

## Can the CEIBA Cocktail Designed for Human Cytochrome P450 Enzymes be Used in the Rat for Drug Interaction Studies?

Paulo Magalhães<sup>a,b,c,d</sup>, Fernando de Andrés<sup>d,e</sup>, Amílcar Falcão<sup>a,b</sup>, Adrián LLerena<sup>d</sup>, Gilberto Alves<sup>b,c</sup>

<sup>a</sup> Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, Coimbra, Portugal. <sup>b</sup> Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal. <sup>c</sup> Health Sciences Research Centre, University of Beira Interior, Rua Marquês d'Ávila e Bolama, Covilhã, Portugal. <sup>d</sup> Clinical Research Centre, Extremadura University Hospital and Medical School, Badajoz, Spain. <sup>e</sup> Department of Analytical Chemistry and Food Technology, Faculty of Pharmacy, University of Castilla-La Mancha, Albacete, Spain.

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**ABSTRACT - Purpose** - The CEIBA cocktail consisting of caffeine (CAF), omeprazole (OZ), dextromethorphan (DM) and losartan (LOS) was previously proposed for the clinical phenotyping of five major human cytochrome P450 (CYP) isoenzymes. This work aimed to assess the usefulness of CEIBA cocktail to study non-clinical drug interactions in the rat. **Methods** - Wistar rats were divided into five groups to receive a single-oral dose of each probe drug (CAF, OZ, LOS, DM), individually or in combination as a cocktail. Plasma concentrations of the probe drugs and their metabolites [paraxanthine (1,7-X), 5-hydroxyomeprazole (5-OZ), losartan carboxylic acid (E-3174), dextrorphan (DX) and 3-methoxymorphinan (3-MM)] were determined by LC-MS/MS, and the corresponding pharmacokinetic parameters were estimated by non-compartmental analysis. The AUC<sub>0-t</sub> and C<sub>max</sub> drug/metabolite ratios (phenotypic metrics) were calculated for each probe drug and compared (probe alone versus cocktail). **Results** - The primary analysis of the pharmacokinetic data suggested the occurrence of pharmacokinetic-based drug interactions when the probe drugs were concurrently administered; such interactions were documented for CAF, 1,7-X, DX and E-3174. Nevertheless, except for the LOS/E-3174 probe drug-metabolite pair ( $p < 0.05$ ), there was little evidence that the probe drugs interacted metabolically as the metabolic ratios calculated were similar in both approaches. Moreover, no evidence was found for relevant pharmacodynamic interactions. **Conclusion** - CEIBA cocktail seems to be a useful tool to investigate drug interactions involving CYP isoenzymes in the rat, particularly at the level of CYP1A2, CYP2D1/2 and CYP2D2 isoforms using the CAF/1,7-X, OZ/5-OZ and DM/DX metabolic ratios, respectively.

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### INTRODUCTION

Nowadays it is consensual that a great part of interindividual variability in drug response is related to differences in the capacity of drug-metabolizing enzymes, especially the cytochrome P450 (CYP) isoenzymes (1–4). Although the differences in drug metabolism capacity is greatly determined by genetic polymorphisms, additional intrinsic and extrinsic factors are able to modulate CYPs activity, contributing thus to the actual drug metabolic phenotype (3–5).

As acknowledged by Vital-Lopez *et al.* (6), there is a gap between gene expression and metabolic phenotype, and the kinetic data may be useful to understand how these processes are connected. Indeed, CYPs genotype cannot accurately predict the actual metabolic phenotype for all individuals (7–9). Therefore, a biomarker

of the functional metabolic state (phenotype) is more valuable than a mere genetic biomarker. Actually, genotyping tests are often limited to distinguish drug response phenotypes because poor CYP genotype/metabolic phenotype correlations can also occur as a result of drug-drug interactions (1,5). Accordingly, extensive efforts have been directed towards development of phenotyping assays to evaluate the actual drug metabolizing activity of CYP isoenzymes. Such assays involve the administration of substrates (probes) which are selectively metabolized by a given CYP isoenzyme, and the substrate/ metabolite concentration ratio, or the metabolic

**Corresponding author:** Gilberto Alves, PharmD, Ph.D.; Faculty of Health Sciences, University of Beira Interior; CICS-UBI – Health Sciences Research Centre, University of Beira Interior Av. Infante D. Henrique; 6200-506 Covilhã, Portugal; E-mail address: gilberto@fcsaude.ubi.pt

ratio, is calculated. Numerous cocktails are reported to phenotype humans (4,10–24), e.g., Cooperstown 5+1 (18), Karolinska (19), Sanofi-Aventis (20), Inje (21), CIME (22), Basel (23), Geneva (24), and CEIBA cocktail (4,25).

The availability of an appropriate cocktail approach to simultaneously assess the activity of different CYP isoenzymes is advantageous not only in clinical setting but also in non-clinical arena, particularly to investigate drug interactions (2,26–30). Nevertheless, CYP-specific probe drug cocktails have been designed to man (2,4,10–24,26), and very few studies have tested their use in animal models (2,26,31). Moreover, a current matter of concern is the emergence in literature of several works conducted in the rat by direct application of probe drug cocktails specifically developed for phenotyping human CYP isoenzymes (32–38). However, humans differ from animals in isoform composition, expression and catalytic activities of drug-metabolising CYP isoenzymes (39), and specific works are required to avoid biased results and/or an erroneous interpretation of the metabolic data obtained.

Consequently, as the rat is certainly the animal model most frequently used (2,26), it is imperative to investigate the extrapolation of a phenotyping cocktail approach designed for human to rat before its widespread use, especially considering the pharmacokinetics behaviour of the probe drugs combined and the potential occurrence of drug-drug interactions (26,31,40). Such idea was already explored for three human cocktails (2,26,31) and it is fully justified for any other cocktail intended to be used in rat species, which constitutes the rational basis for performing the present study.

Recently, we proposed the CEIBA cocktail consisting of four probe drugs [caffeine (CAF), omeprazole (OZ), dextromethorphan (DM) and losartan (LOS)] for the simultaneous phenotyping of the five major human drug-metabolizing CYP isoenzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) in just one assay (3,4,25). Nonetheless, its potential application in the rat has never been studied. Therefore, bearing in mind the aforementioned issues, this work was aimed to evaluate the possibility of applying the CEIBA cocktail approach to study drug interactions in the rat.

## MATERIALS AND METHODS

### Chemicals and materials

DM hydrobromide, dextrophan (DX) tartrate, 3-methoxymorphinan (3-MM) hydrochloride, OZ, CAF, 1,7-dimethylxanthine (paraxanthine; 1,7-X), the internal standard (IS) levallorphan, and  $\beta$ -

glucuronidase were purchased from Sigma-Aldrich (MO, USA). LOS, losartan carboxylic acid (E-3174) and 5-hydroxyomeprazole (5-OZ) sodium salt were obtained from Santa Cruz Biotechnology (CA, USA). LC–MS-grade methanol, acetonitrile and formic acid, and analytical-grade sodium acetate, acetic acid used to prepare the  $\beta$ -glucuronidase treatment buffer, ammonium formate, and SOLA<sup>®</sup> cartridges for solid phase extraction (SPE) were purchased from Fisher Scientific (Geel, Belgium). Analytical-grade potassium dihydrogen phosphate and sodium hydroxide were used to prepare the SPE buffer and were purchased from Merck (Darmstadt, Germany). Deionized water was purified through a water purification system from Millipore (MA, USA). Other compounds used were: carboxymethyl cellulose sodium salt (Sigma-Aldrich, MO, USA), sodium chloride 0.9% solution for injection (Labesfal, Portugal), heparin sodium 5000 I.U./mL for injection (B. Braun Medical, Portugal), and pentobarbital (Eutasil<sup>®</sup> 200 mg/mL, Ceva Saúde Animal) used as anaesthetic drug. Introcan<sup>®</sup> Certo IV in dwelling cannula (22G; 0.9 x 2.5 mm) made of polyurethane from B. Braun Melsungen AG (Melsungen, Germany) and heparin-lithium tubes were also used.

### Animals

Adult male Wistar rats weighting approximately 240-340 g were obtained from local certified animal facilities. The rats were kept in an environmentally controlled room (temperature 20±2°C; relative humidity 55±5%; 12-h light/dark cycle) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy).

To perform a sequential blood sampling over time, a lateral tail vein of each rat was cannulated by an Introcan<sup>®</sup> Certo IV indwelling cannula (22G; 0.9 x 2.5 mm) under anaesthesia with pentobarbital (60 mg/kg, i.p. injection) at night on the day before the administration of the probe drugs. The rats were fasted overnight and 4 h post-dose administration with free access to water. Oral administrations were performed by gavage. All the animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

### Experimental design

Rats ( $n = 25$ ) were randomly divided into five groups. The animals of each group ( $n = 5$ ) were orally treated with a single-dose of the four probe drugs (CAF, OZ, LOS and DM) administered separately (groups 1-4) and in combination as a cocktail (group 5). The probe drugs were

appropriately suspended in a 0.5% carboxymethyl cellulose aqueous solution and administered to rats by gavage. The group 1 was treated with CAF (10 mg/kg), the group 2 with OZ (20 mg/kg), the group 3 with LOS (10 mg/kg), and the group 4 received DM (20 mg/kg). Lastly, all the four-probe drugs were given simultaneously to the group 5, at the same doses as when individually used. For each rat, multiple serial blood samples (approximately 0.3 mL) were collected through the cannula inserted in the tail vein into heparinized tubes before dosing, and at 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h after drug administration. Plasma was subsequently obtained by centrifugation at 4°C and 4000 rpm for 10 min and then stored at -80°C until analysis of the probe drug(s) and the corresponding metabolite(s).

### Drug analysis

Plasma concentration of the probe drugs and their metabolites were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) based on a previously developed methodology (4). Briefly, 100 µL of plasma spiked with levallorphan (IS, 10 ng) were mixed with 400 µL of potassium dihydrogenphosphate 0.1 M at pH 7.5 (extraction buffer). For the solid phase extraction step, the SPE SOLA<sup>®</sup> cartridge was previously conditioned with 500 µL of methanol and 500 µL of extraction buffer and then the sample mixture obtained after the incubation process was loaded. Afterwards, the extraction cartridge was washed with 500 µL of extraction buffer and then the analytes were eluted with 500 µL of methanol. The eluate was evaporated at 40°C under a nitrogen stream and the dried residue was reconstituted in 100 µL of the mobile phase. Aliquots of 10 µL of the reconstituted extract were injected in the LC-MS/MS chromatographic system. The LC-MS/MS chromatographic analysis of the probe drugs and their corresponding metabolites (CAF and 1,7-X, OZ and 5-OZ, LOS and E-3174, DM, DX and 3-MM) was performed on an Agilent 1200 HPLC system (Agilent, CA, USA) using a Poroshell SB-C18 column (75 mm × 3 mm internal diameter; 2.7 µm) at 30 °C and a mobile phase composed by 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 0.4 mL/min and the elution was carried out by means of a linear gradient, with acetonitrile content increasing from 17% to 50% between 0.1 and 4.5 min, maintaining at 50% from 4.5 to 8 min, and then decreased to 17% over 1 min, followed by column equilibration during 4 min. The total run time was, thus, 13 min per sample. Data acquisition was performed in single reaction monitoring mode with a mass

spectrometer (API 2000 triple quadrupole mass spectrometer from AB Sciex, MA, USA) equipped with an electrospray ionization interface (ESI) operating in the positive ion mode for all analytes except E-3174, whose data were acquired in negative mode from 8.5 to 13 min. The mass transitions were as follows: m/z 195.1→138.2 for CAF, m/z 181.2→123.8 for 1,7-X, m/z 346.1→197.9 for OZ, m/z 362.0→214.1 for 5-OZ, m/z 423.0→207.1 for LOS, m/z 434.9→157.0 for E-3174, m/z 272.1→171.1 for DM, m/z 258.1→157.0 for DX, m/z 258.1→128.2 for 3-MM and m/z 284.2→157.1 for levallorphan (IS).

### Pharmacokinetic analysis

The plasma concentration-time profiles of the probe drugs and their metabolites obtained from each rat were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin<sup>®</sup> version 5.2 (Pharsight Co, Mountain View, CA, USA). The maximum concentrations in plasma ( $C_{max}$ ) and the time to reach  $C_{max}$  ( $t_{max}$ ) were directly obtained from the experimental data. Other pharmacokinetic parameters estimated from the individual plasma concentration-time profiles were: the area under the concentration time-curve (AUC) from time zero to the time of the last quantifiable concentration ( $AUC_{0-t}$ ), calculated by the linear trapezoidal rule; AUC from time zero to infinite ( $AUC_{0-\infty}$ ), calculated from  $AUC_{0-t} + (C_{last}/k_{el})$ , where  $C_{last}$  is the last quantifiable concentration and  $k_{el}$  is the apparent elimination rate constant estimated by log-linear regression of the terminal segment of the concentration-time profile; and the apparent terminal elimination half-life ( $t_{1/2}$ ). When the concentration of the analytes was found to be lower than the limit of quantification (LOQ) of the assay, zero value was assigned for the pharmacokinetic calculations.

Additionally, the metabolic ratios (MRs) were determined according to the corresponding  $C_{max}$  and  $AUC_{0-t}$  pharmacokinetic parameters [i.e.,  $C_{max}^{(probe)}/C_{max}^{(metabolite)}$  and  $AUC_{probe}/AUC_{metabolite}$ ] for each probe drug and then compared between treatment conditions (probe alone and cocktail).

### STATISTICAL ANALYSIS

Data were reported as the mean ± standard deviation (percentage of coefficient of variation), except for  $t_{max}$ , expressed as the median value and the associated range (minimum and maximum values).

The Mann-Whitney non-parametric two-tailed test was employed for the analysis of pharmacokinetic differences among the groups of rats that were administered just one probe drug

individually and the group of those rats which received the cocktail of the probe drugs. This statistical approach was separately employed for each of the four-probe drugs included in the CEIBA cocktail, the corresponding metabolites and for the MRs calculated. Statistically significant differences were considered for  $p$  values lower than 0.05 ( $p < 0.05$ ).

## RESULTS

The mean plasma concentration-time profiles of CAF, 1,7-X, OZ, 5-OZ, LOS, E-3174, DM and DX after oral administration of the four probe drugs of the CEIBA cocktail alone or concurrently as a cocktail are shown in Figure 1. The respective main plasma pharmacokinetic parameters were determined and summarised in Table 1. Regarding the 3-MM, several values of concentrations below the LOQ were found in rat plasma, similarly to the phenomenon observed in humans (4,25); thus, the 3-MM was properly quantified just in few samples and, therefore, it was not possible to carry out a suitable pharmacokinetic analysis for this compound (Table 1).

To infer about the potential application of the CEIBA cocktail in the assessment of multiple CYP-mediated drug interactions in Wistar rats, it was first analysed whether pharmacokinetic interactions occur between the probe drugs CAF, OZ, LOS and DM when administered in combination as a cocktail. Overall, considering the mean plasma pharmacokinetic profiles depicted in Figure 1 for each probe drug and the corresponding metabolite when the probes were given individually and as part of the four-probe drug cocktail, it is observable the occurrence of pharmacokinetic-based drug interactions. The concomitant administration of the probe drugs as a cocktail resulted in the reduction of the systemic exposure (as assessed by  $AUC_{0-t}$ , Table 1), except for OZ and its metabolite (5-OZ). More specifically, the following mean decrease values of  $AUC_{0-t}$  were detected: 56.1% for CAF, 58.0% for 1,7-X, 68.8% for DM, 62.3% for DX, 21.5% for LOS and 61.1% for E-3174. On the contrary, an increase of mean  $AUC_{0-t}$  value of 137.7% was observed for OZ, while no significant difference in the systemic exposure for 5-OZ was found when OZ was given alone and as a part of the cocktail. Nevertheless, statistically significant differences were found for CAF, 1,7-X, DX and E-3174 ( $p < 0.05$ ). In agreement with the reduction of the extent of systemic exposure observed for CAF, 1,7-X, DX and E-3174, a statistically significant decrease of the peak systemic exposure (as assessed by  $C_{max}$ ,

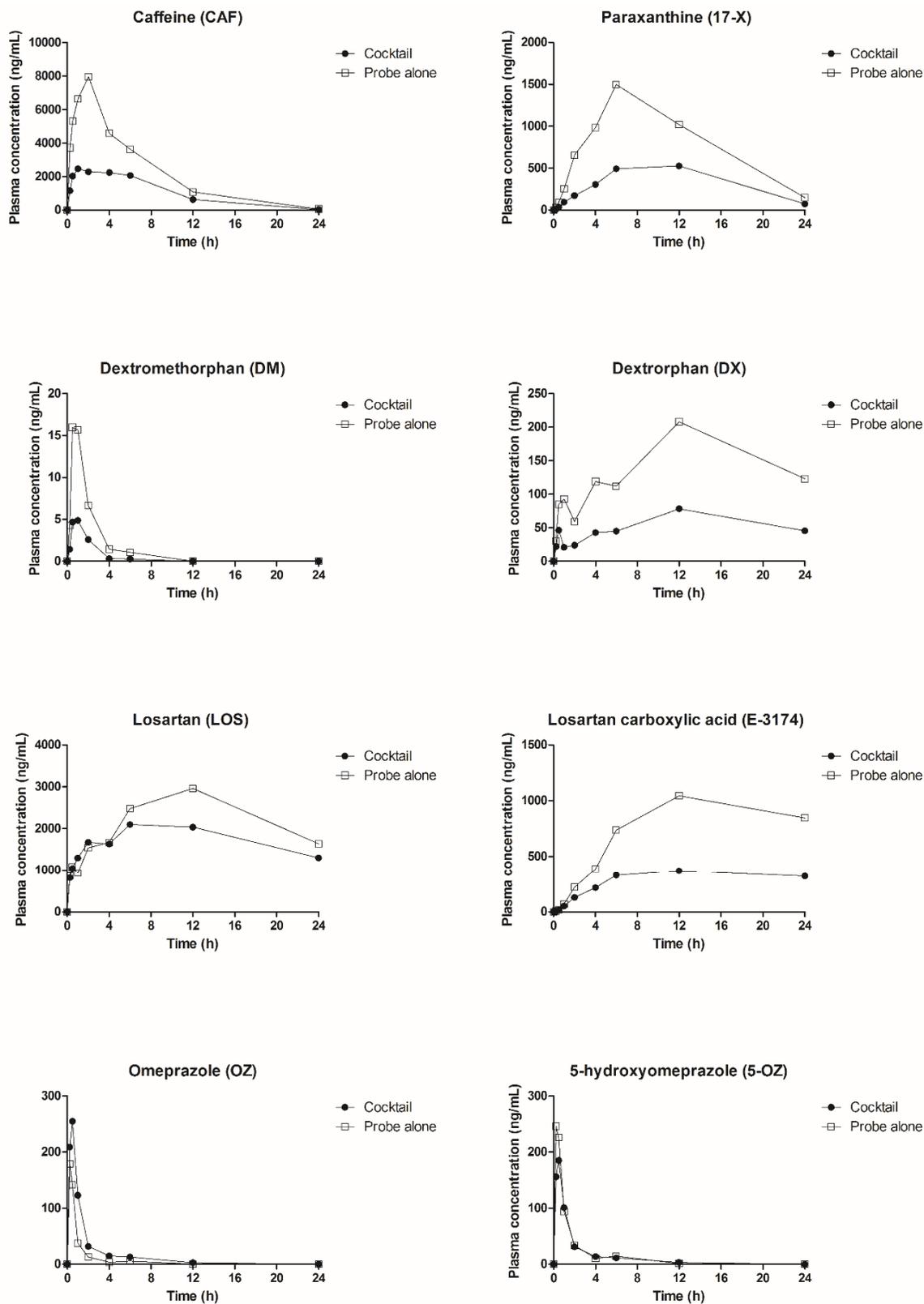
Table 1) was also observed for these compounds when the probe drugs were administered together as a cocktail.

Given the pharmacokinetic interactions documented when the four probe drugs (CAF, OZ, LOS and DM) were co-administered, a secondary analysis of the pharmacokinetic data was performed to evaluate whether metabolic interactions occurred. Thus, the influence of these pharmacokinetic-based drug interactions on the MRs was determined by comparison and statistical analysis of the calculated  $C_{max(\text{probe})}/C_{max(\text{metabolite})}$  and  $AUC_{\text{probe}}/AUC_{\text{metabolite}}$  ratios for each probe drug when administered alone and concurrently as a cocktail (Table 2). More specifically, a statistically significant increase for the LOS/E-3174 probe drug-metabolite pair was found when LOS was administered as a part of the cocktail (Table 2). This result seems to be mainly associated with the significant decrease of the systemic exposure of E-3174 after the administration of LOS as part of the CEIBA cocktail (Table 1).

Moreover, no signal of acute toxicity was detected after the administration of each probe drug alone or together as CEIBA cocktail in any of the rats studied. Therefore, no important pharmacodynamic interactions between any of the probe drugs are anticipated when co-administered to Wistar rats.

## DISCUSSION

The global aim of this work was to assess the usefulness of the CEIBA cocktail in the nonclinical evaluation of metabolic-based drug interactions in Wistar rats, as it was originally designed and utilised to measure the specific-metabolic activity of CYP isoenzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) in humans (41). Several important differences between human and rat CYP isoenzymes involved in the metabolism of the four probe drugs of the CEIBA cocktail were previously confirmed, as shown in Table 3 (26,42–46). In humans, each probe drug is mainly metabolized by a unique CYP isoenzyme, which is not significantly involved in the metabolism of the other probes, whereas this selectivity is not so strict in rats. More specifically, the probe drugs of the CEIBA cocktail share some CYP isoenzymes in the rat and, consequently, various isoenzymes can simultaneously be involved in the metabolism of different probe drugs of the cocktail, enhancing the risk of remarkable metabolic interactions (Table 3).



**Figure 1.** Mean plasma concentration-time profiles of each probe drug and the corresponding metabolites after the oral administration of the probe drugs alone or concomitantly as part of the CEIBA cocktail to Wistar rats.

**Table 1.** Mean pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of each probe administered alone and concomitantly as a cocktail [mean  $\pm$  standard deviation (CV, %) except for  $t_{max}$ , where values are the median (min, max);  $n = 5$  unless otherwise noted].

Probe drug	Treatment	$C_{max}$ (ng/mL)	$t_{max}$ (h)	$AUC_{0-t}$ (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	$t_{1/2}$ (h)
CAF	Probe alone	8284 $\pm$ 1897 (22.9%)	2.00 (0.50, 2.00)	53310 $\pm$ 13008 (24.4%)	53721 $\pm$ 12881 (24%)	2.89 $\pm$ 0.61 (21.1%)
	Cocktail	2621 $\pm$ 440 (16.8%) *	1.00 (0.50, 6.00)	23395 $\pm$ 2342 (10%) *	25356 $\pm$ 3795 (15%) *	3.83 $\pm$ 1.42 (37.1%)
1,7-X	Probe alone	1498 $\pm$ 903 (60.3 %)	6.00	19225 $\pm$ 9749 (50.7 %)	20400 $\pm$ 10219 (50.1%)	5.25 $\pm$ 1.17 (22.2%)
	Cocktail	546 $\pm$ 203 (37.2%) *	6.00 (6.00, 12.00)	8081 $\pm$ 2863 (35.4%) *	7757 $\pm$ 2896 (37.3%) *	7.59 $\pm$ 3.06 (40.3%)
DM	Probe alone	17.4 $\pm$ 20.5 (118%)	0.50 (0.25, 1.00)	32.3 $\pm$ 34.4 (106%)	56.0 $\pm$ 29.4 <sup>d</sup> (52.4%)	1.72 $\pm$ 0.79 <sup>d</sup> (45.9%)
	Cocktail	5.84 $\pm$ 3.78 <sup>c</sup> (64.7%)	0.50 <sup>c</sup>	10.1 $\pm$ 7.28 <sup>c</sup> (72%)	15.2 $\pm$ 9.9 <sup>c</sup> (64.8%)	1.63 $\pm$ 0.55 <sup>c</sup> (33.7%)
DX	Probe alone	215 $\pm$ 103 (48%)	12.00 (0.50, 12.00)	3489 $\pm$ 1490 (42.7%)	ND	33.9 <sup>a</sup>
	Cocktail	85.7 $\pm$ 33.3 (38.9%) *	12.00 (0.50, 12.00)	1314 $\pm$ 529 (40.2%) *	ND	ND
3-MM	Probe alone	34.2 $\pm$ 47.7 (139%)	0.50 (0.25, 6.00)	94.2 <sup>b</sup>	ND	1.33 <sup>a</sup>
	Cocktail	1.87 <sup>b</sup>	2.50 (1.00, 4.00) <sup>b</sup>	ND	ND	ND
LOS	Probe alone	3261 $\pm$ 1169 (35.8%)	6.00 (2.00, 12.00)	53321 $\pm$ 22437 (42.1%)	50981 $\pm$ 22326 <sup>c</sup> (43.8%)	8.74 $\pm$ 1.07 <sup>c</sup> (12.2%)
	Cocktail	2488 $\pm$ 847 (34%)	6.00 (6.00, 24.00)	41847 $\pm$ 16852 (40.3%)	68588 $\pm$ 55872 <sup>c</sup> (81.5%)	13.1 $\pm$ 10.3 <sup>c</sup> (78.3%)
E-3174	Probe alone	1145 $\pm$ 379 (33.1%)	12.00 (6.00, 24.00)	18578 $\pm$ 5505 (29.6%)	18027 <sup>a</sup>	15.0 <sup>a</sup>
	Cocktail	458 $\pm$ 200 (43.7%) *	12.00 (6.00, 24.00)	7233 $\pm$ 2763 (38.2%) *	18259 <sup>b</sup>	26.9 <sup>b</sup>
OZ	Probe alone	179 $\pm$ 116 (65.1%)	0.25	162 $\pm$ 101 (62.1%)	189 $\pm$ 102 <sup>d</sup> (54%)	2.11 $\pm$ 2.01 <sup>d</sup> (95.2%)
	Cocktail	267 $\pm$ 133 (50%)	0.5 (0.25, 0.50)	385 $\pm$ 207 (53.8%)	400 $\pm$ 203 (50.6%)	3.44 $\pm$ 1.78 (51.9%)
5-OZ	Probe alone	254 $\pm$ 203 (79.7%)	0.25 (0.25, 0.50)	317 $\pm$ 203 (64%)	297 $\pm$ 115 <sup>d</sup> (38.6%)	2.95 $\pm$ 2.40 <sup>d</sup> (81.55%)
	Cocktail	199 $\pm$ 64.9 (32.6)	0.50 (0.25, 0.50)	318 $\pm$ 127 (39.9%)	329 $\pm$ 125 (37.9%)	2.98 $\pm$ 1.01 (33.9%)

CAF, caffeine; 1,7-X, 1,7-dimethylxanthine (paraxanthine); DM, dextromethorphan; DX, dextrorphan; 3-MM, 3-methoxymorphinan; LOS, losartan; E-3174, losartan carboxylic acid; OZ, omeprazole; 5-OZ, 5-hydroxyomeprazole;  $C_{max}$ , maximum concentration in plasma;  $t_{max}$ , time to reach  $C_{max}$ ;  $AUC_{0-t}$ , area under the drug concentration time-curve from time zero to the time of the last quantifiable drug concentration;  $AUC_{0-\infty}$ , area under the drug concentration time-curve from time zero to infinite;  $t_{1/2}$ , half-life; \*  $p < 0.05$ , significantly different from the group where the probe was administered alone; <sup>a</sup> $n = 1$ ; <sup>b</sup> $n = 2$ ; <sup>c</sup> $n = 3$ ; <sup>d</sup> $n = 4$ . ND, not determined.

**Table 2.** Metabolic ratios [mean  $\pm$  standard deviation (CV%)] calculated for each probe drug-metabolite pair according to the main pharmacokinetic parameters ( $C_{\max}$  and  $AUC_{0-t}$ ) estimated when each probe was administered alone and concurrently as a cocktail.  $n = 5$  unless otherwise noted.

Probe drug/ metabolite	Treatment	$C_{\max}$ MR	$AUC_{0-t}$ MR
CAF/1,7-X	Probe alone	6.49 $\pm$ 2.517 (38.8%)	3.00 $\pm$ 0.638 (21.3%)
	Cocktail	5.24 $\pm$ 1.569 (30.0%)	3.27 $\pm$ 1.383 (42.3%)
DM/DX	Probe alone	0.11 $\pm$ 0.173 (157%)	0.01 $\pm$ 0.016 (143%)
	Cocktail	0.06 $\pm$ 0.035 (59.5%) <sup>a</sup>	0.01 $\pm$ 0.004 (61.4%) <sup>a</sup>
LOS/E-3174	Probe alone	2.87 $\pm$ 0.462 (16.1%)	2.85 $\pm$ 0.923 (32.4%)
	Cocktail	5.64 $\pm$ 1.074 (19.0%)*	5.78 $\pm$ 0.744 (12.9%)*
OZ/5-OZ	Probe alone	0.76 $\pm$ 0.096 (12.7%)	0.78 $\pm$ 0.774 (99.5%)
	Cocktail	1.27 $\pm$ 0.361 (28.4%)	1.16 $\pm$ 0.299 (25.8%)

CAF, caffeine; 1,7-X, 1,7-dimethylxanthine (paraxanthine); DM, dextromethorphan; DX, dextropropranolol; 3-MM, 3-methoxymorphinan; LOS, losartan; E-3174, losartan carboxylic acid; OZ, omeprazole; 5-OZ, 5-hydroxyomeprazole;  $C_{\max}$ , maximum concentration in plasma;  $AUC_{0-t}$ , area under the drug concentration time-curve from time zero to the time of the last quantifiable concentration. \* $p < 0.05$ , significantly different from the group in which the probe was administered alone; <sup>a</sup> $n = 4$ .

This suggests that potential enzyme competition or saturation phenomena in some of the metabolic pathways may occur in the rat. Hence, these findings raise the need to demonstrate the utility and validity of the CEIBA cocktail in Wistar rats. To the best of our knowledge, no drug-drug interactions involving the probe drugs of the CEIBA cocktail were described in rats. Thus, the present study shows, for the first time, that pharmacokinetic-based drug interactions affecting the CAF, 1,7-X, DX and E-3174 may occur in Wistar rats when the four probe drugs are administered as a cocktail at the selected doses. Regarding the nature of these pharmacokinetic interactions, the differences found in CAF and 1,7-X (probe alone *versus* cocktail) are probably related with drug interactions occurring at the level of gastrointestinal tract affecting the extent of absorption of CAF and, consequently, the extent of its metabolism to 1,7-X; on the contrary, the discrepancy in the results obtained for DX and E-3174 (probe alone *versus* cocktail) seems to be associated with metabolic drug interactions.

For CAF and 1,7-X, a similar decrease in the mean total systemic exposure was observed for both compounds (56.1% and 58.0%, respectively) after the administration of the CEIBA cocktail. Considering that 1,7-X directly depends on the rate and extent of the biotransformation of CAF, the variability observed in this study could be explained by either a global decrease of the absorption of CAF or an increase of the elimination of the drug and metabolite. However, if the reduction in the systemic exposure was mainly due to an increased efficiency of the elimination

process, it would be expected a significant decrease in the  $t_{1/2}$  values and, in contrast, an average increase occurred, although not statistically significant. Therefore, a lower absorption of CAF is likely the main process responsible for the results achieved, having a roughly proportional impact in the values of  $AUC_{0-t}$ ,  $AUC_{0-\infty}$  and  $C_{\max}$  of CAF and, subsequently, of its metabolite 1,7-X (Table 1).

For DX (DM metabolite) and E-3174 (LOS metabolite) a significant decrease in the systemic exposure was also observed when the rats received the probe drugs as a cocktail but, at this point it is noteworthy that no relevant differences were found in the pharmacokinetic profiles of the parent drugs (DM and LOS) (Table 1). Thus, the hypothesis of absorption-related drug interactions is not plausible.

In rat, LOS is mostly metabolized to E-3174 by CYP2C11, an isoenzyme secondarily involved in the biotransformation of CAF to 1,7-X. Thus, the concomitant administration of CAF and LOS can saturate the CYP2C11 metabolic capacity, leading that LOS was predominantly metabolized by a route other than LOS-E-3174. This may explain the decrease of the systemic exposure of E-3174 observed, without significant pharmacokinetic differences for the parent drug (LOS). In addition, the pharmacokinetic profiles of CAF and 1,7-X did not reflect any metabolic interaction, probably because the metabolism of CAF to 1,7-X is mainly mediated by CYP1A2 in rats, an isoenzyme that is unlikely to be subject to a high level of competition and/or saturation phenomena (Table 3).

**Table 3.** Comparison of the human and rat drug-metabolizing CYP isoenzymes for the probe drugs included in the CEIBA cocktail (26,42–46).

Human isoenzyme	Rat isoenzyme	Probe drug/metabolite
CYP1A2	CYP1A2; <i>CYP2C11</i>	CAF/1,7-X
CYP2C9	CYP2C11	LOS/E-3174
CYP2C19	CYP2D1/2; <i>CYP1A1/2</i> ; <i>CYP3A1/2</i>	OZ/5-OZ
CYP2D6	CYP2D2	DM/DX
CYP3A4	CYP3A1/2	DM/3-MM

CYP isoenzymes in italics represent secondary metabolism pathways. CAF, caffeine; 1,7-X, 1,7-dimethylxanthine; DM, dextromethorphan; DX, dextrophan; 3-MM, 3-methoxymorphinan; LOS, losartan; E-3174, losartan carboxylic acid; OZ, omeprazole; 5-OZ, 5-hydroxyomeprazole;

On the other hand, CYP2D2 is the main isoenzyme responsible for the metabolic conversion of DM to DX, although it is also involved in the biotransformation of OZ to 5-OZ. Therefore, the same hypothesis of metabolic competition/saturation may be eligible to explain the significant pharmacokinetic differences found for DX (probe alone *versus* cocktail), but not for DM, OZ and 5-OZ. Similarly, OZ can be metabolized to 5-OZ by other isoenzymes such as CYP1A2 and CYP3A1/2, and DM can be metabolized to other compounds in addition to DX, such as 3-MM (Table 3).

Given that some pharmacokinetic-based drug interactions were detected in Wistar rats, its potential influence on the MRs has been additionally analysed to investigate the usefulness of the CEIBA cocktail in the assessment of CYP-mediated metabolism. For this study,  $AUC_{0-t}$  and  $C_{max}$  were assumed as the main pharmacokinetic parameters that better reflect the drug/metabolite ratio profile for each probe drug and, therefore, they were considered as the best end-points to evaluate the magnitude of such probe drug interactions on the phenotypic metric.

On this matter, the results demonstrate that despite the occurrence of drug-drug interactions at the doses tested, it is not expected that MRs are substantially modified in rats, as the drug/metabolite ratio profiles assessed by  $AUC_{0-t}$  and  $C_{max}$  ratios are similar when the probe drugs were administered alone or concomitantly as a cocktail, except for LOS/E-3174 ( $p < 0.05$ , Table 2). Overall, this means that the CEIBA cocktail can be utilised to study metabolic-based-drug interactions in Wistar rats involving CYP1A2, CYP2D1/2 and CYP2D2, using the CAF/1,7-X, OZ/5-OZ and DM/DX ratios, respectively.

This study also confirms the interspecies differences between rat and human CYP

isoenzymes and highlights the need of interspecies comparisons at pharmacokinetics and on the metabolism of the probe drugs when used as a cocktail. Lastly, it is also important to remark that the current work should be viewed as part of the rational process of extrapolation and optimization of the CEIBA cocktail to rat.

## CONCLUSION

The CEIBA cocktail designed for humans may be used to evaluate metabolic-based drug interactions in the rat involving the CYP1A2, CYP2D1/2 and CYP2D2, by means of the CAF/1,7-X, OZ/5-OZ and DM/DX ratios, respectively.

Since very few CYP phenotyping cocktail approaches are described to the rat, the present study is of great value as it demonstrates the validity of the CEIBA cocktail to study *in vivo* drug interactions in this species. However, to extend the applicability of the CEIBA cocktail to a greater number of CYP isoenzymes some optimization is required, particularly the selection of more appropriate probe drugs for CYP3A1/2 and CYP2C11 isoenzymes.

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