoquine (5 mg)	oral	urine	0–6 h	Debrisoquine/hydroxy-debrisoquine ratio	[7]
CYP2E1	Chlorzoxazone (250 mg)	oral	plasma	2 h 32 min	6-hydroxy chlorzoxazone/ chlorzoxazone ratio	[15]
CYP3A4	Midazolam (0.025 mg kg^{-1})	iv	plasma	0, 1, 2, 3, 4, 5, 6, 12 h	Midazolam AUC_{last}	[16]

Debrisoquine The procedure for the determination of debrisoquine and 4-hydroxydebrisoquine in urine (50 ml) was based on sample dilution followed by injection on to a reverse-phase C_8 high performance liquid chromatography (HPLC) system (mobile phase: 70% 50 mM ammonium acetate pH 9.0; 30% acetonitrile). Debrisoquine and 4-hydroxydebrisoquine were chromatographically separated from other urine constituents and selectively detected by monitoring specific parent-daughter ion transitions using a triple quadrupole mass spectrometer (MS). The respective lower limit of quantification for debrisoquine and 4-hydroxydebrisoquine was 0.50 and 0.1 mg ml^{-1}. The precision of the debrisoquine assay was 4.5% and 2.5% at respective concentrations of 1.0 and 16.0 mg ml^{-1}. At the concentrations of 0.20 and 4.0 mg ml^{-1} the respective precision of the 4-hydroxydebrisoquine assay was 9.3% and 7.3%.

Chlorzoxazone The procedure for the determination of chlorzoxazone and 6-hydroxychlorzoxazone in plasma (500 ml) involved enzyme hydrolysis with b-glucoronidase followed by solid phase extraction. Extracts were then injected onto a reverse phase C_{18} HPLC system (mobile phase: 72% 0.1 M ammonium acetate; 22.5% acetonitrile; 5.5% tetrahydrofuran) with UV detection at 283 nm. The lower limit of quantification for chlorzoxazone and 6-hydroxychlorzoxazone was 0.10 mg ml^{-1}. The precision of the chlorzoxazone assay was 5.7% and 1.9% at respective concentrations of 0.10 and 3.0 mg ml^{-1}. At the same concentrations the precision of the 6-hydroxychlorzoxazone assay was 6.2% and 8.2%, respectively.

Data analysis

Pharmacokinetic The respective phenotypic indexes that were measured for each of the probes are given in Table 1. A review of the literature indicates that these indexes were reflective of the systemic clearance of the probe drug and hence the activity of the CYP pathway under investigation [7, 13–16]. To account for the presence of paraxanthine and caffeine prior to dosing, a correction factor was introduced into the calculation of the paraxanthine to caffeine ratio. For this purpose, the half-life values of caffeine and paraxanthine were assumed to be 4 and 3 h, respectively [17]. Using these values, the residual caffeine concentration from the predose concentrations remaining 6 h after dosing was estimated. These corrected concentrations of caffeine and paraxanthine were then used to calculate metabolite to parent ratios. For midazolam, AUC_{last} was calculated by noncompartmental analysis using 'WinNonLin' (Version 2.0).

Statistical analysis The present study was exploratory in nature and as such there was no formal hypothesis testing. To assess the effect of the cocktail on the metabolism of each probe, the geometric mean of the log-transformed phenotypic index for an individual probe was calculated when it was given alone and as part of the cocktail. Geometric mean ratios (GM) and 90% confidence intervals for the phenotypic index were then calculated, from the GM obtained after administration of the probe as part of the cocktail divided by the GM when the probe was given alone.

Results

Pharmacokinetics

Geometric mean ratios of the metabolic indexes for each probe and 90% CI are given in Table 2. Data are only presented from those subjects who completed a cocktail limb and a respective probe alone limb. Figures 1–5 depict the respective phenotypic indexes for each probe alone and as part of the cocktail. For debrisoquine and chlorzoxazone (six and two individuals, respectively) the required metric could not be measured, because concentrations were below the lower limit of quantification of the assay method. A large number of individuals had detectable concentrations of caffeine and paraxanthine in the predose samples, despite the 24 h caffeine restriction (for the first six subjects) prior to dosing. The intro-

Table 2
Geometric mean ratios and 90% confidence intervals of the phenotype indexes for the probe drugs given alone and as part of a cocktail

Probe	CYP	n	Geometric mean ratio[1]	90% Confidence interval Lower	Upper
caffeine	1A2	12	0.86	0.67	1.10
midazolam	3A4	12	0.96	0.74	1.24
tolbutamide	2C9	12	0.86	0.72	1.03
debrisoquine	2D6	6	1.04	0.97	1.12
chlorzoxazone	2E1	10	0.95	0.86	1.05

[1]*The ratio of the geometric mean of the phenotypic index, obtained after administration of the probe as part of the cocktail and when given alone.*

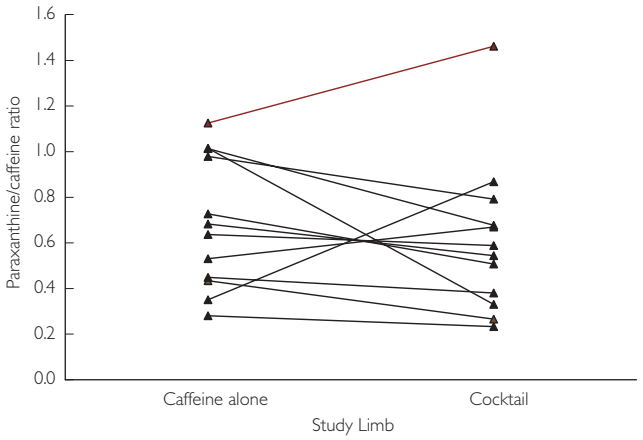

Figure 1
Paraxanthine/caffeine ratios in plasma when caffeine was given alone and as part of the five drug cocktail

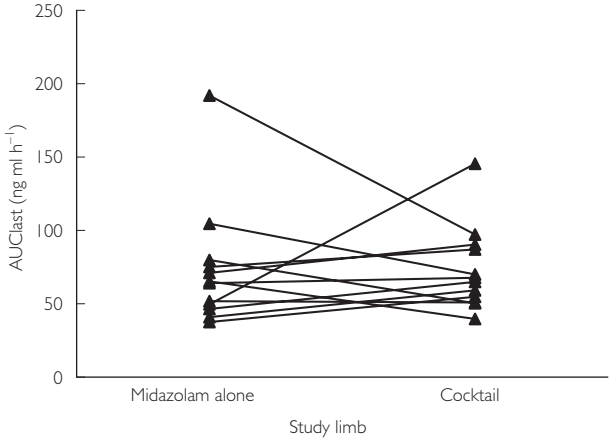

Figure 2
Plasma midazolam AUC_{last} when it was given alone and as part of the five drug cocktail

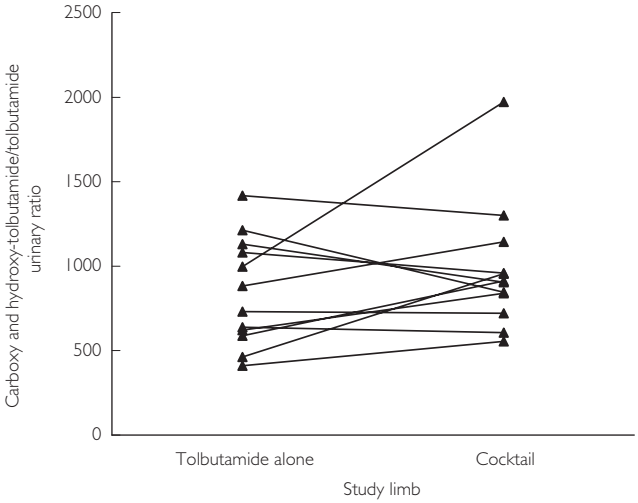

Figure 3
Hydroxytolbutamide plus carboxytolbutamide/tolbutamide urinary ratios when tolbutamide was given alone and as part of the five drug cocktail

Figure 4
Debrisoquine/4-hydroxy debrisoquine urinary ratios when debrisoquine was gvien alone and as part of the five drug cocktail

duction of a longer caffeine restriction (48 h) prior to dosing (for the later subject cohort) resulted in lower concentrations of caffeine and paraxanthine in the pre-dose samples (Table 3). Pre-dose residual caffeine and paraxanthine was noted in three subjects and one subject respectively after a 24 and 48 h caffeine restriction.

Pharmacodynamics and adverse events

No clinically important changes were seen in mean heart rate, systolic and diastolic blood pressure. Isolated low blood pressures were noted but none occurred in the cocktail limb.

Three subjects had asymptomatic low blood glucose concentrations following administration of the cocktail. In all cases the hypoglycaemia was considered to be of negligible clinical significance. Dextrose was given as rescue medication on each occasion as a precautionary procedure.

Dizziness was experienced following administration of midazolam and debrisoquine alone (n = 9 and 1, respectively) and the cocktail (n = 8). All these events were considered to be related to the administration of probe drugs.

Discussion

The aim of the present study was to assess the utility of a five-drug cocktail to measure the intrinsic activity of five cytochrome P450 enzymes, namely CYP1A2,

Table 3
Pre- and post-dose concentrations of caffeine and paraxanthine after a period of 12 h or 24 h abstinence from caffeine

Period of abstinence	Subject	Pre- dose concentration μg ml^{-1}		Post- dose concentration μg ml^{-1}	
		Caffeine	Paraxanthine	Caffeine	Paraxanthine
24 h	1	0.27	0.22	2.74	0.67
	2	1.23	1.25	2.07	1.39
	3	< 0.1	0.19	1.66	0.89
	4	0.59	0.43	2.47	0.85
	5	< 0.1	< 0.1	1.58	0.42
	6	< 0.1	0.26	0.66	1.03
48 h	7	< 0.1	< 0.1	0.53	0.46
	8	< 0.1	< 0.1	1.01	0.80
	9	0.12	0.15	1.47	0.58
	10	< 0.1	< 0.1	0.96	0.65
	11	< 0.1	< 0.1	0.68	0.37
	12	< 0.1	< 0.1	1.02	0.60

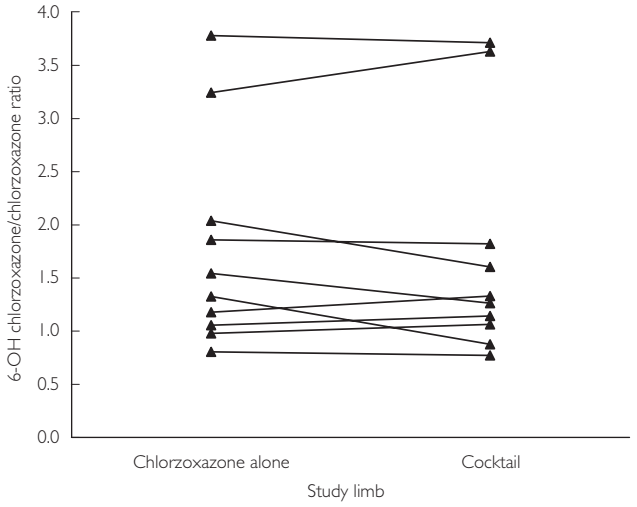

Figure 5
6-Hydroxychlorzoxazone/chlorzoxazone plasma ratios when chlorzoxazone was given alone and as part of the five drug cocktai

CYP3A4, CYP2C9, CYP2D6 and CYP2E1. For the methodology to become a valuable tool during drug development, there should be no concerns over safety. Pharmacokinetic and pharmacodynamic interaction between any of the five probe drugs when administered simultaneously would also be unacceptable.

Potentially hypotension could affect blood flow to the gut and liver with consequent changes in absorption and metabolism, respectively. We could find no published data relating falls in systemic blood pressure to changes in hepatic blood flow in healthy subjects, although one paper [18] reports that cardiac index was weakly but positively correlated with indocyanine green clearance. Probe drugs could also have differential effects on splanchnic and/or hepatic blood flow. In one report, caffeine has been shown to reduce liver blood flow in healthy subjects [18], disparate effects of midazolam on the systemic and hepatic circulation in the dog [19] have also identified. Equally, overt drug-induced hypoglycaemia could cause release of sympathetic hormones that can affect cardiovascular parameters. The wide usage of the probes in this study suggests that there are no concerns regarding these issues when each drug is given alone. The current study found no evidence that changes in physiological parameters are any more marked when the probes were given in combination.

There was little evidence of pharmacokinetic interaction between any of the five probe drugs, although in some instances there was large interindividual variability, which is probably reflective of the small data sets for some of the probes. In particular, data for debrisoquine was obtained from only six subjects, because of limiting assay sensitivity. To minimise the likelihood of pharmacodynamic interaction, the dose of debrisoquine used was 5 mg, compared to the standard dose (10 mg) generally used to assess CYP2D6 phenotype [11]. Since the present cocktail was tolerated well, it should now be possible to increase the dose of debrisoquine in future probe cocktail studies in order to maximize the data return. Alternatively, it should be possible to improve the assay sensitivity for this probe. It is recognized that the pharmacokinetics of iv midazolam only reflect the hepatic activity of CYP3A4. Midazolam would need to be given both orally and iv to differentiate between gut and hepatic CYP3A4 activity [20]. Furthermore, midazolam does not discriminate between CYP3A4 and CYP3A5, though it is probably the best available probe

for CYP3A4 [4]. For example, the use of erythromycin, which is a more selective CYP3A4 substrate, is confounded by its interaction with P-glycoprotein.

An interaction between chlorzoxazone and midazolam has been reported when the two probes are used as part of a cocktail [8]. The interaction was thought to result from chlorzoxazone affecting the first pass metabolism of midazolam through inhibition of gut CYP3A. In the present study, midazolam was given intravenously and no such interaction was observed.

To obtain a reliable value for AUC, the best index of CYP3A activity, serial blood sampling for 12 h after dosing with midazolam was performed in the present study. This is inconvenient for the subjects and costly from a bioanalysis viewpoint. Work has recently been published that validated a limited sampling approach for the measurement of midazolam AUC [16]. Adopting such a method for future cocktail studies may prove to be more cost effective and convenient than the standard full-pharmacokinetic profile strategy used generally.

A number of assumptions were made during the analysis of the caffeine data. These were necessary to account for the residual caffeine and paraxanthine found in pre-dose plasma despite caffeine abstinence. Thus, metabolic ratios were corrected using half-life values and assuming a mono-exponential decline in caffeine and paraxanthine concentration. A potentially more accurate method for dealing with residual caffeine and paraxanthine has been reported. It uses the difference between the 2- and 5-h postdose metabolite to parent ratio as the surrogate metric for CYP1A2 activity [21]. Several extrinsic factors such as smoking and diet can induce CYP1A2 [22]. In the present study it is possible that such factors may have contributed to the variability noted in the caffeine data. Therefore, particular attention should be paid to the control of such extrinsic causes of variability when caffeine is used as probe for CYP1A2 in any future cocktail studies.

The lack of a probe for CYP2C19 is a potential weakness of the present cocktail. Validated probes for CYP2C19 include S-mephenytoin and omeprazole. Two studies [5, 23] report the successful use of these probes when used as a part of a cocktail. However, omeprazole is more readily available than mephenytoin and may prove to be a better probe for CYP2C19 in the long term.

Consideration needs to be given to how probe cocktails should be used in clinical development. For example, should a cocktail that contains probes for all the major drug metabolizing enzymes be administered clinically, irrespective of the CYPs identified as being inhibited using *in vitro* microsomal systems. This topic has been the subject of debate in the literature [4]; we favour a pragmatic approach using the *in vitro* data as a guide to which probes should be used in the clinic.

In conclusion, the probe cocktail investigated in the present study has the potential to become a useful tool to aid the detection of clinically important DDIs during drug development. The five probe drugs were safe when administered in combination and there was no overt evidence of metabolic interaction. For the methodology to be used as a robust tool in drug development then a number of further investigations are required. Estimation of the intrasubject variability expected for a particular probe drug's phenotypic index needs to be identified. This will aid the determination of potentially clinically significant changes to the metabolism of the probe drug. Furthermore, the ability of the present cocktail to detect known drug interactions needs to be demonstrated.

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