Appropriate Phenotyping Procedures for Drug Metabolizing Enzymes and Transporters in Humans and Their Simultaneous Use in the "Cocktail" Approach

U Fuhr[1,2], A Jetter[1] and J Kirchheiner[3]

Phenotyping for drug metabolizing enzymes and transporters is used to assess quantitatively the effect of an intervention (*e.g.*, drug therapy, diet) or a condition (*e.g.*, genetic polymorphism, disease) on their activity. Appropriate selection of test drug and metric is essential to obtain results applicable for other substrates of the respective enzyme/transporter. The following phenotyping metrics are recommended based on the level of validation and on practicability: CYP1A2, paraxanthine/caffeine in plasma 6 h after 150 mg caffeine; CYP2C9, tolbutamide plasma concentration 24 h after 125 mg tolbutamide; CYP2C19, urinary excretion of 4′-OH-mephenytoin 0–12 h after 50 mg mephenytoin; CYP2D6, urinary molar ratio debrisoquine/4-OH-debrisoquine 0–8 h after 10 mg debrisoquine; and CYP3A4, plasma clearance of midazolam after 2 mg midazolam (all drugs given orally).

Phenotyping procedures may be combined in a "cocktail" to assess a metabolic profile. This is used primarily to examine the drug–drug interaction potential of a new drug. Many useful cocktails have been reported without mutual interaction between probe drugs and with good tolerability. Still, there is ample space for improvement by replacing individual probe drugs, by use of better validated metrics, by reducing the doses, and/or by simplifying of the procedures. The identification of metrics with low intraindividual variability allows assessing the effect of influencing factors in crossover cocktail studies with a sample size of 12 volunteers only. Phenotyping drug cocktails are a valuable, safe, and scientifically sound tool to characterize drug metabolism. They will have a permanent place in phase I of clinical drug development.

BASICS OF PHENOTYPING FOR A DRUG METABOLIZING ENZYME OR A DRUG TRANSPORTER

Phenotyping for drug metabolizing enzymes or transporters is defined as measuring its actual *in vivo* activity in an individual. This is performed by administration of a selective substrate for this enzyme and subsequent determination of appropriate pharmacokinetic parameters, or by using metabolism or transport of endogenous substrates. The metric used may be systemic clearance of a drug eliminated exclusively by the respective enzyme, partial clearance for a metabolic pathway, or absorption rate in the case of a transporter. Other parameters such as single point concentrations or ratios of metabolite over parent concentrations in plasma, saliva, and/or urine are also often used.[1–3]

As a general requirement for the use of phenotyping in comparative clinical trials or for making therapeutic decisions, it is essential that individual enzyme/transporter activity is stable. It is well known that for almost any drug, intraindividual variability in pharmacokinetics is clearly lower than interindividual variability. In a retrospective comparison of between- and within-person variances of several metrics for CYP3A4 activity in 161 subjects, the contribution of interindividual variation to overall variation exceeded 89% (point estimate) for validated CYP3A4 metrics and 83% for others.[4] This example shows that individual enzyme activity is indeed quite stable, even for an enzyme without major genetic polymorphisms, *i.e.*, without genetically fixed determinants of activity. Although there are no respective systematic compilations on enzymes and transporters, studies with repeated administrations of probe drugs suggest that individual stability of enzyme activity in general is high.[5–7]

[1]Department of Pharmacology, Clinical Pharmacology Unit, University of Cologne, Cologne, Germany; [2]ITECRA Institute for Tailored Early Clinical Research and Advice GmbH & Co. KG, Cologne, Germany; [3]Department of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, Ulm, Germany. Correspondence: U Fuhr (uwe.fuhr@uk-koeln.de)

doi:10.1038/sj.clpt.6100050

There are several potential applications for phenotyping, most of which remain to be established. Phenotyping may be used to assess basal enzyme activity in the absence of influencing factors (needing, *e.g.*, discontinuation of therapeutic drugs) or to assess actual enzyme in the presence of such factors. As a prognostic factor for the outcome of drug therapy and as information to be used for individual dose adjustments phenotyping may have a place in the hierarchy of the measures to control drug therapy. These span from taking covariates into account (*e.g.*, severity of disease) over phenotyping and therapeutic drug monitoring to clinical monitoring. Currently, the direct clinical application of phenotyping in patients is established only in a single case, which is measuring thiopurine *S*-methyltransferase activity in individuals undergoing treatment with thiopurines.[8] Phenotyping may also be used as a selection criterion of participants in clinical trials, *e.g.*, if poor metabolizers are to be excluded. By comparing the results of phenotyping to pharmacokinetic parameters of other drugs, enzymes, and transporters with a major role in the pharmacokinetics of these drugs may be identified.

One application, however, has already made its way to widespread use. This is identification of factors influencing enzyme and/or transporter activity. Once identified, these subsequently may be used to optimize individual therapy. The main application in this area is to quantify drug–drug interactions, *i.e.*, possible inhibition or induction of enzymes or transporters caused by the drug to be characterized. To this end, crossover studies are carried out with administration of the respective phenotyping agents in both periods, combined with co-administration of the drug to be tested in one period [2] (see also the 1999 FDA document "Guidance for industry: *in vivo* drug metabolism/drug interaction studies"). Because this procedure addresses the mechanism of an interaction, the results are supposed to be predictive for pharmacokinetic interactions with other substrates of the respective structure.[9] Accordingly, drug companies, as well as only regulatory authorities, increasingly favor this approach over testing possible interactions of a new drug only with drugs that will be frequently co-administered for therapeutic reasons. Many pharmaceutical companies have included phenotyping interactions studies in phase I of clinical development; often, simultaneous administration of several phenotyping drugs (the cocktail approach) is used to this end.

Beyond drug–drug interactions, the identification of other factors and the quantification of their effects on enzyme/transporter activity by phenotyping is a prerequisite for using these factors to adjust doses. The main factors examined comprise some disease states (*e.g.*, hepatic or renal failure, intensive care),[10] genetic variants,[11,12] sex,[13] age (newborns/old age), environmental exposure (*e.g.*, diet, smoking),[13,14] and ethnicity, which is related to both genetic and environmental influences.[15]

Requirements for Appropriate Phenotyping Procedures

Many properties, which in part are contradictory, are wanted for an optimal phenotyping procedure (**Tables 1** and **2**). The most important requirement is that it provides an accurate estimate of the activity to be characterized, but this may have to be balanced against other issues.

As a rule, there is no substrate to a transporter or an enzyme that is completely specific. Thus, a sufficient degree of selectivity is essential, showing that the phenotyping metric used reflects enzyme/transporter activity in various settings. Many validation criteria for sufficient selectivity have been defined, such as a close correlation of the metric to *in vivo* expression and activity of the respective protein in the corresponding tissue (*e.g.*, liver or small intestine mucosa).[16,17] Furthermore, the metrics should be changed correspondingly by inhibitors and inducers and should also reflect genetic polymorphisms, if any are known (**Table 1**). Still, for many metrics, including those used in extensively used drug cocktails, no complete validation has been carried out, and only a few metrics fulfill most of these criteria (see below).

Tolerability of the phenotyping drug is the second major issue. For instance, tolbutamide clearance following a 500 mg test dose is a selective CYP2C9 metric, but it requires blood glucose monitoring and/or glucose supplementation to avoid hypoglycemia.[18] Other phenotyping agents with safety concerns include digoxin, mephenytoin, midazolam, and alfentanil. One solution to this problem often is to administer very low doses; in the case of tolbutamide, a 125 mg dose could be used in combination with the improved analytical sensitivity of a liquid chromatography/tandem mass spectrometry method.[19] It should be taken into account that a dose change may cause a change in selectivity. Fortunately, lower

Table 1 Validation criteria for phenotyping metrics (modified from refs. 16, 17)

1. Changes in metric when patients are treated with inhibitors/inducers of the enzyme/transporter
2. Differences between healthy subjects and patients with liver disease (if the enzyme is expressed primarily in the liver)
3. Correlation of metabolite formation/transport rate with activity and content of enzyme/transporter in subcellular fractions (*e.g.*, human liver microsomes) and/or cellular systems
4. Proven in vitro specificity of the metabolic step/transport
5. High contribution of the metabolic step/transport to overall drug metabolism
6. Correlation of metric with the partial clearance for the respective specific metabolic step/transport
7. Reproducibility (a low coefficient of variation for repeated tests)
8. Correlation of the metric with the AUC of parent substrate
9. Correlation of the metric with other validated metrics
10. Metric reflects known genetic polymorphism
11. Metric does not depend on other factors not related to enzyme activity (urinary pH, urinary flow, renal function)

Table 2 Further desirable properties of phenotyping metrics (modified from refs. 16, 17)

1. Good availability of the drug (registration as a therapeutic drug)
2. Good availability of information on the drug, including preclinical data
3. Low invasiveness of the method (low risk for adverse effects, no blood sampling needed)
4. Ease of procedure for the subjects and the investigator
5. Low risk for errors during sample collection (*e.g.*, metabolic ratio in a single sample superior to urine collection in fixed intervals)

substrate concentrations usually are linked to an increased selectivity because of the decreasing contribution of enzymes with low affinity but high capacity.

Phenotyping drugs are not licensed and marketed for phenotyping, but as therapeutic drugs. Excellent phenotyping drugs are not necessarily successful therapeutic drugs and, therefore, may occasionally be withdrawn from the market or may not be available in all countries. Availability problems exist, *e.g.*, debrisoquine, chlozoxazone, mephenytoin, sparteine, and tolbutamide. Furthermore, in some instances, special preparations are required or wanted, such as mass-labeled substances, which are not readily available. Another possible limitation is the limited availability of preclinical data and of clinical safety information for individual phenotyping agents because some of these have been used as therapeutic drugs for decades and up-to-date information has never been compiled.

Phenotyping should be simple from an organizational and technical point of view. Thus, oral administration should be preferred over intravenous (i.v.) administration. The most convenient approach would be the use of dietary constituents (*e.g.*, caffeine for *N*-acetyltransferase type 2 (NAT2) phenotyping. see ref. 20) or of endogenous substrates (*e.g.*, cortisol[21] or cholesterol[22] for CYP3A4). It is desirable to use a simple metric, *e.g.*, a metabolic ratio in a spot urine sample or a single point plasma concentration is preferred over complete concentration vs time profile. However, the use of single measurements must also take into account that a single error in this case has much more impact than a single error within an entire concentration vs time profile. A short period of observation from administration of the probe drug to the point of time for sampling is an advantage. A long elimination half-life may also be a major obstacle for repeated phenotyping, *e.g.*, for using the mephenytoin metabolite nirvanol to phenotype for CYP2B6.[23] It is desirable that sampling is non-invasive, and urine and saliva concentrations should be preferred to plasma samples. Non-invasiveness may be essential for epidemiological studies.

Reliable analytical methods are essential for phenotyping, especially if low doses of probe drugs are administered. Often, the methods need to quantify very low concentrations of metabolites in the presence of other substances with similar physicochemical properties including those with the same molecular mass. Standard requirements for analytical methods, precision, and accuracy better than 20% at the lower limit of quantification may not be sufficient. The combination of chromatographic separation with tandem mass spectrometry is the method of choice; however, the selectivity brought about by mass spectrometry alone is often not sufficient, for example, for the quantification of enantiomers ((*S*)- and (*R*)-mephenytoin[24] or of metabolites hydroxylated at different positions).[25] For the quantification of metabolites, availability of reference substances may also be a limitation for the use of phenotyping procedures. Finally, it is sometimes difficult in the literature to track down whether (especially) urine samples have been treated by glucuronidase (+ sulfatase) before quantification, but this knowledge is essential.

Of outstanding importance for the use of a phenotyping metric is a low intraindividual variability for repeated tests. This property determines the sample size in comparative studies,[26] *e.g.*, in drug interaction studies, and therefore metrics with much nonspecific noise should be avoided. For instance, although urinary ratios of metabolites over the parent compound in the case of caffeine and tolbutamide clearly reflect the activity of CYP1A2 and CYP2C9, respectively, they have a much higher intraindividual CV than single point plasma ratio metrics.[18,19,27,28]

Some metrics are confounded by processes other than the activities these metrics are used to characterize, but these confounding factors can be quantified. Taking these confounding factors into account may considerably improve the validity of a metric. For instance, as glomerular filtration also affects digoxin C_{max}, taking creatinine clearance into account may improve the ability of this metric to assess *P*-glycoprotein activity and to differentiate between genotypes.[29] Likewise, taking urinary pH into account may improve dextromethorphan metabolic ratios as metrics for CYP2D6 activity.[30]

Appropriate Phenotyping Procedures for Individual Drug Metabolizing Enzymes and Transporters

Although the use of phenotyping might be interesting for any drug metabolizing enzyme or drug transporter, only a few enzymes or transporters are currently monitored in activity by the use of phenotyping. The reasons are, on the one hand, that for some proteins, their role in drug disposition is limited or remains to be elucidated; on the other hand, no selective substrates have been identified or validated so far. Among the rate-limiting enzymes, cytochrome P450 enzymes are key players. Intestinal and hepatic CYP3A4/5, CYP2D6, and CYP2C9 are known to mediate the metabolism of a broad range of drugs. CYP1A2, CYP2B6, CYP2C8, CYP2C19, and CYP2E1 are relevant for several drugs, but appropriate phenotyping procedures with a sufficient degree of validation are not available for some of these enzymes. In the case of CYP2B6, mephenytoin and bupropion are being evaluated as phenotyping drugs.[23,31] Currently, there is no substrate with sufficient selectivity for CYP2C8 relative to CYP2C9 to be used for phenotyping in humans.[32] No major role in drug

metabolism is apparent for CYP1A1, CYP1B1, and CYP2A6. (The two known human drugs primarily metabolized by CYP2A6, nicotine and coumarin, are also used for phenotyping[33,34].) For glucuronosyltransferases, glutathione transferases, and sulfotransferases, no appropriate phenotyping methods are in use. NAT2, thiopurine *S*-methyltransferase, and dihydropyrimidine dehydrogenase activities can be assessed reliably, but these enzymes are of outstanding importance for a few drugs only. A caffeine-based metric for xanthine oxidase is notoriously included in phenotyping evaluations, but in this case also, both validation and clinical relevance are very limited.

It is probable that drug transporters are involved in transmembrane trafficking of most drugs. Some information on transporter substrates is summarized in the appendix to the September 2006 FDA draft guidance "Guidance for industry: drug interaction studies—study design, data analysis, and implications for dosing and labeling". However in contrast to drug metabolism, the role of individual transporter activity for the pharmacokinetics of a given drug is poorly understood, in part because of the lack of selectivity.[35] Also, the concept of phenotyping has not yet been applied to transporter function as in the case of CYPs, with the single exception of *P*-glycoprotein.

With respect to regulatory affairs, it should also be mentioned that the list of enzyme substrates proposed in the actual 1999 FDA document "Guidance for industry: *in vivo* drug metabolism/drug interaction studies" (midazolam, buspirone, felodipine, simvastatin, or lovastatin for CYP3A4; theophylline for CYP1A2; *S*-warfarin for CYP2C9; and desipramine for CYP2D6) represents a compromise between clinical importance and enzyme selectivity. In the new September 2006 draft guidance "Guidance for industry: drug interaction studies—study design, data analysis, and implications for dosing and labeling", not much has changed: the primary recommendations are midazolam for CYP3A, theophylline for CYP1A, repaglinide for CYP2C, *S*-warfarin for CYP2C, omeprazole for CYP2C; and desipramine for CYP2D6. Therefore, not all of these drugs may therefore be considered as optimal for phenotyping.

In the following, individual phenotyping procedures are briefly discussed and limited to those also used in typical phenotyping cocktails. A judgment is provided based on the criteria given in **Tables 1** and **2** and their usefulness in the cocktail settings; for a more extensive discussion of these and other procedures (see refs. 1, 3, 36). The resulting assessment is summarized in **Table 3**, where the most appropriate phenotyping methods are listed.

CYP1A2

Caffeine is the only CYP1A2 phenotyping agent for which respective metrics are fully validated. The most convenient of the validated metric is the plasma ratio of paraxanthine over caffeine 5–7 h after administration of a 100–200 mg caffeine test dose.[27,37,38] Caffeine clearance with full concentration vs time profile (with somewhat less variation but many more expenses) or saliva ratios (slightly higher variation) may also be used. Urinary metabolic ratios are plagued by much more nonspecific noise and/or bias[27,38] and should therefore be avoided. The caffeine dose may be administered as caffeine-containing drinks such as instant coffee[27] or cola. Because of many dietary sources, caffeine abstinence required for at least 12 h (better for 36 h) is not always easy to adhere to. Currently, mass-labeled caffeine to avoid diet interference is not readily available.

CYP2C9

An intraindividual comparison of *in vitro* enzyme activity to phenotyping metrics *in vivo*, which is the most reliable validation procedure, is not available for any of the CYP2C9 phenotyping agents. Tolbutamide has been validated by comparison to CYP2C9 genotype and by co-administration of selective inhibitors. Both tolbutamide plasma clearance and tolbutamide plasma concentrations 24 h after administration have been shown to closely reflect CYP2C9 activity.[18,19] In contrast, urinary metabolic ratios[28] have a much weaker correlation to enzyme activity and are also not correlated to plasma-based metrics.[19] The 125 mg dose should be preferred over the standard 500 mg doses because the low dose does not produce relevant hypoglycemia.[19] Currently, tolbutamide is available only as a generic in the United States; the original manufacturer discontinued marketing of its preparation probably for lack of commercial relevance, suggesting that availability of the drug is also endangered.

S-warfarin plasma area under the curve after a 10 mg dose of racemic warfarin, usually given in combination with 10 mg of vitamin K to prevent anticoagulation, is used in several phenotyping cocktails. Primarily based on pronounced effects of CYP2C9 variants on warfarin pharmacokinetics[39] and on the effects of selective CYP2C9 inhibitors *in vivo*,[40] this parameter may also be considered as a valid CYP2C9 probe. However, plasma needs to be sampled for at least 72 h to this end.[40,41] Furthermore, the need for analytical separation of warfarin enantiomers is a limitation for the development of methods with simultaneous quantification of several phenotyping drugs and their metabolites by LC–MS/MS.

Compared to tolbutamide and warfarin, less clear data are available for losartan and flurbiprofen metrics used. In one study, losartan pharmacokinetics showed pronounced differences between CYP2C9 genotypes,[42] but there was no effect of CYP2C9 inhibition.[43] Flurbiprofen clearance was clearly decreased by co-administration of fluconazole, a partially selective CYP2C9 inhibitor;[44] however, it is, unclear to what extent this applies to the flurbiprofen urinary metabolic ratio used in a cocktail.[45] Because in a direct comparison, the effect of CYP2C9 variants was less for losartan and flurbiprofen than for tolbutamide, the former were considered as inferior phenotyping agents.[46]

In one cocktail, diclofenac has been included.[47] Although diclofenac 4′-hydroxylation *in vitro* is an established marker for CYP2C9 activity, the lack of any effect of CYP2C9

Table 3 Selected phenotyping procedures considered as most appropriate

Enzyme/transporter	Substrate (p.o. if not indicated otherwise)	Metric(s) (in italics if validation needs amendment)
CYP1A2	Caffeine 100–200 mg	Ratio paraxanthine/caffeine in a single plasma or saliva sample 4–8 h postdose Caffeine clearance/*F*, problem: many samples required
CYP2C9	Tolbutamide 125–500 mg	Tolbutamide concentration in a single plasma sample 24 h postdose Tolbutamide clearance/*F*, problem: many samples required
	Warfarin 10 mg+vitamin K 10 mg	Warfarin AUC, problem: long sampling period of at least 72 h required
CYP2C19	Mephenytoin 50–100 mg	Urinary excretion of (*R*, *S*)-4′-OH-mephenytoin 0–12 h postdose, problem: limited availability of the substance
	Omeprazole 20–40 mg	*Ratio omeprazole/5-OH-omeprazole in a single plasma sample 2–3 h postdose*
CYP2D6	Debrisoquine 10 mg	Urinary ratio 4-OH-debrisoquine/(4-OH-debrisoquine+ debrisoquine) 0–8 h postdose, problem: limited availability of the substance
	Dextromethorphan-HBr 30 mg	Urinary ratio dextromethorphan/dextrorphan 0–8(−24) h postdose, problems: effect of urinary pH and flow on metric, high intraindividual variability *dextromethorphan clearance/F, problem: many samples required*
CYP2E1	Chlorzoxazone 200–500 mg (should not be included in a cocktail)	Ratio 6-OH-chlorzoxazone/chlorzoxazone in a single plasma sample 2–4 h postdose, problem: chlorzoxazone inhibits CYP3A4 chlorzoxazone clearance/*F*, problems: chlorzoxazone inhibits CYP3A4, many samples required
CYP3A4 (intestinal, hepatic)	Midazolam 0.075–1 mg i.v.	clearance of i.v. midazolam (hepatic CYP3A4), problem: many samples required, but limited sampling strategy available
	Midazolam 2 mg p.o.	Clearance/*F* of oral midazolam (overall CYP3A4), problem: many samples required, but limited sampling strategy available. *Intestinal extraction of oral midazolam (intestinal CYP3A4), problems: valid only in conjunction with i.v. midazolam, many samples required*
NAT2 (*N*-acetyl-transferase 2)	Caffeine 100–200 mg	Urinary metabolic ratio (AFMU+AAMU)/(AFMU+AAMU+1X+1U) appr. 4–6 h postdose (some other similar ratios are also appropriate)
P-glycoprotein	Digoxin 0.5 mg	*Digoxin C_{max}, adjusted to differences in creatinine clearance; problem: high intraindividual variability*

genotype on diclofenac pharmacokinetics *in vivo* suggests that this drug should not be used for CYP2C9 phenotyping.[48,49]

CYP2C19

Traditionally, the third-line antiepileptic mephenytoin has been used for CYP2C19 phenotyping. Available validation is primarily based on *in vitro* results and on the effect of CYP2C19 genetic variant.[1] The most appropriate parameter is the fraction of the dose eliminated as 4′-OH-mephenytoin in urine from dosing up to 12 h postdose.[23,25] Sampling periods shorter than 8 h increase variability considerably. Most of this metabolite is glucuronidated. For acceptable variation, treatment with glucuronidase is essential. Because of concerns with respect to tolerability, the usual dose of 100 mg has been decreased to 50 mg, with no impact on the results.[25]

As the availability of mephenytoin became limited (only marketed by Gerot, Austria), omeprazole is often used instead. Although clearly a CYP2C19 substrate, omeprazole undergoes a complex metabolism and is also both an inhibitor[50] and an inducer[51] of CYP1A2. Also, validation of omeprazole-derived metrics is limited and such metrics may be influenced by several factors other than CYP2C19 activity.[1]

CYP2D6

This enzyme was among the first for which a genetic polymorphism has been detected. First, a polymorphism was recognized in the phenotype, much earlier than the molecular background of this polymorphism has been understood. Debrisoquine, the drug that gave the first clue, is still around and is an excellent CYP2D6 phenotyping drug with extensive validation. The ratio debrisoquine over 4-OH-debrisoquine in urine collected up to 8 h postdose is the standard metric. Unfortunately, availability is a major problem, debrisoquine is currently only marketed in a few countries, and it is not certain for how long may be available at all. Sparteine is no longer marketed as a drug. As a standard of convenience, dextromethorphan is used increasingly. Formation of dextrorphan is clearly a selective CYP2D6 pathway, and tolerability of dextromethorphan is excellent. The molar ratio of dextromethorphan over dextrorphan in urine collected 0–8 h postdose has been validated extensively under controlled conditions. However, the formation of 3-methoxymorphinan is an alternative pathway, mediated mainly by CYP3A4,[1] and as an additional confounder, urinary pH has a major effect on the urinary metabolic ratio.[30,52] Although the urinary ratio is clearly able to discern

between genetically defect CYP2D6 poor metabolizers and extensive metabolizers, it has a relatively high variability with a mean intraindividual CV of above 35%,[5,53] and minor changes of CYP2D6 activity may go unnoticed when using this metric.[25] The idea of taking urinary pH into account as a covariate[30] remains to be validated. Thus, plasma concentrations or respective ratios may be superior, but quantification of the very low concentrations requires LC–MS/MS analytics, and evaluation is still ongoing.

Metoprolol is also used extensively for CYP2D6 phenotyping. The effect of CYP2D6 genotypes on metoprolol-based metrics is less compared to debrisoquine and dextromethorphan,[54] and validation by comparison to other metrics gave equivocal results.[1] Therefore, no metoprolol-based metric is recommended.

CYP2E1

The clearance of chlorzoxazone and the ratio 6-OH-chlorzoxazone over chlorzoxazone in plasma 2 to 4 h after oral administration of a 250–500 mg dose are validated CYP2E1 markers.[55] In contrast to the respective ratios in urine, the correlation of plasma ratios to the "gold standard" metric, *i.e.*, fractional clearance of chlorzoxazone to 6-OH-chlorzoxazone,[1] was excellent.[56] However, as chlorzoxazone inhibits CYP3A4 *in vivo*,[57] this component should not be included in a phenotyping cocktail. This problem cannot be amended by dose reduction because this results in a change of selectivity.[55] Presently, there are no other CYP2E1 markers available.

CYP3A4 (intestinal and hepatic)

As there are many CYP3A4 substrates, there are also many CYP3A4 phenotyping procedures in use. To assess CYP3A4 activity *in vivo*, endogenous substrates such as cortisol[21] and cholesterol[22] are available, but because of considerable intraindividual variation for cortisol-based metrics[58] and insufficient validation of cholesterol-based metrics, these are not recommended for phenotyping. Most of the metrics used are not fully specific for CYP3A4 vs CYP3A5, although midazolam and erythromycin pharmacokinetics probably do not depend much on CYP3A5.[59,60] The most frequently used test substrates include midazolam, erythromycin, dapsone, quinine, and nifedipine. Midazolam is a benzodiazepine drug that undergoes considerable first pass metabolism by both intestinal and hepatic CYP3A4 and thereafter is cleared selectively by the hepatic enzyme. Very extensive validation is available for clearance midazolam after i.v. bolus administration to assess hepatic CYP3A4 activity.[16,61,62] This metric has a low intraindividual coefficient of variation of about 11%.[6] Simultaneous (differently mass-labeled)[63] or sequential administration of oral and i.v. midazolam[25,64,65] was used to obtain separate estimates of hepatic and intestinal CYP3A4 activities, which are regulated independently.[66] Intestinal extraction calculated from both oral and i.v. administration[25,63] is used as a metric for intestinal enzyme activity. This is a logical approach supported by the results of inhibition studies,[61,67] but remains to be examined further by *in vivo* vs *in vitro* comparison. From oral administration alone, clearance (CL)/*F* is used as a global CYP3A4 metric, which also reflects hepatic CYP3A4 activity, but less so than CL after i.v. administration.[68] Plasma concentrations and urine concentrations in single samples have been used as simplified metrics also after oral dosing. Although urinary ratios and ratios including the main metabolite 1-OH-midazolam were not closely related to plasma area under the curve,[69] these strategies were valid for plasma midazolam.[62] The initially high doses of 5 mg and more were related to relevant sedation, but currently lower doses down to 0.075 mg[70] have eliminated this problem.

Erythromycin *N*-demethylation is mediated specifically by CYP3A4.[59] The erythromycin breath test is an extensively validated CYP3A4 phenotyping procedure. Therefore, it was initially surprising that phenotyping results with midazolam and erythromycin were not concordant.[71] The important role of *P*-glycoprotein in erythromycin pharmacokinetics is an explanation for this finding.[72] Erythromycin also increases gastrointestinal motility. We therefore recommend not to include it as part of a phenotyping cocktail.

Dapsone, a CYP3A4 phenotyping drug used in many cocktails, has never been validated as a selective substrate. It may not be appropriate even from theoretical considerations (for details, see refs. 73, 74) and also failed to show the expected changes in pharmacokinetics upon co-treatment with CYP3A4 inhibitors.[75] Quinine, which is also used in phenotyping cocktails, may undergo considerable metabolism by enzymes other than CYP3A4, is a *P*-glycoprotein substrate, has only limited validation and also inhibits CYP2D6.[76,77] Therefore, it was administered separately from the other drugs in a cocktail and probably is not the optimal CYP3A4 phenotyping drug in this setting. Nifedipine, used in an earlier cocktail, is a CYP3A4 substrate *in vitro*, but has never been assessed as a valid CYP3A4 metric.[16]

NAT2

Several different urinary caffeine metabolic ratios are included in cocktails, mainly because caffeine is administered anyway as a CYP1A2 substrate. Despite the complex secondary metabolism of caffeine, which is not fully understood, all these ratios are useful to identify genetic NAT2 variants.[20,78,79] For the metabolic ratio (AFMU + AAMU)/(AFMU + AAMU + 1X + 1U), steady values were reached and the CV was below 16% for any sampling interval after 4 h.[80] Dietary caffeine sources without administration of a test dose were sufficient for most individuals, making this ratio useful in epidemiological studies.[80]

Likewise, dapsone used for CYP3A4 phenotyping generates an NAT2 metric as a byproduct. Although dapsone is used extensively to assess the acetylator phenotype,[81] like caffeine-based metrics, the only validation is by comparison to NAT2 genotypes. It is not clear how changes in NAT2 activity would relate to changes in the respective metrics.

The level of validation is similar for a sulfamethazine metabolic ratio in serum, 6 h after dosing.[82]

NAT2 activity is important for the metabolism of only a few drugs; therefore, it is of limited importance to include a respective metric in phenotype cocktails unless a specific question is addressed.

P-glycoprotein

P-glycoprotein is involved in the membrane transport of many drugs;[83] thus, a metric for this process is highly desirable. As the transporter is expressed in the gut, in the liver, in the kidney and also in target tissues for drug therapy, a single metric probably not provide much information. However, it may be useful for a qualitative identification of influencing factors. Digoxin is a relatively specific substrate,[84] and pharmacokinetics, especially C_{max}, is affected by genetic variants[85] and by the inducer rifampicin.[86] Thus, this may be considered as a potential metric for mainly intestinal *P*-glycoprotein, but its usefulness remains to be determined. Results for further *P*-glycoprotein substrates such as talinolol and fexofenadine are equivocal.[87–89]

USE OF PHENOTYPING INFORMATION TO PREDICT PHARMACOKINETICS OF SUBSTRATES FOR RESPECTIVE ENZYMES

One would expect that validated phenotyping parameters reflecting enzyme activity may be translated directly to pharmacokinetics of other substrates. However, individual pharmacokinetic properties of the test drugs, as well as of the other substrates, need to be taken into account. For instance, pharmacokinetics of tizanidine, a CYP1A2 substrate, is much more sensitive to alterations in CYP1A2 activity than validated caffeine-based metrics.[90] The explanation is that tizanidine—in contrast to caffeine—undergoes extensive first pass metabolism. Only with this information, the effect of CYP1A2 inhibition on tizanidine pharmacokinetics can be predicted correctly. More obviously, substrates that are also subject to extensive metabolism by other than the index enzyme are less prone to changes in enzyme activity than highly selective substrates. Vice versa, results obtained with less selective phenotyping drugs may be difficult to extrapolate on highly selective drug substrates.

A further problem is that enzymes and enzyme variants may handle different substrates in a different way. It has been shown that CYP2C9 variants, which have a major impact on tolbutamide metabolism,[18] do not affect diclofenac 4-hydroxylation, which is also clearly a CYP2C9 pathway.[49] In the case of CYP3A4, substrates and inhibitors may bind to different subdomains of the CYP3A4 binding site.[91] This behavior of enzymes may also limit the predictivity of phenotyping.

RATIONALE FOR COMBINED ADMINISTRATION OF PHENOTYPING AGENTS

There are several situations where information about the activity of more than a single enzyme/transporter is relevant; for example, to assess the interaction potential of a new drug or the effect of disease on drug metabolism in general. Traditionally, a whole series of crossover studies of phenotyping would be needed here. As an alternative, simultaneous administration of several probe substrates is used in the "cocktail" approach. This approach had been proposed initially by Schellens *et al.*[92] in Leiden. It was subsequently reactivated by Frye *et al*,[73] and is now being used increasingly as understanding of the role of individual proteins in drug metabolism and transport has increased and analytical methods have considerably improved (LC–MS/MS). With the "cocktail" approach, a single study is sufficient, resulting in a considerable reduction of direct costs and, even more important, of the time required to compile the information. It may even be an advantage from an ethical point of view to include a much lower number of individuals in clinical studies to resolve a question. Variation that may occur in many processes with impact on pharmacokinetics, which would become relevant in a series of studies, may be avoided by simultaneous administration. Beyond combined administration, LC–MS/MS analysis also allows the simultaneous quantification of concentrations, further reducing expenses.[25,93] A modular composition of cocktails is possible for specific questions. Finally, a cocktail may contain components that are evaluated conditionally on the results for other components:[25] If, for instance, a new drug inhibits several CYPs *in vitro* with the lowest K_i value for CYP2D6, an extensive cocktail may be administered, but concentrations for metrics other than CYP2D6 activity may be quantified only if there is a relevant effect on CYP2D6.

POSSIBLE PROBLEMS BY COMBINED ADMINISTRATION OF PHENOTYPING AGENTS

As mentioned above, phenotyping has an inherent limitation. To obtain basal activity, often there is the necessity of stopping other drug treatments. This also applies for cocktail phenotyping and may make it difficult to assess the impact of disease on drug metabolism by this tool.

The benefits of combining several substrates must be balanced against potential problems. The main issue already addressed from the beginning,[73,92] is the possibility that the various substrates cause mutual pharmacokinetic drug–drug interactions. To date, metrics for most cocktails following separate and combined administration of the components have been compared and a relevant mutual interaction was not observed in most cases, although sample sizes in these studies are often low and existing negative studies suffer from a lack of power and even of power calculations.[73,74] The probability of such interactions may be minimized by the use of low doses in combination with highly sensitive analytical methods. Beyond pharmacokinetic interactions, pharmacodynamic interactions may also be present. For instance, midazolam, dextromethorphan, and mephenytoin all have some sedating properties. It is difficult to predict the combined effect of such drugs, and therefore, subjects need to be monitored intensively when such combinations are used for the first time.[25] Once again, the use of low doses has

been shown to avoid such problems.[25] One may speculate that even more severe and unexpected toxicity may be elicited by the combined administration, but currently there is no evidence for this. Preclinical data for such combinations are not available; however, these are also not required for co-administration of therapeutic drugs. In summary, mutual interactions probably are not a relevant problem for current cocktails used.

It is unclear to what extent the data of isolated and combined phenotyping are reliable in extreme situations, such as severe disease, renal insufficiency, and pronounced inhibition of some enzymes. It is conceivable that such factors may change phenotyping metrics by a mechanism other than directly altering index enzyme activity. Analytical interactions ceased to be a major issue once LC–MS/MS analytics became available,[93,94] but even with conventional high-pressure liquid chromatography, separation of components is possible.[73,95]

A major issue for pharmaceutical companies in the past has been acceptance of cocktail data by regulatory authorities. However, nowadays, several examples for acceptance are available, and we recommend seeking scientific advice before the study if any doubts remain. We also hope that this publication will promote regulatory acceptance of cocktail studies.

DESIGN OF COCKTAIL STUDIES

To quantify the effect of a condition or an intervention on drug metabolism, intraindividual crossover studies are the gold standard whenever a period without the condition/intervention is available. In this case, the use of a placebo intervention is desirable to achieve equal conditions. To quantify the extent of effect, the bioequivalence approach should be used.[96] The null hypothesis in this case is "there is a relevant effect of the intervention", which may be rejected if the 90% confidence intervals for the metric ratio with or without it is completely inside predefined limits. Limits for the rejection of the null hypothesis do not need to be identical to those used in bioequivalence testing (*i.e.*, 0.8–1.25), but should be justified. Together with the expected effect size and the intraindividual variability of the metric (see section on individual metrics and **Table 4**), this is also the basis for estimation of the sample size needed.[26] When using standard bioequivalence criteria, a sample size of 12 is sufficient in such studies if the appropriate low variability metrics are combined in a cocktail (see **Table 4**). If crossover studies are not possible, respective confidence intervals for between-group comparisons based on the Hodges–Lehmann estimator[97] may be used.

If an intervention (*e.g.*, co-administration of a drug) is to be studied, the extent of intervention should be according to the clinical situation to be assessed. For chronic drug treatment as an intervention, administration of the drug thus should be at the standard therapeutic dose and last until steady state for both the drug (approximately five half-lives) and, if any, the changes of enzyme activities are reached. One week appears to be sufficient for enzyme induction, which usually results in slower changes than enzyme inhibition.[14] The exposure to the interacting drug should be monitored by quantification of the drug and its relevant metabolites.

For conditions such as diseases, it appears reasonable to study various degrees of severity.

INDIVIDUAL DRUG COCKTAILS

Numerous drug cocktails have been published. Earlier cocktails often used less selective marker drugs because the molecular identity of the respective enzymes or transporters was unclear. In all cocktails, the selection of metrics is determined by several factors, including the specific objective of the respective study, the availability of analytical methods, and the balance between expense and validation. Still, even recent phenotyping cocktails often contain substances or use phenotyping metrics that are not optimal. Therefore, we aimed at providing an overview of the characteristics of typical existing cocktails to provide guidance for users. In this review, we will consider selected cocktails that include at least three selective phenotyping drugs. An overview of these cocktails is given in **Table 4**, where the references are also included. For identification, the primary affiliation towns of the authors were used. The Pittsburgh and the Cooperstown cocktails have been used most often.

The Changsha cocktail

This cocktail covers most CYPs and uses a convenient sampling strategy, *that is*, urine collection 0–8 h postdose and plasma sampling 1, 4, and 6 h postdose, but includes two metrics that are not fully validated and chlorzoxazone as a CYP3A4 inhibitor.

The Cologne cocktail

It may not be surprising that the authors tried to optimize their own cocktail with respect to all important criteria. The advantages are that only fully validated metrics have been used whenever these have been available, that this is the only cocktail with separate estimation of hepatic and intestinal CYP3A4 activity, and that this is the only cocktail including a metric for CYP2B6 (nirvanol excretion in urine up to 168 h as percentage of 50 mg mephenytoin dose) and for *P*-glycoprotein (C_{max} of 0.5 mg digoxin). However, we are aware that these additional parameters are at a different level of validation; thus the cocktail is a mix of validated and experimental procedures, providing a mix of confirmatory and exploratory assessments. Drawbacks are that in its current version, many samples have to be measured to this end, and that the possibility of interactions has not been tested explicitly for digoxin. Also, from the scientific point of view, debrisoquine might be a better choice than dextromethorphan until more information of plasma dextromethorphan metrics is available.

The Cooperstown (5 + 1) cocktail

This cocktail covers most of the relevant CYPs plus NAT2. It has been used extensively and includes mainly fully validated

Table 4 Comparison of typical phenotyping cocktails with at least three selective probe drugs

Cocktail identification	Enzymes/transporters covered, probe drugs and doses, metrics (% intraindividual CV if available) (bold if selected procedure+metric is sufficiently validated, empty box if absent)										
	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP2E1	iCYP3A4	hCYP3A4	XO	NAT2	P-gp
Changsha[103,104]	**CA 100 mg** **MR P** **6 h**			**MP 100 mg** **Ae 4′OHM** **0–8 h**	MT 100 mg MR U 8 h	**CZ 200 mg** **MR P** **4 h**	MI 7.5 mg MR P 1 h				
Cologne[19,23,25,29,64,80] (i.v. MI 4 h later)	**CA 150 mg** **MR P** **6 h (16%)**	MP 50 mg Ae NIR 0-168 h (19%)	**TO 125 mg** C **P TOL** **24 h (8%)**	**MP 50 mg** **Ae 4′OHM** **0–12 h (13%)**	**DX 30 mg** **MR U** **8 h (30%)**		MI 2 mg Fi P 0-12 h (18%)	**MI i.v 1 mg** **CL P** **0–12 h (7%)**		**(CA)** **MR U** **6 h (15%)**	DI 0.5 mg C_{max} P 0–12 h (49%)
Cooperstown (5+1)[40,100,105] (oral MI on separate day[106])	CA 2 mg/kg **MR U** **0–12 h**		**WA 10 mg** S–**WA AUC P** **0–96 h**	OM 40 mg MR P 2 h	**DX 30 mg** **MR U** **0–12 h**			**MI i.v** **25–75 μg/kg,** **CL P** 0–6(−12)h	(CA) MR U 0–12 h	**(CA)** **MR U** **0–12 h**	
Darmstadt[47] (MT on separate day)	**CA 100 mg** **AUC P** **0–24 h**		DC 50 mg AUC P 0–24 h	**MP 100 mg** **Ae 4′OHM** **0–8 h**	MT 100 mg AUC P 0–72 h		**MI 7.5 mg** **AUC P** **0–24 h**				
Indianapolis (modular composition, *e.g.*, refs. 41, 107) (oral MI on separate day)	**CA** **100–200 mg,** MR P 6 h		**TO 500 mg** **CL/F P TO** **24 h**		**DX 30 mg** **MR U** **24 h**		**MI 5 mg** **CL/F P** **0–12 h**	**MI i.v** **50 μg/kg** **CL P** **0–12 h**			
Jena[108] (1 g MM additionally)	**CA 200 mg** **AUC P** **0–12 h**				**DB 10 mg** **MR U** **0–6 h**					SM 500 mg MR U;P 0–6 h; 6 h	
Karolinska[77] (QU on separate occasion)	**CA 100 mg** **MR P** **4 h (12%)**		LO 25 mg MR U 8 h (22%)	OM 20 mg MR P 3 h (25%)	**DB 10 mg** **MR U** **8 h (22%)**		QU 250 mg MR P 16 h (8%)				
Leiden[92,101]				**MP 100 mg** **Ae 4′OHM** **0–8 (−48)h**	**SP 25–90 mg,** **MR U** **0–8 h**		NI 5–20 mg CL/*F* P 0–8 h				
Loughborough[99]	**CA 50 mg** **MR P** **6.5 h**		TO 250 mg MR U 6–12 h		**DB 5 mg** **MR U** **0–6 h**	**CZ 250 mg** **MR P** **2.5 h**	**MI i.v 25 μg/kg** **CL P** **0–12 h**				
Pittsburgh (*e.g.*, refs. 45, 73, 109–114)	**CA 100 mg** **MR P** **8 h (15%)**		FL 50 mg MR U 0–8 h (9%)	**MP 100 mg** **Ae 4′OHM** **0–8 h (7–12%)**	**DB 10 mg/** **DX 30 mg** **MR U** **0–8 h** **(DB 5-8%)**	**CZ 250 mg** MR P **4 h (17%)**	DP 100 mg MR U 0–8 h (5–8%)			(DP) MR U 0–8 h (4–9%)	
Quebec[74]	CA 100 mg MR U 0–8 h (16%)		TO 250 mg MR U 0–8 h (22%)		MT 25 mg MR U 0–8 h (18%)	CZ 250 mg MR U 0–8 h (13%)	DP 100 mg MR U 0–8 h (33%)		(CA) MR U 0–8 (5%)	**(CA)** **MR U** **0–8 h** **(13%)**	

Ae, amount excreted; C, concentration; CA, caffeine; CL, clearance; CYP, cytochrome P450; DB, debrisoquine; DC, diclofenac; DI, digoxin; DP, dapsone; DX, dextromethorphan; F, bioavailability; Fi, intestinal availability; FL, flurbiprofen; iCYP3A4, intestinal CYP3A4; hCYP3A4, hepatic CYP3A4; LO, losartan; MI, midazolam; MM, metamizol; MP, mephenytoin; MR, metabolic ratio; MT, metoprolol; NI, nifedipine; NIR, nirvanol; OM, omeprazole; P, plasma; QU, quinine; SM, sulfamethazine ; SP, sparteine; TO, tolbutamide; U, urine; WA, warfarin+ vitamin K. Cocktails evolved over time, and some modifications with respect to individual doses, metrics, and inclusion of components have been reported for most cocktails; each detail could not be included in this table (see references). Doses were administered orally if not indicated otherwise.

metrics, with the exception of CYP1A2 and CYP2C19. Although plasma samples were also drawn, a highly variable urinary metabolic ratio was used as CYP1A2 metric but this could easily be amended by use of the metabolic ratio in plasma.

The Darmstadt cocktail

In a study to assess the interaction profile of sarizotan, a new cocktail was composed which includes metrics for the five major CYPs. Metoprolol area under the curve up to 72 h was used as a CYP2D6 metric; to this end, the drug was given on a separate day. This unvalidated procedure is very laborious and overcomes some, but not all, theoretical limitations of metoprolol as a CYP2D6 marker drug, but may have been selected because of the probability of clinical co-medication. Diclofenac probably is not a good choice for CYP2C9 phenotyping, whereas the metrics used for CYP1A2, CYP2C19, and CYP3A4 are also costly, but valid.

The Indianapolis cocktail

This cocktail is focused on CYP1A2, CYP2C9, and CYP2D6, and includes only fully validated metrics. Sample collection up to 96 h postdose was done to thoroughly, assess metrics, but can easily be simplified. Data on intraindividual variability are not given.

The Jena cocktail(s)

In the early 1990s, a German group developed a cocktail with selective metrics for CYP2D6, NAT2, and CYP1A2, and a non-selective metamizole-based metric (**Table 4**). A few years later, the same group reported a cocktail for CYP2A6 (coumarin), CYP2D6 (dextromethorphan), and CYP2C19 (mephenytoin), based on urinary metrics only.[98] Mutual interactions as well as simplified sampling strategies were considered. Both cocktails were reasonably combined, but did not cover all of the important enzymes, and further applications have not been reported.

The Karolinska cocktail

This cocktail covers most of the relevant CYPs. NAT2 and XO activities were measured additionally. It uses mainly fully validated metrics—with the exception of the quinine MR, for which only limited data are available. In addition, quinine is administered on a separate occasion, because some inhibition of CYPs other than CYP3A4 is expected. Furthermore, intraindividual CV exceeds 20% for CYP2D6, CYP2C9, and CYP2C19 metrics.

The Leiden cocktail

This prototype cocktail was already quite advanced; at that time, it became obvious that the substances used were selective substrates for individual CYPs *in vitro* and *in vivo*, but validation as a phenotyping metric had just begun. In one study,[92] the non-selective CYP substrate antipyrine was also included. Several doses were tested, and many different metrics were calculated and compared, taking the effect of inhibitors and genetic polymorphisms into account. The current unavailability for sparteine, the coverage of only three CYPs, and the lack of validation of nifedipine metrics are the major limitations of this cocktail.

The Loughborough cocktail

This cocktail includes the classical urinary CYP2C9 ratio that does not really reflect enzyme activity. It also uses a low debrisoquine dose, which may confer tolerability advantages.

The Pittsburgh cocktail

This cocktail covers most of the relevant CYPs. Initally, there was no metric for CYP2C9 included, but recently, flurbiprofen 50 mg was added.[45] However, there is limited experience with this phenotyping drug. Urine is collected up to 8 h postdose, and two plasma samples are drawn at 4 h and 8 h. Most metrics used are sufficiently validated, and because of the low intraindividual variability (CV below 15.2% and lower), the sample size is sufficient to prove lack of interaction between the components, which has been tested extensively. Drawbacks are the inclusion of dapsone as an unvalidated and probably inappropriate CYP3A4 substrate, and of chlorzoxazone, which is inhibitory for CYP3A4.

The Quebec cocktail

This cocktail is peculiar because it is based on the measurement of urinary metabolic ratios after oral self-administration of the drugs in the evening and overnight urine sampling. The advantage is the ease of the procedure, the low number of samples, and the widespread availability of the substances as therapeutic drugs. However, most of these ratios are only partially validated and/or have been shown to only poorly reflect enzyme activity. Extensive comparisons of the metrics between various combinations of the phenotyping drugs have been made to exclude mutual interactions, but because of the high intraindividual coefficients of variation in some cases (up to 33%), the power may not be sufficient to prove lack of interaction with the sample size of $n = 10$ used.[26] This may also further explain why the known effect of chlorzoxazone on CYP3A4[57] was not observed here. Still, this cocktail may be very useful whenever strictly controlled experimental conditions cannot be applied.

ADVERSE EVENTS OF PHENOTYPING COCKTAILS

Published information on cocktail safety is limited. Tolerability was described as excellent for the Pittsburgh cocktail [73] as well as for the Quebec cocktail.[74] No severe or serious adverse events were reported for the Darmstadt cocktail and presentation of data was limited ("well tolerated"), but a 100 mg metoprolol dose caused a pharmacodynamic response and sedation.[47] Dizziness caused by 25 μg/kg i.v. of midazolam and hypoglycemia related to the 250 mg oral dose of tolbutamide was reported for the Loughborough

cocktail.[99] No hypoglycemic effect was seen by the low 125 mg tolbutamide dose for the Cologne cocktail, but the low 1 mg i.v. bolus of midazolam caused short-term mild sedation.[25] Sedation was also reported for the Cooperstown (5 + 1) cocktail related to the 25 μg/kg i.v. dose, but pulse oximetry was unaffected.[40,100] No effect of 10 mg warfarin + 10 mg vitamin K on INR was seen.[40] No changes in clinical safety laboratory parameters, electrocardiograms, physical examinations, or vital signs were reported,[100] and also have not been observed in the five studies conducted thus far using variants of the Cologne cocktail.

CONCLUSION AND OUTLOOK

In 1993, after the first cocktail studies had been published,[92,101] Paolini *et al.*,[102] in a comment, expressed their concerns with respect to the practical utility of the cocktail approach. Ten years later, Tanaka *et al.*[36] judged the available phenotyping procedures as not really useful. However, a thorough review of the data shows that for most of the enzymes addressed, there are phenotyping procedures that provide valid information on individual activity. However, selection of an appropriate test drug and metric is essential. Just combining procedures reported for individual drugs does not yet make a valid phenotyping cocktail, even if there is no mutual interaction between components to be expected.

Most existing phenotyping cocktails are appropriate for their intended use. Still, there is ample space for improvement by replacement of individual probe drugs, by use of a better validated metric, by reducing the doses, and/or by simplification of the procedures. Low doses and successful validation of limited sampling strategies make the use of cocktails increasingly convenient. The identification of metrics with low intraindividual variability allows the assessment of the effect of influencing factors with a very limited sample size (12 in most cases).[26] Therefore, phenotyping drug cocktails are valuable, safe, cheap, and scientifically sound tools for characterizing drug metabolism. Specifically, they will have a permanent place in phase I of drug development to assess the metabolic interaction profile of drugs.

It is desirable to register and market phenotyping agents also with the indication of phenotyping in order to make phenotyping procedures independent of the current availability of the respective substances as therapeutic agents.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

1. Streetman, D.S., Bertino, J.S. Jr & Nafziger, A.N. Phenotyping of drug-metabolizing enzymes in adults: a review of *in-vivo* cytochrome P450 phenotyping probes. *Pharmacogenetics* **10,** 187–216 (2000).
2. Tucker, G.T., Houston, J.B. & Huang, S.M. Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential-toward a consensus. *Clin. Pharmacol. Ther.* **70,** 103–114 (2001).
3. Wilkinson, G.R. *In Vivo Probes for Studying Induction and Inhibition of Cytochrome P450 Enzymes in Humans*, (Marcel Dekker, Inc., New York, (2001).
4. Özdemir, V., Kalow, W., Tang, B.K., Paterson, A.D., Walker, S.E. & Endrenyi, L. *et al.* Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics* **10,** 373–388 (2000).
5. Kashuba, A.D., Nafziger, A.N., Kearns, G.L., Leeder, J.S., Shirey, C.S. & Gotschall, R. *et al.* Quantification of intraindividual variability and the influence of menstrual cycle phase on CYP2D6 activity as measured by dextromethorphan phenotyping. *Pharmacogenetics* **8,** 403–410 (1998).
6. Kashuba, A.D., Bertino, J.S. Jr, Rocci, M.L. Jr, Kulawy, R.W., Beck, D.J. & Nafziger, A.N. Quantification of 3-month intraindividual variability and the influence of sex and menstrual cycle phase on CYP3A activity as measured by phenotyping with intravenous midazolam. *Clin. Pharmacol. Ther.* **64,** 269–277 (1998).
7. Kashuba, A.D., Bertino, J.S. Jr, Kearns, G.L., Leeder, J.S., James, A.W. & Gotschall, R. *et al.* Quantitation of three-month intraindividual variability and influence of sex and menstrual cycle phase on CYP1A2, *N*-acetyltransferase-2, and xanthine oxidase activity determined with caffeine phenotyping. *Clin. Pharmacol. Ther.* **63,** 540–551 (1998).
8. Wang, L. & Weinshilboum, R. Thiopurine *S*-methyltransferase pharmacogenetics: insights, challenges and future directions. *Oncogene* **25,** 1629–1638 (2006).
9. Fuhr, U., Weiss, M., Kroemer, H.K., Neugebauer, G., Rameis, H. & Weber, W. *et al.* Systematic screening for pharmacokinetic interactions during drug development. *Int. J. Clin. Pharmacol. Ther.* **34,** 139–151 (1996).
10. Park, G.R. Molecular mechanisms of drug metabolism in the critically ill. *Br. J. Anaesth.* **77,** 49 (1996).
11. Daly, A.K. Pharmacogenetics of the major polymorphic metabolizing enzymes. *Fundam. Clin. Pharmacol.* **17,** 27–41 (2003).
12. Kirchheiner, J., Fuhr, U. & Brockmoller, J. Pharmacogenetics-based therapeutic recommendations – ready for clinical practice? *Nat. Rev. Drug. Discov.* **4,** 639–647 (2005).
13. Tantcheva-Poor, I., Zaigler, M., Rietbrock, S. & Fuhr, U. Estimation of cytochrome *P*-450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine test. *Pharmacogenetics* **9,** 131–144 (1999).
14. Faber, M.S. & Fuhr, U. Time response of cytochrome P450 1A2 activity on cessation of heavy smoking. *Clin. Pharmacol. Ther.* **76,** 178–184 (2004).
15. Aklillu, E., Herrlin, K., Gustafsson, L.L., Bertilsson, L. & Ingelman-Sundberg, M. Evidence for environmental influence on CYP2D6-catalysed debrisoquine hydroxylation as demonstrated by phenotyping and genotyping of Ethiopians living in Ethiopia or in Sweden. *Pharmacogenetics* **12,** 375–383 (2002).
16. Watkins, P.B. Noninvasive tests of CYP3A enzymes. *Pharmacogenetics* **4,** 171–184 (1994).
17. Zaigler, M., Tantcheva-Poor, I. & Fuhr, U. Problems and perspectives of phenotyping for drug-metabolizing enzymes in man. *Int. J. Clin. Pharmacol. Ther.* **38,** 1–9 (2000).
18. Kirchheiner, J, Bauer, S., Meineke, I., Rohde, W., Prang, V. & Meisel, C. *et al.* Impact of CYP2C9 and CYP2C19 polymorphisms on tolbutamide kinetics and the insulin and glucose response in healthy volunteers. *Pharmacogenetics* **12,** 101–109 (2002).
19. Jetter, A., Kinzig-Schippers, M., Skott, A., Lazar, A., Tomalik-Scharte, D. & Kirchheiner, J. *et al.* Cytochrome P450 2C9 phenotyping using low-dose tolbutamide. *Eur. J. Clin. Pharmacol.* **60,** 165–171 (2004).
20. Jetter, A., Kinzig-Schippers, M., Illauer, M., Hermann, R., Erb, K. & Borlak, J. *et al.* Phenotyping of *N*-acetyltransferase type 2 by caffeine from uncontrolled dietary exposure. *Eur. J. Clin. Pharmacol.* **60,** 17–21 (2004).
21. Galteau, M.M. & Shamsa, F. Urinary 6beta-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. *Eur. J. Clin. Pharmacol.* **59,** 713–733 (2003).
22. Bodin, K., Bretillon, L., Aden, Y., Bertilsson, L., Broome, U. & Einarsson, C. *et al.* Antiepileptic drugs increase plasma levels of 4beta-hydroxy-cholesterol in humans: evidence for involvement of cytochrome p450 3A4. *J. Biol. Chem.* **276,** 38685–38689 (2001).

23. Klaassen, T., Jetter, A., Tomalik-Scharte, D., Kasel, D., Harlfinger, S. & Kirchheiner, J. *et al.* Urinary metrics to phenotype for CYP2C19 and CYP2B6. *Clin. Exp. Pharmacol. Physiol.* **31**(Suppl): A145 (2004).
24. Laine, K., Tybring, G. & Bertilsson, L. No sex-related differences but significant inhibition by oral contraceptives of CYP2C19 activity as measured by the probe drugs mephenytoin and omeprazole in healthy Swedish white subjects. *Clin. Pharmacol. Ther.* **68,** 151–159 (2000).
25. Tomalik-Scharte, D., Jetter, A., Kinzig-Schippers, M., Skott, A., Sorgel, F. & Klaassen, T. *et al.* Effect of propiverine on cytochrome P450 enzymes: a cocktail interaction study in healthy volunteers. *Drug Metab. Dispos.* **33,** 1859–1866 (2005).
26. Diletti, E., Hauschke, D. & Steinijans, V.W. Sample size determination for bioequivalence assessment by means of confidence intervals. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **29,** 1-8 (1991).
27. Fuhr, U., Rost, K.L., Engelhardt, R., Sachs, M., Liermann, D. & Belloc, C. *et al.* Evaluation of caffeine as a test drug for CYP1A2, NAT2 and CYP2E1 phenotyping in man by *in vivo* versus *in vitro* correlations. *Pharmacogenetics* **6,** 159–176 (1996).
28. Veronese, M.E., Miners, J.O., Randles, D., Gregov, D. & Birkett, D.J. Validation of the tolbutamide metabolic ratio for population screening with use of sulfaphenazole to produce model phenotypic poor metabolizers. *Clin. Pharmacol. Ther.* **47,** 403–411 (1990).
29. Jabrane, W., Malchau, G., Kinzig-Schippers, M., Tönnes, E., Jetter, A. & Lück, H. *et al.* A pilot study to address the role of renal function in phenotyping for intestinal transporter activity using oral marker substrates Proc. of the 6th Congress of the European Association for Clinical Pharmacology and Therapeutics (EACPT), Istanbul. 2003.
30. Özdemir, M., Crewe, K.H., Tucker, G.T. & Rostami-Hodjegan, A. Assessment of *in vivo* CYP2D6 activity: differential sensitivity of commonly used probes to urine pH. *J. Clin. Pharmacol.* **44,** 1398–1404 (2004).
31. Kirchheiner, J., Klein, C., Meineke, I., Sasse, J., Zanger, U.M. & Murdter, T.E. *et al.* Bupropion and 4-OH-bupropion pharmacokinetics in relation to genetic polymorphisms in CYP2B6. *Pharmacogenetics* **13,** 619–626 (2003).
32. Kirchheiner, J., Roots, I., Goldammer, M., Rosenkranz, B. & Brockmoller, J. Effect of genetic polymorphisms in cytochrome p450 (CYP) 2C9 and CYP2C8 on the pharmacokinetics of oral antidiabetic drugs: clinical relevance. *Clin. Pharmacokinet.* **44,** 1209–1225 (2005).
33. Dempsey, D., Tutka, P., Jacob, P. III, Allen, F., Schoedel, K. & Tyndale, R.F. *et al.* Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. *Clin. Pharmacol. Ther.* **76,** 64–72 (2004).
34. Pelkonen, O., Rautio, A., Raunio, H. & Pasanen, M. CYP2A6: a human coumarin 7-hydroxylase. *Toxicology* **144,** 139–147 (2000).
35. Ho, R.H. & Kim, R.B. Transporters and drug therapy: implications for drug disposition and disease. *Clin. Pharmacol. Ther.* **78,** 260–277 (2005).
36. Tanaka, E., Kurata, N. & Yasuhara, H. How useful is the ''cocktail approach'' for evaluating human hepatic drug metabolizing capacity using cytochrome P450 phenotyping probes *in vivo*? *J. Clin. Pharm Ther.* **28,** 157–165 (2003).
37. Fuhr, U. & Rost, K.L. Simple and reliable CYP1A2 phenotyping by the paraxanthine/caffeine ratio in plasma and in saliva. *Pharmacogenetics* **4,** 109–116 (1994).
38. Rostami-Hodjegan, A., Nurminen, S., Jackson, P.R. & Tucker, G.T. Caffeine urinary metabolite ratios as markers of enzyme activity: a theoretical assessment. *Pharmacogenetics* **6,** 121–149 (1996).
39. Linder, M.W., Looney, S., Adams, J.E. III, Johnson, N., Antonino-Green, D. & Lacefield, N. *et al.* Warfarin dose adjustments based on CYP2C9 genetic polymorphisms. *J. Thromb. Thrombolysis.* **14,** 227–232 (2002).
40. Chainuvati, S., Nafziger, A.N., Leeder, J.S., Gaedigk, A., Kearns, G.L. & Sellers, E. *et al.* Combined phenotypic assessment of cytochrome p450 1A2, 2C9, 2C19, 2D6, and 3A, *N*-acetyltransferase-2, and xanthine oxidase activities with the ''Cooperstown 5+1 cocktail''. *Clin. Pharmacol. Ther.* **74,** 437–447 (2003).
41. Bruce, M.A., Hall, S.D., Haehner-Daniels, B.D. & Gorski, J.C. *In vivo* effect of clarithromycin on multiple cytochrome P450s. *Drug Metab. Dispos.* **29,** 1023–1028 (2001).
42. Yasar, U., Forslund-Bergengren, C., Tybring, G., Dorado, P., Llerena, A. & Sjoqvist, F. *et al.* Pharmacokinetics of losartan and its metabolite E-3174 in relation to the CYP2C9 genotype. *Clin. Pharmacol. Ther.* **71,** 89–98 (2002).
43. Meadowcroft, A.M., Williamson, K.M., Patterson, J.H., Hinderliter, A.L. & Pieper, J.A. The effects of fluvastatin, a CYP2C9 inhibitor, on losartan pharmacokinetics in healthy volunteers. *J. Clin. Pharmacol.* **39,** 418–424 (1999).
44. Greenblatt, D.J., von Moltke, L.L., Perloff, E.S., Luo, Y., Harmatz, J.S. & Zinny, M.A. Interaction of flurbiprofen with cranberry juice, grape juice, tea, and fluconazole: *in vitro* and clinical studies. *Clin. Pharmacol. Ther.* **79,** 125–133 (2006).
45. Zgheib, N.K., Frye, R.F., Tracy, T.S., Romkes, M. & Branch, R.A. Validation of incorporating flurbiprofen into the Pittsburgh cocktail. *Clin. Pharmacol. Ther.* **80,** 257–263 (2006).
46. Lee, C.R., Pieper, J.A., Frye, R.F., Hinderliter, A.L., Blaisdell, J.A. & Goldstein, J.A. Tolbutamide, flurbiprofen, and losartan as probes of CYP2C9 activity in humans. *J. Clin. Pharmacol.* **43,** 84–91 (2003).
47. Krösser, S., Neugebauer, R., Dolgos, H., Fluck, M., Rost, K.L. & Kovar, A. Investigation of sarizotan's impact on the pharmacokinetics of probe drugs for major cytochrome P450 isoenzymes: a combined cocktail trial. *Eur. J. Clin. Pharmacol.* **62,** 277–284 (2006).
48. Yasar, U., Eliasson, E., Forslund-Bergengren, C., Tybring, G., Gadd, M. & Sjoqvist, F. *et al.* The role of CYP2C9 genotype in the metabolism of diclofenac *in vivo* and *in vitro*. *Eur. J. Clin. Pharmacol.* **57,** 729–735 (2001).
49. Kirchheiner, J., Meineke, I., Steinbach, N., Meisel, C., Roots, I. & Brockmoller, J. Pharmacokinetics of diclofenac and inhibition of cyclooxygenases 1 and 2: no relationship to the CYP2C9 genetic polymorphism in humans. *Br. J. Clin. Pharmacol.* **55,** 51–61 (2003).
50. Rost, K.L., Fuhr, U., Thomsen, T., Zaigler, M., Brockmoller, J. & Bohnemeier, H. *et al.* Omeprazole weakly inhibits CYP1A2 activity in man. *Int. J. Clin. Pharmacol. Ther.* **37,** 567–574 (1999).
51. Rost, K.L., Brosicke, H., Heinemeyer, G. & Roots, I. Specific and dose-dependent enzyme induction by omeprazole in human beings. *Hepatology* **20,** 1204–1212 (1994).
52. Borges, S., Li, L., Hamman, M.A., Jones, D.R., Hall, S.D. & Gorski, J.C. Dextromethorphan to dextrorphan urinary metabolic ratio does not reflect dextromethorphan oral clearance. *Drug Metab. Dispos.* **33,** 1052–1055 (2005).
53. Zhou, H., Tong, Z. & McLeod, J.F. ''Cocktail'' approaches and strategies in drug development: valuable tool or flawed science? *J. Clin. Pharmacol.* **44,** 120–134 (2004).
54. McGourty, J.C., Silas, J.H., Lennard, M.S., Tucker, G.T. & Woods, H.F. Metoprolol metabolism and debrisoquine oxidation polymorphism–population and family studies. *Br. J. Clin. Pharmacol.* **20,** 555–566 (1985).
55. Mishin, V.M., Rosman, A.S., Basu, P., Kessova, I., Oneta, C.M. & Lieber, C.S. Chlorzoxazone pharmacokinetics as a marker of hepatic cytochrome P4502E1 in humans. *Am. J. Gastroenterol.* **93,** 2154–2161 (1998).
56. Frye, R.F., Adedoyin, A., Mauro, K., Matzke, G.R. & Branch, R.A. Use of chlorzoxazone as an *in vivo* probe of cytochrome P450 2E1: choice of dose and phenotypic trait measure. *J. Clin. Pharmacol.* **38,** 82–89 (1998).
57. Palmer, J.L., Scott, R.J., Gibson, A., Dickins, M. & Pleasance, S. An interaction between the cytochrome P450 probe substrates chlorzoxazone (CYP2E1) and midazolam (CYP3A). *Br. J. Clin. Pharmacol.* **52,** 555–561 (2001).
58. Chen, Y.C., Gotzkowsky, S.K., Nafziger, A.N., Kulawy, R.W., Rocci, M.L. Jr & Bertino, J.S. Jr *et al.* Poor correlation between 6beta-hydroxycortisol: cortisol molar ratios and midazolam clearance as measure of hepatic CYP3A activity. *Br. J. Clin. Pharmacol.* **62,** 187–195 (2006).
59. Wrighton, S.A., Brian, W.R., Sari, M.A., Iwasaki, M., Guengerich, F.P. & Raucy, J.L. *et al.* Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). *Mol. Pharmacol.* **38,** 207–213 (1990).
60. He, P., Court, M.H., Greenblatt, D.J. & Von Moltke, L.L. Genotype–phenotype associations of cytochrome P450 3A4 and 3A5 polymorphism with midazolam clearance *in vivo*. *Clin. Pharmacol. Ther.* **77,** 373–387 (2005).
61. Tsunoda, S.M., Velez, R.L., von Moltke, L.L. & Greenblatt, D.J. Differentiation of intestinal and hepatic cytochrome P450 3A activity with use of midazolam as an *in vivo* probe: effect of ketoconazole. *Clin. Pharmacol. Ther.* **66,** 461–471 (1999).

62. Chaobal, H.N. & Kharasch, E.D. Single-point sampling for assessment of constitutive, induced, and inhibited cytochrome P450 3A activity with alfentanil or midazolam. *Clin. Pharmacol. Ther.* **78,** 529–539 (2005).
63. Gorski, J.C., Jones, D.R., Haehner-Daniels, B.D., Hamman, M.A., O'Mara, E.M. Jr & Hall, S.D. The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. *Clin. Pharmacol. Ther.* **64,** 133–143 (1998).
64. Jabrane, W., Fuhr, U., Kinzig-Schippers, M., Jetter, A., Hering, U. & Walchner-Bonjean, M. *et al.* Sequential i.v. and oral administration of midazolam to estimate hepatic and intestinal CYP3A4 activity. *Eur. J. Clin. Pharmacol.* **57,** A35 (2001).
65. Lee, J.I., Chaves-Gnecco, D., Amico, J.A., Kroboth, P.D., Wilson, J.W. & Frye, R.F. Application of semisimultaneous midazolam administration for hepatic and intestinal cytochrome P450 3A phenotyping. *Clin. Pharmacol. Ther.* **72,** 718–728 (2002).
66. von Richter, O., Burk, O., Fromm, M.F., Thon, K.P., Eichelbaum, M. & Kivisto, K.T. Cytochrome P450 3A4 and *P*-glycoprotein expression in human small intestinal enterocytes and hepatocytes: a comparative analysis in paired tissue specimens. *Clin. Pharmacol. Ther.* **75,** 172–183 (2004).
67. Veronese, M.L., Gillen, L.P., Burke, J.P., Dorval, E.P., Hauck, W.W. & Pequignot, E. *et al.* Exposure-dependent inhibition of intestinal and hepatic CYP3A4 *in vivo* by grapefruit juice. *J. Clin. Pharmacol.* **43,** 831–839 (2003).
68. Wandel, C., Bocker, R.H., Bohrer, H., deVries, J.X., Hofmann, W. & Walter, K *et al.* Relationship between hepatic cytochrome P450 3A content and activity and the disposition of midazolam administered orally. *Drug Metab. Dispos.* **26,** 110–114 (1998).
69. Rogers, J.F., Nafziger, A.N., Kashuba, A.D., Streetman, D.S., Rocci, M.L. Jr & Choo, E.F. *et al.* Single plasma concentrations of 1′-hydroxymidazolam or the ratio of 1′-hydroxymidazolam:midazolam do not predict midazolam clearance in healthy subjects. *J. Clin. Pharmacol.* **42,** 1079–1082 (2002).
70. Eap, C.B., Buclin, T., Cucchia, G., Zullino, D., Hustert, E. & Bleiber, G. *et al.* Oral administration of a low dose of midazolam (75 microg) as an *in vivo* probe for CYP3A activity. *Eur. J. Clin Pharmacol.* **60,** 237–246 (2004).
71. Kinirons, M.T., O'Shea, D., Kim, R.B., Groopman, J.D., Thummel, K.E. & Wood, A.J. *et al.* Failure of erythromycin breath test to correlate with midazolam clearance as a probe of cytochrome P4503A. *Clin. Pharmacol. Ther.* **66,** 224–231 (1999).
72. Kurnik, D., Wood, A.J. & Wilkinson, G.R. The erythromycin breath test reflects *P*-glycoprotein function independently of cytochrome P450 3A activity. *Clin. Pharmacol. Ther.* **80,** 228–234 (2006).
73. Frye, R.F., Matzke, G.R., Adedoyin, A., Porter, J.A. & Branch, R.A. Validation of the five-drug "Pittsburgh cocktail" approach for assessment of selective regulation of drug-metabolizing enzymes. *Clin. Pharmacol. Ther.* **62,** 365–376 (1997).
74. Sharma, A., Pilote, S., Belanger, P.M., Arsenault, M. & Hamelin, B.A. A convenient five-drug cocktail for the assessment of major drug metabolizing enzymes: a pilot study. *Br. J. Clin. Pharmacol.* **58,** 288–297 (2004).
75. Gass, R.J., Gal, J., Fogle, P.W., Detmar-Hanna, D. & Gerber, J.G. Neither dapsone hydroxylation nor cortisol 6beta-hydroxylation detects the inhibition of CYP3A4 by HIV-1 protease inhibitors. *Eur. J. Clin. Pharmacol.* **54,** 741–747 (1998).
76. Mirghani, R.A., Ericsson, O., Tybring, G., Gustafsson, L.L. & Bertilsson, L. Quinine 3-hydroxylation as a biomarker reaction for the activity of CYP3A4 in man. *Eur. J. Clin. Pharmacol.* **59,** 23–28 (2003).
77. Christensen, M., Andersson, K., Dalen, P., Mirghani, R.A., Muirhead, G.J. & Nordmark, A. *et al.* The Karolinska cocktail for phenotyping of five human cytochrome P450 enzymes. *Clin. Pharmacol. Ther.* **73,** 517–528 (2003).
78. Kalow, W. & Tang, B.K. The use of caffeine for enzyme assays: a critical appraisal. *Clin. Pharmacol. Ther.* **53,** 503–514 (1993).
79. Nyeki, A., Buclin, T., Biollaz, J. & Decosterd, L.A. NAT2 and CYP1A2 phenotyping with caffeine: head-to-head comparison of AFMU vs AAMU in the urine metabolite ratios. *Br. J. Clin. Pharmacol.* **55,** 62–67 (2003).
80. Jetter, A., Kinzig-Schippers, M., Illauer, M., Tomalik-Scharte, D., Sörgel, F. & Fuhr, U. When should urine samples for NAT2 phenotyping with caffeine be collected? *Clin. Pharmacol. Ther.* **75,** 69 (2004).
81. Xie, H.G., Xu, Z.H., Ou-Yang, D.S., Shu, Y., Yang, D.L. & Wang, J.S. *et al.* Meta-analysis of phenotype and genotype of NAT2 deficiency in Chinese populations. *Pharmacogenetics* **7,** 503–514 (1997).
82. Meisel, P., Arndt, D., Scheuch, E., Klebingat, K.J. & Siegmund, W. Prediction of metabolic activity from genotype: the gene–dose effect of *N*-acetyltransferase. *Ther. Drug Monit.* **23,** 9–14 (2001).
83. Ieiri, I., Takane, H. & Otsubo, K. The MDR1 (ABCB1) gene polymorphism and its clinical implications. *Clin. Pharmacokinet.* **43,** 553–576 (2004).
84. Taipalensuu, J., Tavelin, S., Lazorova, L., Svensson, A.C. & Artursson, P. Exploring the quantitative relationship between the level of MDR1 transcript, protein and function using digoxin as a marker of MDR1-dependent drug efflux activity. *Eur. J. Pharm. Sci.* **21,** 69–75 (2004).
85. Johne, A., Kopke, K., Gerloff, T., Mai, I., Rietbrock, S. & Meisel, C. *et al.* Modulation of steady-state kinetics of digoxin by haplotypes of the *P*-glycoprotein MDR1 gene. *Clin. Pharmacol. Ther.* **72,** 584–594 (2002).
86. Greiner, B., Eichelbaum, M., Fritz, P., Kreichgauer, H.P., von Richter, O. & Zundler, J. *et al.* The role of intestinal *P*-glycoprotein in the interaction of digoxin and rifampin. *J. Clin. Invest.* **104,** 147–153 (1999).
87. Drescher, S., Schaeffeler, E., Hitzl, M., Hofmann, U., Schwab, M. & Brinkmann, U. *et al.* MDR1 gene polymorphisms and disposition of the *P*-glycoprotein substrate fexofenadine. *Br. J. Clin. Pharmacol.* **53,** 526–534 (2002).
88. Hamman, M.A., Bruce, M.A., Haehner-Daniels, B.D. & Hall, S.D. The effect of rifampin administration on the disposition of fexofenadine. *Clin. Pharmacol. Ther.* **69,** 114–121 (2001).
89. Zhang, W.X., Chen, G.L., Zhang, W., Tan, Z.R., Liu, J. & Zhou, G. *et al.* MDR1 genotype do not influence the absorption of a single oral dose of 100 mg talinolol in healthy Chinese males. *Clin. Chim. Acta.* **359,** 46–52 (2005).
90. Granfors, M.T., Backman, J.T., Neuvonen, M., Ahonen, J. & Neuvonen, P.J. Fluvoxamine drastically increases concentrations and effects of tizanidine: a potentially hazardous interaction. *Clin. Pharmacol. Ther.* **75,** 331–341 (2004).
91. Galetin, A., Ito, K., Hallifax, D. & Houston, J.B. CYP3A4 substrate selection and substitution in the prediction of potential drug-drug interactions. *J. Pharmacol. Exp. Ther.* **314,** 180–190 (2005).
92. Schellens, J.H., van der Wart, J.H., Brugman, M. & Breimer, D.D. Influence of enzyme induction and inhibition on the oxidation of nifedipine, sparteine, mephenytoin and antipyrine in humans as assessed by a "cocktail" study design. *J. Pharmacol. Exp. Ther.* **249,** 638–645 (1989).
93. Scott, R.J., Palmer, J., Lewis, I.A. & Pleasance, S. Determination of a 'GW cocktail' of cytochrome P450 probe substrates and their metabolites in plasma and urine using automated solid phase extraction and fast gradient liquid chromatography tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **13,** 2305–2319 (1999).
94. Yin, O.Q., Lam, S.S., Lo, C.M. & Chow, M.S. Rapid determination of five probe drugs and their metabolites in human plasma and urine by liquid chromatography/tandem mass spectrometry: application to cytochrome P450 phenotyping studies. *Rapid Commun. Mass Spectrom.* **18,** 2921–2933 (2004).
95. Jerdi, M.C., Daali, Y., Oestreicher, M.K., Cherkaoui, S. & Dayer, P. A simplified analytical method for a phenotyping cocktail of major CYP450 biotransformation routes. *J. Pharm. Biomed. Anal.* **35,** 1203–1212 (2004).
96. Steinijans, V.W., Hartmann, M., Huber, R. & Radtke, H.W. Lack of pharmacokinetic interaction as an equivalence problem. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **29,** 323–328 (1991).
97. Hollander, M. & Wolfe, D.A. *Nonparametric statistical methods*, Wiley, New York, (1973).
98. Endres, H.G., Henschel, L., Merkel, U., Hippius, M. & Hoffmann, A. Lack of pharmacokinetic interaction between dextromethorphan, coumarin and mephenytoin in man after simultaneous administration. *Pharmazie* **51,** 46–51 (1996).
99. Blakey, G.E., Lockton, J.A., Perrett, J., Norwood, P., Russell, M. & Aherne, Z. *et al.* Pharmacokinetic and pharmacodynamic assessment of a five-probe metabolic cocktail for CYPs 1A2, 3A4, 2C9, 2D6 and 2E1. *Br. J. Clin. Pharmacol.* **57,** 162–169 (2004).

100. Ma, J.D., Nafziger, A.N., Villano, S.A., Gaedigk, A. & Bertino, J.S. Jr Maribavir pharmacokinetics and the effects of multiple-dose maribavir on cytochrome P450 (CYP) 1A2, CYP 2C9, CYP 2C19, CYP 2D6, CYP 3A, *N*-acetyltransferase-2, and xanthine oxidase activities in healthy adults. *Antimicrob. Agents Chemother.* **50,** 1130–1135 (2006).
101. Schellens, J.H., Ghabrial, H., van der Wart, H.H., Bakker, E.N., Wilkinson, G.R. & Breimer, D.D. Differential effects of quinidine on the disposition of nifedipine, sparteine, and mephenytoin in humans. *Clin. Pharmacol. Ther.* **50,** 520–528 (1991).
102. Paolini, M., Biagi, G.L., Bauer, C. & Cantelli-Forti, G. Cocktail strategy: complications and limitations. *J. Clin. Pharmacol.* **33,** 1011–1012 (1993).
103. Zhu, B., Ou-Yang, D.S., Chen, X.P., Huang, S.L., Tan, Z.R. & He, N. *et al.* Assessment of cytochrome P450 activity by a five-drug cocktail approach. *Clin. Pharmacol. Ther.* **70,** 455–461 (2001).
104. Chen, X.P., Tan, Z.R., Huang, S.L., Huang, Z., Ou-Yang, D.S. & Zhou, H.H. Isozyme-specific induction of low-dose aspirin on cytochrome P450 in healthy subjects. *Clin. Pharmacol. Ther.* **73,** 264–271 (2003).
105. Streetman, D.S., Bleakley, J.F., Kim, J.S., Nafziger, A.N., Leeder, J.S. & Gaedigk, A. *et al.* Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, *N*-acetyltransferase-2, and xanthine oxidase with the Cooperstown cocktail. *Clin. Pharmacol. Ther.* **68,** 375–383 (2000).
106. Yeh, R.F., Gaver, V.E., Patterson, K.B., Rezk, N.L., Baxter-Meheux, F. & Blake, M.J. *et al.* Lopinavir/ritonavir induces the hepatic activity of cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP1A2 but inhibits the hepatic and intestinal activity of CYP3A as measured by a phenotyping drug cocktail in healthy volunteers. *J. Acquir. Immune Defic. Syndr.* **42,** 52–60 (2006).
107. Wang, Z., Gorski, J.C., Hamman, M.A., Huang, S.M., Lesko, L.J. & Hall, S.D. The effects of St John's wort (*Hypericum perforatum*) on human cytochrome P450 activity. *Clin. Pharmacol. Ther.* **70,** 317–326 (2001).
108. Henschel, L. & Hoffmann, A. Assessment of biotransformation capacity following oral administration of various model substances as cocktail). *Z. Gastroenterol.* **29,** 645–649 (1991).
109. Adedoyin, A., Frye, R.F., Mauro, K. & Branch, R.A. Chloroquine modulation of specific metabolizing enzymes activities: investigation with selective five drug cocktail. *Br. J. Clin. Pharmacol.* **46,** 215–219 (1998).
110. Adedoyin, A., Stiff, D.D., Smith, D.C., Romkes, M., Bahnson, R.C. & Day, R. *et al.* All-*trans*-retinoic acid modulation of drug-metabolizing enzyme activities: investigation with selective metabolic drug probes. *Cancer Chemother. Pharmacol.* **41,** 133–139 (1998).
111. Branch, R.A., Adedoyin, A., Frye, R.F., Wilson, J.W. & Romkes, M. *In vivo* modulation of CYP enzymes by quinidine and rifampin. *Clin. Pharmacol. Ther.* **68,** 401–411 (2000).
112. Frye, R.F., Schneider, V.M., Frye, C.S. & Feldman, A.M. Plasma levels of TNF-alpha and IL-6 are inversely related to cytochrome P450-dependent drug metabolism in patients with congestive heart failure. *J. Card. Fail.* **8,** 315–319 (2002).
113. Bebia, Z., Buch, S.C., Wilson, J.W., Frye, R.F., Romkes, M. & Cecchetti, A. *et al.* Bioequivalence revisited: influence of age and sex on CYP enzymes. *Clin. Pharmacol. Ther.* **76,** 618–627 (2004).
114. Zhang, X., Lalezari, J.P., Badley, A.D., Dorr, A., Kolis, S.J. & Kinchelow, T. *et al.* Assessment of drug–drug interaction potential of enfuvirtide in human immunodeficiency virus type 1-infected patients. *Clin. Pharmacol. Ther.* **75,** 558–568 (2004).