BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **35**: 228–236 (2014)
Published online 4 February 2014 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/bdd.1888

Simultaneous and comprehensive *in vivo* analysis of cytochrome P450 activity by using a cocktail approach in rats

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ABSTRACT: A cocktail approach can detect the activities of multiple cytochrome P450 (CYP) isoforms following the administration of multiple CYP-specific substrates in a single experiment. This study aimed to develop a simultaneous and comprehensive *in vivo* analysis of CYP activity in rats. The rats received an oral administration of losartan (10 mg/kg) and omeprazole (40 mg/kg). Caffeine (1 mg/kg), dextromethorphan (10 mg/kg) and midazolam (10 mg/kg) were administered 15 min later. In the drug-interaction phase, the rats were treated orally with dexamethasone (80 mg/kg) 24 h before, or with ketoconazole (10 mg/kg), fluvoxamine (100 mg kg) or fluconazole (10 mg/kg) 1 h before the administration of cocktail drugs. The concentrations of the drugs and their metabolites were determined by LC/MS/MS. Plasma concentrations of five CYP substrates and their metabolites were simultaneously evaluated after the oral drug administration. Fluvoxamine and fluconazole significantly increased the C_{max} and *AUC* of caffeine, and the *AUC* of omeprazole and midazolam. Dexamethasone significantly increased C_{max} and *AUC* of losartan, while it decreased the C_{max} of midazolam. Ketoconazole showed no significant effect on the pharmacokinetic parameters of the tested drugs. In conclusion, a cocktail approach was developed for simultaneous and comprehensive analysis of the activities of multiple CYP isoforms in rats. In this approach, the effects of inhibitors and an inducer of various CYP isoforms were examined. Although further studies are necessary to predict the effects in humans, this approach may be expected to serve as a convenient method for detecting drug–drug interactions in rats. Copyright © 2014 John Wiley & Sons, Ltd.

Key words: cytochrome P450; cocktail approach; drug interaction; rat; pharmacokinetics

Introduction

Cytochrome P450 (CYP) enzymes are involved in the metabolism of drugs that are used clinically worldwide [1,2]. The CYPs that are most commonly described as contributors to drug clearance are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5. Approximately 50% of the drugs metabolized by CYPs are metabolized by CYP3A4 [3]; however, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 also play important roles in drug metabolism. The activities of these CYP isoforms are reported to show large inter- and intra-individual variation, particularly associated with age, gender and genetic polymorphism [4–6]. In addition, certain medications can induce or inhibit the activity of drug-metabolizing enzymes, resulting in drug–drug interactions. Drug interactions that alter CYP activity can result in variations in substrate pharmacokinetics, which in turn may change the pharmacodynamics of these drugs. Thus, determining the pharmacokinetics and pharmacodynamics of CYP substrates is important for predicting potential drug interactions and for dose optimization.

There are multiple approaches for studying drug–drug interactions that focus on CYP activity,

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Received 6 August 2013
Revised 16 December 2013
Accepted 23 December 2013

including *in vitro* studies using liver microsomes, *in vivo* pharmacokinetic studies using animals such as rats, and clinical studies in healthy subjects or patients that investigate the pharmacokinetics of CYP isoform substrates [7–11]. In addition, recent studies have used a modeling and simulation technique that predicts the results of drug–drug interactions in humans on the basis of findings of *in vitro* studies [12,13]. Among these various approaches, evaluation by clinical studies tends to have the greatest impact, in that it is not affected by the problems of species difference and the results can thus be extrapolated directly to humans. However, *in vivo* pharmacokinetic studies using animals remain the most common approach for evaluating drug interactions, largely because of the inherent limitations of clinical studies. Furthermore, most *in vivo* drug interaction studies in either humans or rats usually examine the activity of a single CYP isoform by using a specific CYP probe. In this approach, multiple drug administrations are required, thus necessitating pharmacokinetic studies for determining the activity of each CYP isoform. On the other hand, a cocktail approach can help to measure the activity of multiple CYP isoforms after the administration of multiple CYP-specific substrates in a single experiment [14–17]. Thus, the cocktail approach is a powerful tool for the simultaneous and comprehensive evaluation of drug–drug interactions involving multiple CYP isoforms. However, few studies have reported the results of a comprehensive analysis of CYP activity in rats, although several cocktail studies have been reported in humans, such as the Cooperstown 5+1 cocktail [15], the Karolinska cocktail [16] and the Inje cocktail [17].

This study aimed to develop a simultaneous and comprehensive *in vivo* analysis of CYP activity in rats. The Inje cocktail of substrate drugs was administered to rats to assess the effects of various CYP inducers and inhibitors on the activity of multiple CYP isoforms.

Materials and Methods

Chemicals

Caffeine, losartan, omeprazole, dextromethorphan, midazolam, dextrorphan, 1′-hydroxymidazolam and nitrazepam were purchased from Sigma-Aldrich (St Louis, MO). Paraxanthine, E3174 and 5-hydroxyomeprazole were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). All other chemicals were obtained from commercial sources.

Animals

Male Sprague-Dawley rats aged 7–9 weeks (Japan SLC Inc., Shizuoka, Japan) were housed under a 12 h light/dark cycle in a room with controlled temperature (24 ± 2 °C) and humidity (55% ± 5%). Food and water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Shizuoka.

Experimental design

Before administration of drugs, the femoral artery was cannulated with an SP-31 polyethylene tube (Natsume Seisakusho Co. Ltd, Tokyo, Japan) under anesthesia with pentobarbital (40 mg/kg, i.p.). The

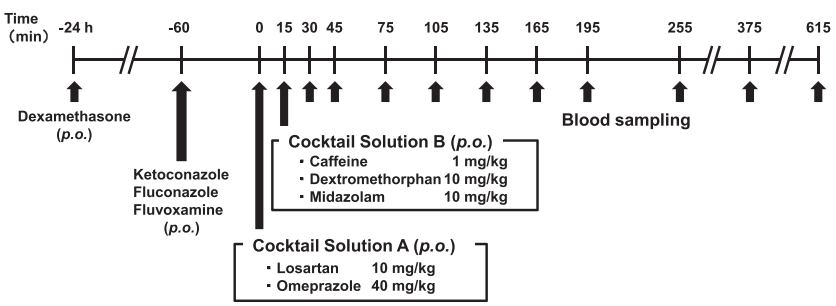

Figure 1. The experimental design of the cocktail approach tested in rats

DOI: 10.1002/bdd

experimental design of the cocktail approach is shown in Figure 1. In the control phase, the rats were fasted overnight, and they received an oral administration of losartan (10 mg/kg) and omeprazole (40 mg/kg) suspended in 0.5% carboxymethylcellulose (cocktail solution A). Then, caffeine (1 mg/kg), dextromethorphan (10 mg/kg) and midazolam (10 mg/kg) suspended in 1% carboxymethylcellulose (cocktail solution B) were administered orally 15 min later. Blood samples (0.15 ml) were collected from the arterial catheter at 30, 45, 75, 105, 135, 165, 195, 255, 375 and 615 min after the administration of cocktail solution A. Plasma samples were isolated from whole blood by centrifugation, and stored at −70 °C until the drug concentrations were determined.

In the drug-interaction phase, the rats were orally treated with dexamethasone (80 mg/kg) 24 h before or ketoconazole (10 mg/kg), fluvoxamine (100 mg/kg) or fluconazole (10 mg/kg) 1 h before the administration of cocktail solution A (Figure 1). Dexamethasone, ketoconazole and fluvoxamine were suspended in 1% carboxymethylcellulose and fluconazole was dissolved in water as a solvent. The control rats received the vehicle only. Drug administrations and blood sampling were performed in the same manner as the control phase.

Determination of drugs and their metabolites

Plasma concentrations of cocktail drugs (caffeine, losartan, omeprazole, dextromethorphan and midazolam) and their metabolites (paraxanthine, E-3174, 5-hydroxyomeprazole, dextrorphan and 1′-hydroxymidazolam, respectively) were determined using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), following solid phase extraction using an Ostro 96-well plate (Waters, Milford, MA). The plasma samples (50 µl) were spiked with an internal standard (50 µl of 100 ng/ml nitrazepam), and mixed with 150 µl of acetonitrile. The samples were then added to the 96-well plate, and 10 µl of the residue was injected into the chromatographic system for analysis.

Liquid chromatography (LC) was performed on an Agilent 1100 LC system (Agilent Technologies, Wilmington, DE). The isolation of the five CYP probe drugs and their metabolites was performed on a CAPCELL PAK C18 MGIII column (150 mm × 2 mm; i.d., 5 µm) (Shiseido Co. Ltd, Tokyo, Japan). The column temperature was maintained at 40 °C. The mobile phase A was 10 mM ammonium acetate, and mobile phase B was acetonitrile. The total flow rate of the mobile phases was 0.2 ml/min, and a gradient of 22.5–90% B was applied over a period of 6 min for sample elution. The total LC run time was 12 min. A mass spectrometer (API 3000; Applied Biosystems, Foster City, CA) equipped with an electrospray ionization interface operating in the positive ion mode was used to characterize the CYP probe drugs and their metabolites. The mass transitions were as follows: m/z 195 → 138 for caffeine, m/z 181 → 124 for paraxanthine, m/z 423 → 207 for losartan, m/z 437 → 235 for E-3174, m/z 346 → 198 for omeprazole, m/z 362 → 214 for 5-hydroxyomeprazole, m/z 272 → 171 for dextromethorphan, m/z 258 → 199 for dextrorphan, m/z 326 → 291 for midazolam and m/z 342 → 324 for 1′-hydroxymidazolam. The lower limit of detection was 2 ng/ml for dextromethorphan, dextrorphan, midazolam and 1′-hydroxymidazolam, and 10 ng/ml for caffeine, paraxanthine, losartan, E3174, omeprazole and 5-hydroxyomeprazole. Precision indicated as the coefficient validation was less than 15.3% for all compounds. The accuracy ranged from 86.4% to 113.7% for all compounds in the determination.

Data analysis

The pharmacokinetic parameters of the drugs were estimated by non-compartmental analysis. The maximum concentrations of drugs in plasma (C_{max}) were used as the observed data. The area under the plasma concentrations of drugs versus time curve (*AUC*) was obtained by the trapezoidal rule for the last determined points for each drug ($AUC_{0\text{-}360}$ for caffeine, dextromethorphan and midazolam; $AUC_{0\text{-}375}$ for omeprazole; and $AUC_{0\text{-}615}$ for losartan). The *AUC* of metabolites was calculated for the same time points as their parent drugs. The *AUC* ratio was then defined as $AUC_{metabolite}/AUC_{drug}$.

All data are presented as mean ± SD. GraphPad Prism (version 5.0; GraphPad Software, San Diego, California, USA) was used for all statistical analyses. Statistical analysis of the data

DOI: 10.1002/bdd

was performed using Dunnett's test for multiple comparisons. Statistical significance was accepted at $p < 0.05$.

Results

Plasma concentration time profiles of five different CYP substrates and their metabolites

The plasma concentrations of five CYP substrates and their metabolites were simultaneously evaluated after oral drug administration in rats. As shown in Figure 2, the concentrations of caffeine, omeprazole, dextromethorphan and midazolam in plasma were determined at the indicated time points until 360 min after administration. In contrast, the plasma losartan concentration remained above the limit of quantification up to 600 min after administration. Similarly, the metabolites of five different CYP substrates were determined at the same sampling time points as each corresponding drug. The pharmacokinetic parameters of all CYP substrates and metabolites were also calculated.

Effects of ketoconazole, fluconazole, fluvoxamine and dexamethasone on plasma concentrations of five different CYP substrates

Figure 3 shows the time course of plasma concentrations of five different CYP substrates from 30 to 600 min after oral administration in rats pretreated with ketoconazole, fluconazole, fluvoxamine, dexamethasone and vehicle (control). The pharmacokinetic parameters of drugs and metabolites were calculated from these plasma concentration–time profiles (Table 1).

Fluvoxamine significantly increased both the C_{max} and $AUC_{0\text{-}360}$ of caffeine by 1.7- and 3.5-fold, respectively (Table 1). However, the *AUC* ratio of caffeine to paraxanthine in fluvoxamine-treated rats was not significantly different from that in the control rats. There were no significant differences in the pharmacokinetic parameters of caffeine between control rats and rats treated with ketoconazole, fluconazole or dexamethasone.

After pretreatment with dexamethasone, the C_{max} and $AUC_{0\text{-}615}$ of losartan were significantly increased by 2.0- and 1.9-fold, respectively, and

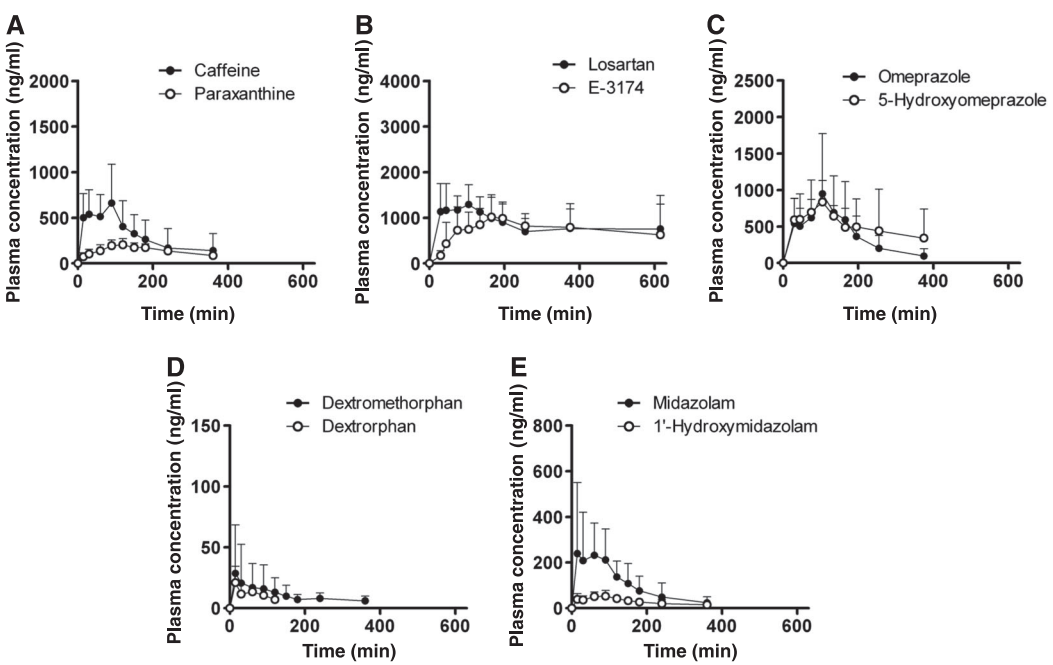

Figure 2. Plasma concentrations of caffeine/paraxanthine (A), losartan/E-3174 (B), omeprazole/5-hydroxyomeprazole (C), dextromethorphan/dextrorphan (D) and midazolam/1′-hydroxymidazolam (E) after oral administration in rats. Each point represents the mean ± SD of samples from seven rats

DOI: 10.1002/bdd

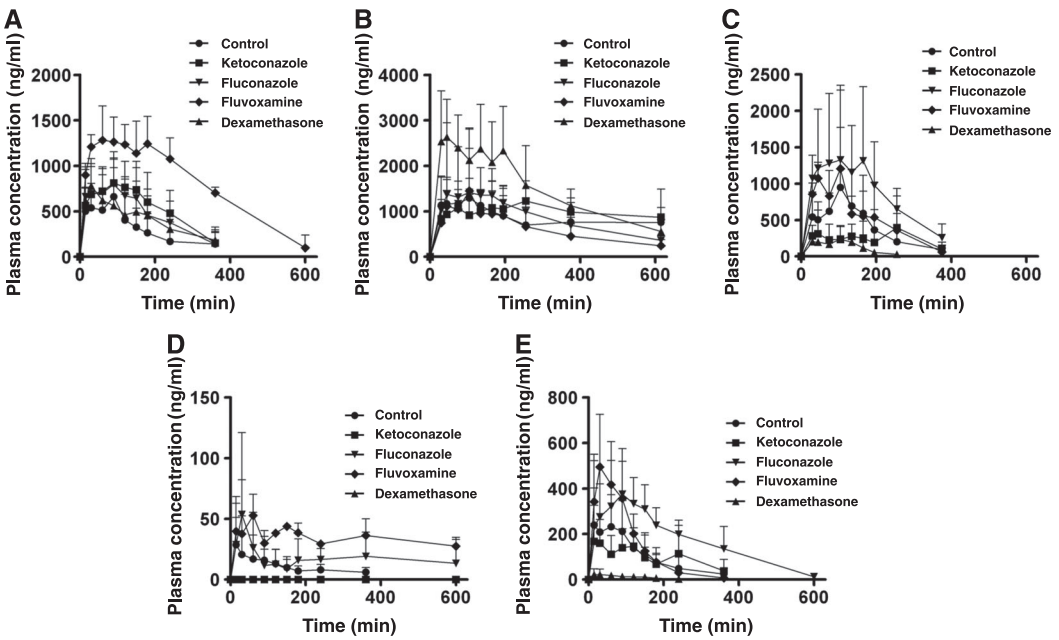

Figure 3. Plasma concentrations of caffeine (A), losartan (B), omeprazole (C), dextromethorphan (D) and midazolam (E) after oral administration in rats pretreated with ketoconazole, fluconazole, fluvoxamine, dexamethasone or vehicle (control). Each point represents the mean ± SD of samples from 4–7 rats

the *AUC* ratio of losartan to E-3174 was markedly decreased (by 81%). In addition, the *AUC* ratio of losartan was significantly decreased (by 56%) upon pretreatment with fluvoxamine. There were no significant differences in the pharmacokinetic parameters of losartan between control rats and rats treated with ketoconazole or fluconazole.

The $AUC_{0\text{-}375}$ of omeprazole was increased by 2.2-fold after pretreatment with fluconazole, while the C_{max} and *AUC* ratio of omeprazole to 5-hydroxyomeprazole were not significantly changed by fluconazole. The pharmacokinetic parameters of omeprazole were not significantly altered after treatment with ketoconazole, fluvoxamine or dexamethasone.

There were no significant differences in the pharmacokinetic parameters of dextromethorphan between control rats and rats treated with fluconazole or fluvoxamine. Plasma concentrations of dextromethorphan were under the limit of quantification 30 min after administration, and its pharmacokinetic parameters could not be determined in the rats treated with ketoconazole or dexamethasone.

The $AUC_{0\text{-}360}$ of midazolam was significantly increased (by 133%) after the administration of fluconazole. The C_{max} and *AUC* ratio of midazolam to 1′-hydroxymidazolam showed a tendency to increase and decrease, respectively. The C_{max} and $AUC_{0\text{-}360}$ were decreased by 92% after pretreatment with dexamethasone, and the *AUC* ratio was significantly increased by 3.4-fold. Ketoconazole and fluvoxamine showed no significant effect on the pharmacokinetic parameters of midazolam.

Discussion

The aim of this study was to develop a cocktail approach in rats to detect drug–drug interactions associated with five different CYP isoforms. To date, few studies have applied a cocktail approach to animal experiments. In the present study, the concentrations of the substrates of various CYP isoforms (i.e. caffeine, losartan, omeprazole, dextromethorphan and midazolam) and their major metabolites were measured simultaneously after oral administration of a cocktail containing five

DOI: 10.1002/bdd

Table 1. Maximum plasma concentration (C_{max}) and *AUC* of caffeine, losartan, omeprazole, dextromethorphan and midazolam after oral administration of five different CYP substrates in rats treated with vehicle (control), ketoconazole, fluconazole, fluvoxamine or dexamethasone

Treatment	C_{max} (ng/ml)	*AUC* (min · μg/ml)	$AUC_{metabolite}/AUC_{drug}$
Caffeine (CYP1A)		(0–360 min)	
Control	851 ± 299	110.4 ± 61.9	0.810 ± 0.625
Ketoconazole	845 ± 313	192.6 ± 76.3	0.242 ± 0.062
Fluconazole	986 ± 291	173.2 ± 63.5	0.356 ± 0.114
Fluvoxamine	1451 ± 215*	384.8 ± 62.4*	0.059 ± 0.030
Dexamethasone	830 ± 180	151.7 ± 84.6	0.540 ± 0.841
Losartan (CYP2C)		(0–615 min)	
Control	1599 ± 527	503.1 ± 187.9	0.996 ± 0.374
Ketoconazole	1363 ± 362	596.6 ± 182.3	0.659 ± 0.254
Fluconazole	1850 ± 1366	528.7 ± 216.2	1.239 ± 0.311
Fluvoxamine	1560 ± 1299	378.5 ± 232.6	0.440 ± 0.103*
Dexamethasone	3182 ± 711*	909.7 ± 195.5*	0.185 ± 0.087*
Omeprazole (CYP2C)		(0–375 min)	
Control	1028 ± 712	145.2 ± 71.2	1.220 ± 0.655
Ketoconazole	600 ± 385	93.1 ± 57.0	1.221 ± 0.302
Fluconazole	1840 ± 940	322.4 ± 143.0*	0.857 ± 0.238
Fluvoxamine	1482 ± 962	199.0 ± 92.1	1.061 ± 0.453
Dexamethasone	264 ± 115	34.1 ± 12.6	1.544 ± 0.658
Dextromethorphan (CYP2D)		(0–360 min)	
Control	30 ± 39	3.2 ± 3.8	0.447 ± 0.281
Ketoconazole	n.d.	n.d.	n.d.
Fluconazole	62 ± 60	6.3 ± 4.8	0.596 ± 0.270
Fluvoxamine	46 ± 43	8.9 ± 7.7	0.682 ± 0.235
Dexamethasone	n.d.	n.d.	n.d.
Midazolam (CYP3A)		(0–360 min)	
Control	336 ± 267	37.2 ± 23.8	0.338 ± 0.135
Ketoconazole	246 ± 138	36.7 ± 22.7	0.367 ± 0.295
Fluconazole	450 ± 156	86.6 ± 27.5*	0.291 ± 0.143
Fluvoxamine	594 ± 65	55.9 ± 13.9	0.555 ± 0.163
Dexamethasone	28 ± 20*	3.0 ± 1.7	1.479 ± 0.464*

Values are mean ± SD (n = 4–7).
*Significant difference ($p < 0.05$) between control and ketoconazole, fluconazole, fluvoxamine or dexamethasone. n.d., not detected.

different CYP substrates. Thus, by using this approach, it was shown that it is possible to use a cocktail approach in rats to study drug metabolism and interaction *in vivo*, a method which has been rarely described.

The activity of CYP1A was examined using caffeine and paraxanthine. In this study, oral administration of fluvoxamine significantly increased both the C_{max} and $AUC_{0\text{-}360}$ of caffeine by 2-fold and 3.5-fold, respectively. Culm-Merdek *et al*. reported that fluvoxamine markedly reduced the apparent oral clearance of caffeine and prolonged its elimination in a clinical study [18]. In addition, Kot *et al*. suggested caffeine as a marker substrate for CYP1A in both rats and humans [19]. Thus, the results of our present study suggest that the activity of CYP1A could be detected using plasma caffeine concentration in our cocktail approach, and that the effects of inhibitors, such as fluvoxamine, can be analysed.

Midazolam is a well-known substrate of CYP3A and is used as a specific CYP3A probe in drug interaction studies in both rats and humans. Fluconazole increased $AUC_{0\text{-}360}$ of midazolam by 2-fold, and the *AUC* ratio of midazolam to 1′-hydroxymidazolam decreased after drug administration. In contrast, dexamethasone reduced the C_{max} and $AUC_{0\text{-}360}$ of midazolam, while reducing the *AUC* ratio. These results are consistent with those of previous studies of specific CYP isoforms in both rats and humans [10,20,21]. These results suggest that the pharmacokinetics of midazolam could reflect the activity of CYP3A, and that drug interactions can be assessed by the

DOI: 10.1002/bdd

cocktail approach as well as the specific CYP isoform approach. On the other hand, in this study, no changes were observed in pharmacokinetic parameters following the pre-administration of ketoconazole, which is known as a potent inhibitor of CYP3A in humans. However, the effect of ketoconazole on CYP3A in rats is currently controversial. Ketoconazole has been reported to increase the C_{max} and *AUC* of both nevirapine and midazolam in rats [20,21]. On the other hand, some studies have reported that ketoconazole inhibition of CYP3A is weaker in rats than in humans [22]. It is likely that doses of ketoconazole higher than those used in this study may inhibit CYP3A because Kishimoto *et al*. reported that its inhibitory effect increased from 5 mg/ml to 50 mg/ml in a dose-dependent manner [20].

CYP2C11 is the predominant isoform in male rat liver, although the CYP2C family includes several isoforms, such as CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP2C22 and CYP2C23 in rats [23,24]. Thus, the activity of CYP2C was assessed by using both losartan and omeprazole, which are selective probes of CYP2C9 and CYP2C19, respectively, in humans. The *AUC* ratio of losartan was decreased following pretreatment with fluvoxamine, which is a potent inhibitor of CYP1A2 and CYP2D6, as well as a moderate inhibitor of CYP2C19, suggesting that fluvoxamine may inhibit the activity of CYP2C in rats. Interestingly, the C_{max} and $AUC_{0\text{-}615}$ of losartan markedly increased following pretreatment with dexamethasone. To date, the inhibitory effect of dexamethasone on CYP isoforms has not been reported, although there are several reports showing its effect on CYP induction. Although the mechanism of the increased plasma losartan concentration is unclear, it may be due partially to inhibition by residual plasma dexamethasone after the relatively high pretreatment dose (80 mg/kg).

The $AUC_{0\text{-}375}$ of omeprazole was significantly increased and the *AUC* ratio of omeprazole to 5-hydroxyomeprazole tended to decrease after treatment with fluconazole. An *in vitro* study performed using human liver microsomes showed that fluconazole inhibits several CYP isoforms including CYP2C9, CYP2C19 and CYP3A4 [25]. Thus, the increased $AUC_{0\text{-}375}$ of omeprazole may result from the inhibition of CYP2C following the administration of fluconazole. However, dexamethasone decreased the $AUC_{0\text{-}375}$ of omeprazole; this effect was similar to its effect on the *AUC* of midazolam, which is a CYP3A substrate, although the difference was not statistically significant. In addition to CYP2C19, omeprazole is partly metabolized by CYP3A4 in humans [26]. In this context, it cannot be ruled out that CYP3A in addition to CYP2C may be involved in the metabolic pathway of omeprazole.

Dextromethorphan and its major metabolite, dextrorphan, were used in this study as probe drugs to evaluate the activity of the CYP2D isoform, which was reported previously to have a high sequence identity between rat and human subtypes [27]. In the present study, no significant differences were observed in the pharmacokinetic parameters of dextromethorphan. Because the plasma concentrations of dextromethorphan and dextrorphan remained at low levels compared with those of the other drugs, and these drugs are excreted into the urine after glucuronidation, evaluation of CYP activity using dextromethorphan is sometimes performed using urine samples. Thus, it may be necessary to use urine samples to evaluate CYP2D activity with dextromethorphan in rats.

Conclusion

A simultaneous and comprehensive analysis of the activity of multiple CYP isoforms in rats was developed using a cocktail approach. In addition, the effects of inhibitors and an inducer of different CYP isoforms, ketoconazole, fluconazole, fluvoxamine and dexamethasone were assessed. Further studies using this approach in rats are necessary to determine the effects of increases in the various types of CYP inhibitors and inducers in order to predict effects in humans because of species differences in CYP. However, this approach is expected to serve as a convenient method for detecting potential drug–drug interactions in rats.

Acknowledgements

This work is supported by Japan Research Foundation for Clinical Pharmacology. The authors

DOI: 10.1002/bdd

are grateful to Mr Motoyasu Miura, Mr Masaki Yamamura, Ms Yukie Fukai and Ms Natuki Sakurada for their excellent technical assistance.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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DOI: 10.1002/bdd