Development of the "Inje Cocktail" for High-throughput Evaluation of Five Human Cytochrome P450 Isoforms *in vivo*

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To develop and validate an *in vivo* cocktail method for high-throughput phenotyping of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A, 12 healthy subjects received five probe drugs alone or simultaneously. The *in vivo* phenotyping index of CYP2C9, the ratio of 8 h urine concentration of losartan to its metabolite after a single administration of losartan, was not significantly different from that obtained using the five-drug cocktail. Similarly, the ratios of [omeprazole]/[5-hydroxyomeprazole] (CYP2C19) and [paraxanthine]/[caffeine] (CYP1A2) in 4 h plasma samples and the log ratio of [dextromethorphan]/[dextrorphan] (CYP2D6) in 8 h urine samples and the 4 h plasma concentrations of midazolam (CYP3A) after single administration or well-established three-drug cocktail of caffeine, omeprazole, and dextromethorphan were not significantly different from those after the new five-drug cocktail. In conclusion, the new five-drug cocktail regimen, named the "Inje cocktail," can be used as a tool to phenotype *in vivo* enzyme activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A with only 4 h blood sampling and 8 h urine collection following simultaneous administration of the five probe drugs.

The cytochrome P450 (CYP) system is the major drug-metabolizing enzyme system in humans[1] and is involved in the metabolism of numerous endogenous compounds and xenobiotics.[2] The metabolizing activities of the CYP isoforms show wide interindividual variations; these variations originate from genetic variants, such as genetic polymorphism, environmental factors, dietary components, medicines, and endogenous mediators, such as hormones and cytokines.[3–6] The differences in metabolizing activities of the CYP isoforms result in interindividual differences in plasma drug concentrations and may cause serious side effects or no effect. Thus, the evaluation of CYP enzyme activities in human subjects is of great importance.

Two common approaches are used to assess *in vivo* CYP enzyme activity. One is a selective phenotyping method involving administration of one CYP-specific probe, and the other is a cocktail phenotyping method that concurrently detects the activity of multiple CYP enzymes following simultaneous administrations of multiple CYP-specific probes. The biggest advantage of the cocktail approach is that it enables real-time assessment of the activity of various drug-metabolizing enzymes with a single experiment.[7–13] However, it also has certain limitations, such as mutual interactions between probe drugs, side effects of probe drugs, and analytical complexities.[13] For example, interactions between caffeine (CYP1A2) and chlorzoxazone (CYP2E1)[14] and between dextromethorphan (CYP2D6) and chloroguanide (CYP2C19)[15] have been reported. Thus, pharmacokinetic and pharmacodynamic interactions between probe drugs should be considered and evaluated in developing cocktail combinations.[16] Moreover, the selection of the proper phenotyping index for each enzyme activity and the establishment of high-throughput analytical methods for multiple probe drugs are important.

Many cocktail methods have been developed since the "Pittsburgh cocktail".[7] Currently, well-known cocktail approaches include the "Cooperstown cocktail"[9] and the "Karolinska cocktail",[11] with each having both advantages and disadvantages. Probe drugs in the Cooperstown cocktail, such as caffeine (CYP1A2), omeprazole (CYP2C19), dextromethorphan (CYP2D6), and intravenous midazolam (CYP3A), have no interactions with each other.[9] However, the enzyme activity of CYP3A in the gastrointestinal tract cannot be evaluated with the Cooperstown cocktail because

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of the intravenous administration of midazolam (probe for CYP3A). Moreover, the cocktail does not contain a probe drug for CYP2C9, which accounts for approximately 18% of the CYP protein in humans.[17] This limitation was ameliorated by using warfarin for CYP2C9 in the "Cooperstown 5 + 1 cocktail."[12] The Karolinska cocktail[11] used caffeine (CYP1A2), losartan (CYP2C9), omeprazole (CYP2C19), debrisoquin (CYP2D6), and quinine (CYP3A) as probe drugs. However, a significant increase of metabolic ratio of debrisoquin was observed when these probe drugs were administered simultaneously, thus they have decided to separate the oral intake of debrisoquin from other probe drugs. It also has some limitations to be used in Korea because debrisoquin and quinine are not popular drugs and are not available.

Thus, we sought to develop a new five-drug cocktail regimen overcoming these limitations. We added two probe drugs (losartan for CYP2C9 and oral midazolam for CYP3A) to the three-drug cocktail regimen of caffeine (CYP1A2), omeprazole (CYP2C19), and dextromethorphan (CYP2D6). These drugs are all commercially available, possess low adverse effect profiles, and are specific for the CYP enzymes.

RESULTS

Twelve subjects participated in this study and successfully finished all study schedules. No clinically significant adverse effects were observed during single drug or drug cocktail administration (that is, none of the volunteers had a fall in blood pressure or change in pulse rate during drug administration and no sedative effect occurred after oral administration of midazolam).

Genotyping results for *CYP2C9* and *CYP2D6* showed no *CYP2C9*2/*2*, **2/*3*, or **3/*3*, and no *CYP2D6*5/*5*, indicating that the genetically defined poor metabolizers for CYP2C9 and CYP2D6 were not detected in the participants. However, one *CYP2C19*2/*3* was found in genotyping for *CYP2C19*. The subject with *CYP2C19*2/*3* was correctly confirmed as a poor metabolizer of omeprazole. Thus, we excluded this subject from the statistical analysis of CYP2C19 phenotyping.

Table 1 Geometric mean ratios and its 90% CI for the plasma AUC of probe drugs and their metabolites and the 8 h urinary metabolic ratio of dextromethorphan (*n*=12)

Enzyme	Parameters	Mean ratio	90% CI (%)
CYP3A	AUC_{inf} of midazolam	1.05	92–118
CYP2C9	AUC_{last} of losartan	0.93	85–101
	AUC_{last} of E-3174	0.99	93 –105
CYP1A2	AUC_{last} of caffeine	0.96	89–104
	AUC_{last} of paraxanthine	0.92	82–103
CYP2C19	AUC_{last} of omeprazole (n=11)	1.05	88–124
	AUC_{last} of 5-hydroxyomeprazole (*n*=11)	1.01	87–118
CYP2D6	$Log([DMP]/[DP])_{8h}$	1.06	98–113

AUC, area under the plasma–time curve; CI, confidence interval; DP, dextrorphan; DMP, dextromethorphan.

Among the five probe drugs, dextromethorphan, losartan, midazolam, and their metabolites in biological samples were analyzed separately. Caffeine, omeprazole, and their metabolites were analyzed simultaneously. During the drug analysis procedure, no interfering peaks were observed in chromatograms of plasma and urine samples obtained after the five-drug cocktail administration (data not shown).

The 90% confidence intervals (CI) about the geometric mean ratios of the area under the plasma concentration–time curve (AUC) of midazolam and the plasma AUC of drug and metabolite of losartan, omeprazole, and caffeine fell entirely within the range of 80–125%. Also, the 90% CI for the 8 h urinary metabolic ratio of dextromethorphan was within 80–125% (**Table 1**). These results suggest that lack of interaction that may cause the change of CYP enzyme activity was presented between probe drug administrations.

The correlations between the plasma concentrations of each sampling time and the AUC_{inf} of midazolam are shown in **Figure 1**. The correlation data at 8 h and above were not available because several concentrations were below the detection limit of midazolam. The concentrations of midazolam at each time point after 0.5 h were highly correlated with the AUC_{inf} of midazolam ($r > 0.7$, $P < 0.001$). The correlation coefficients (r) at 4, 5, and 6 h were greater than 0.9 (each $P < 0.001$). Significant correlations were found between the plasma losartan/E-3174 AUC_{last} ratios and the urinary losartan/E-3174 ratios after 0–8 and 0–12 h ($r > 0.7$, $P < 0.001$) (**Figure 2**). The plasma concentration ratios of paraxanthine/caffeine at each time point after 1 h were also significantly correlated with the AUC_{last} ratios of paraxanthine/caffeine ($r > 0.8$, $P < 0.001$) (**Figure 3**). The correlations between the plasma concentration ratio of omeprazole/5-hydroxyomeprazole concentration ratios at 3, 4, 5, and 6 h and the plasma omeprazole/5-hydroxyomeprazole AUC_{last} ratio are shown in **Figure 4** ($r > 0.9$, $P < 0.001$). The plasma concentrations of omeprazole at other time points could not be quantified in some subjects.

To determine proper phenotyping index, several time points at which phenotyping indices favorably reflected each enzyme activity were selected based on the coefficient of determination (r^2) and root-mean-squared error (%RMSE) (**Table 2**). Phenotyping indices at 4 h were appropriate for CYP3A, CYP1A2, and CYP2C19 activities, which were selected based on the r^2 and %RMSE (**Table 2**). Urinary metabolic ratio of losartan and dextromethorphan for 0–8 h postdose were selected for CYP2C9 and CYP2D6 activities.

These selected phenotyping indices of the five probes were not significantly different between single- or three-drug administration and five-drug cocktail (**Figure 5**, **Table 3**). **Table 3** presents the 90% CI for the geometric mean ratios of the phenotyping indices of midazolam, losartan, caffeine, omeprazole, and dextromethorphan. For midazolam, caffeine, and dextromethorphan, the 90% CI fell within the range of 80–125%. However, for losartan and omeprazole, the 90% CI were not entirely within 80–125% (71–112 and 89–130%, respectively).

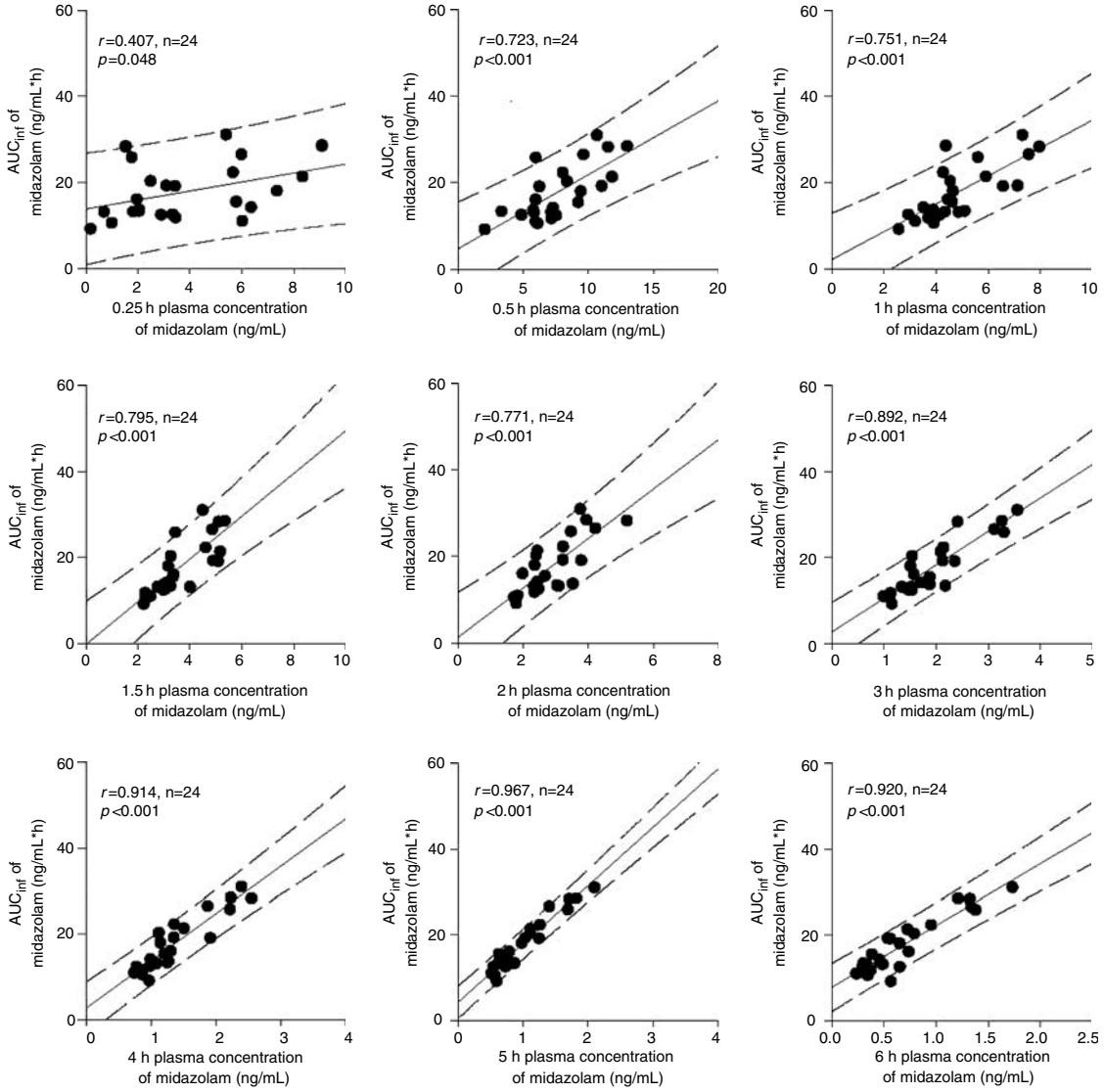

Figure 1 Correlation between the plasma AUC_{inf} of midazolam and the plasma concentration at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h after oral administration of midazolam (2 mg).

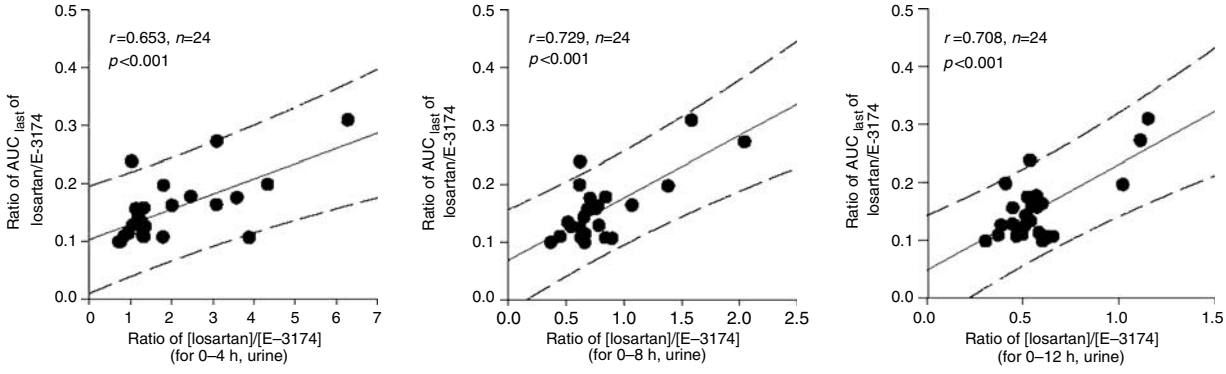

Figure 2 Correlation between the AUC_{last} ratio of losartan/E-3174 and the urinary ratio in urine samples collected for 0–4, 0–8, and 0–12 following oral administration of losartan (50 mg).

DISCUSSION

The cocktail approach to evaluate CYP enzyme activity requires consideration of pharmacokinetic and pharmacodynamic interactions between cocktail probe drugs. In this study, we validated a new five-drug cocktail regimen of caffeine, losartan, omeprazole, dextromethorphan, and oral

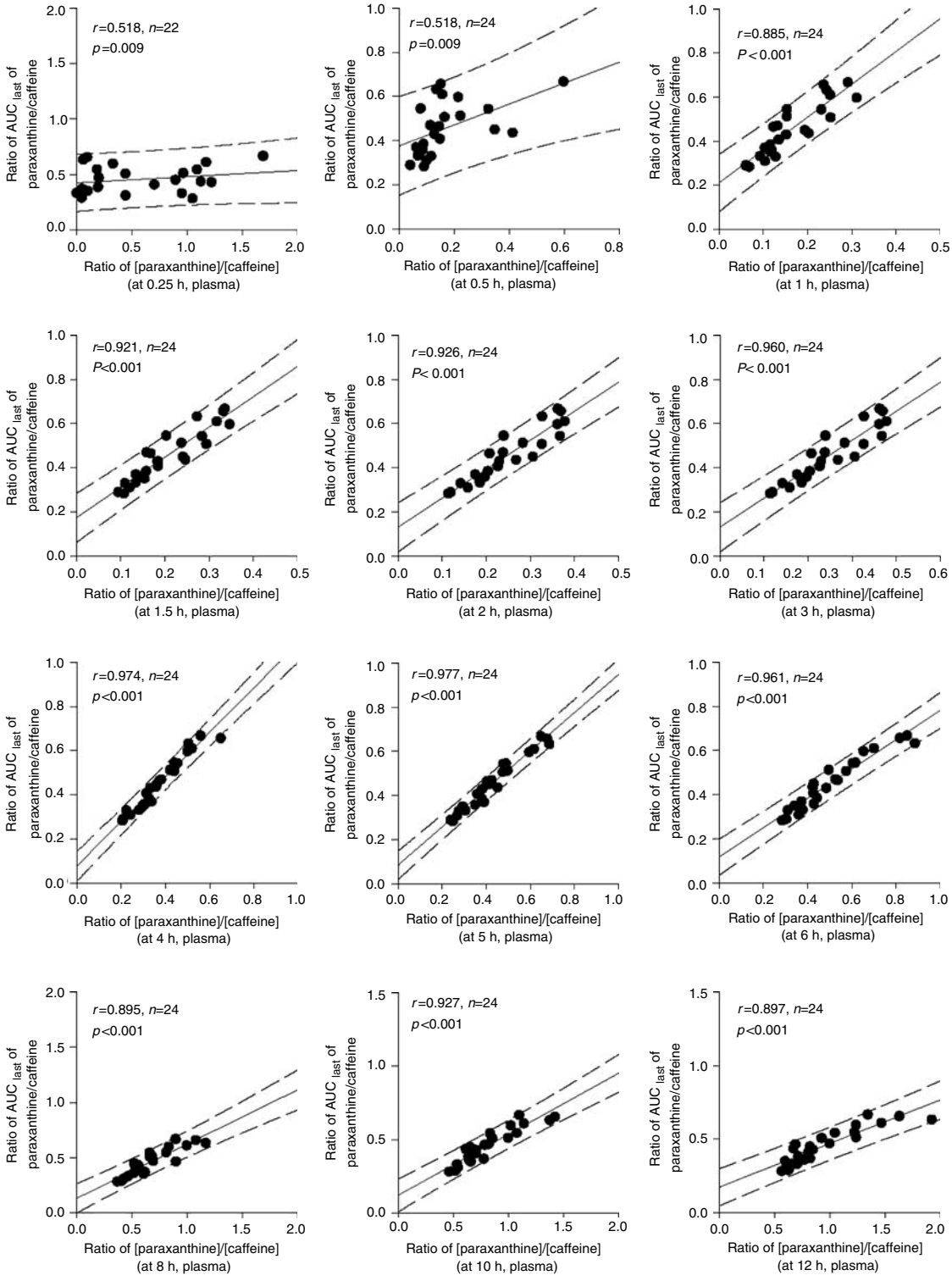

Figure 3 Correlation between the AUC_{last} ratio of paraxanthine/caffeine and the plasma concentration ratio at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h following caffeine administration (93 mg).

midazolam for determining the metabolizing activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A, respectively. We found no evidence of pharmacokinetic or pharmacodynamic interactions compared with well established single- or three-drug cocktail phenotyping.

Caffeine has been widely used as a probe drug to evaluate CYP1A2 enzyme activity and various metabolic indices have been studied.[9–12,18–21] Among them, the paraxanthine/caffeine ratio in plasma samples is a representative metabolic index for CYP1A2 activity. Many studies have shown that this

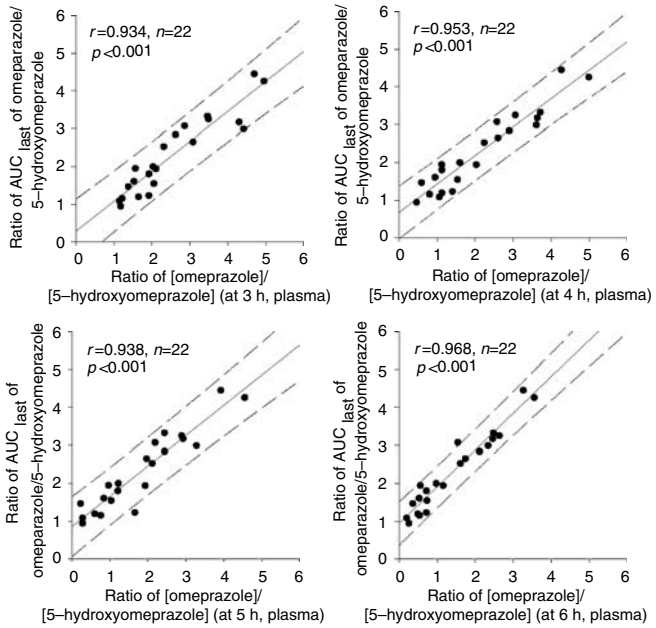

Figure 4 Correlation between the AUC_{last} ratio of omeprazole/5-hydroxyomeprazole and the plasma concentration ratio at 3, 4, 5, and 6 h following omeprazole administration (20 mg).

Table 2 r^2 and %RMSE

Phenotyping indices	Sampling time (h)	r^2	%RMSE (⩽15%)	*P*-value
[Midazolam] in plasma	3	0.80	16.6	<0.001
	4	0.84	14.9	<0.001
	5	0.94	9.4	<0.001
	6	0.85	14.4	<0.001
[Paraxanthine]/[caffeine] in plasma	3	0.92	7.5	<0.001
	4	0.95	6.0	<0.001
	5	0.95	5.7	<0.001
	6	0.92	7.4	<0.001
[Omeprazole]/[5-hydroxyomeprazole] in plasma	3	0.87	16.3	<0.001
	4	0.91	13.9	<0.001
	5	0.88	15.8	<0.001
	6	0.94	11.5	<0.001
[Losartan]/[E-3174] in urine	0–4	0.43	27.9	<0.001
	0–8	0.53	25.5	<0.001
	0–12	0.50	26.0	<0.001

%RMSE, root-mean-squared error; r^2, coefficient of determination.

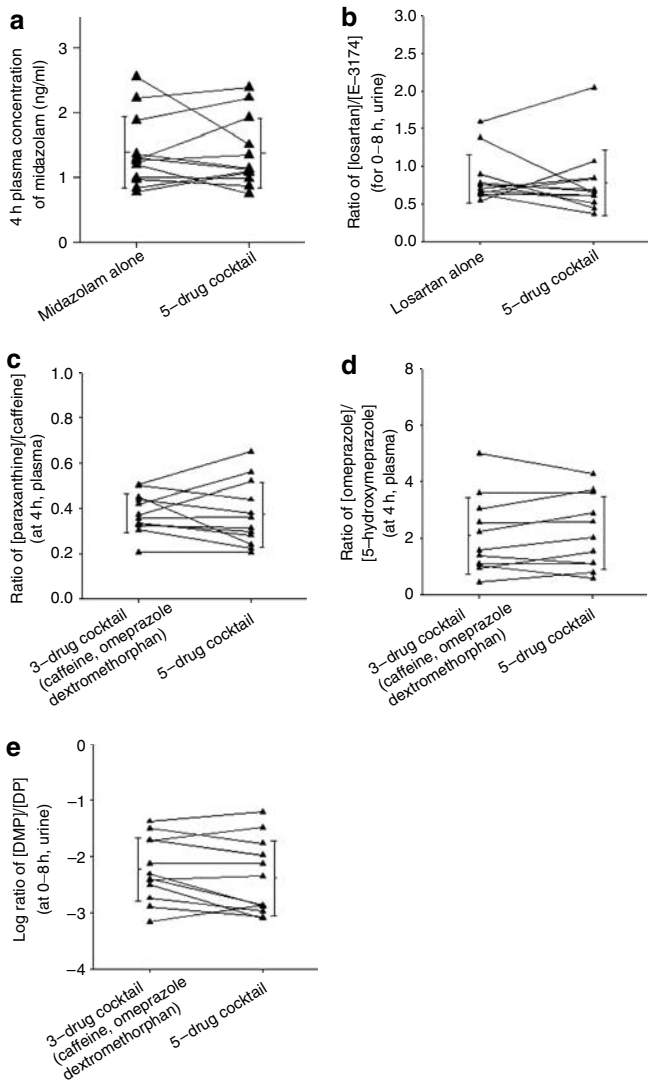

Figure 5 (**a**) Comparison of a phenotyping index of CYP3A activity (4 h plasma concentration of midazolam) after administration of midazolam alone versus the five-drug cocktail (midazolam, losartan, caffeine, omeprazole, dextromethorphan). (**b**) Comparison of a phenotyping index of CYP2C9 activity (0–8 h urinary ratio of losartan/E3174) after administration of losartan alone versus the five-drug cocktail. (**c**) Comparison of phenotyping indices of CYP1A2 activity, the ratio of [paraxanthine]/[caffeine] in the 4 h plasma sample, (**d**) CYP2C19 activity, the ratio of [omeprazole]/[5-hydroxyomeprazole] in the 4 h plasma sample, and (**e**) CYP2D6 activity, the log ratio of [dextromethorphan (DMP)]/[dextrorphan (DP)] in the urine sample collected for 8 h after administration of a three-drug cocktail (caffeine, omeprazole, dextromethorphan) versus the five-drug cocktail.

ratio is closely correlated with caffeine plasma systemic clearance.[8,18,19] In the Karolinska cocktail, the cocktail uses the paraxanthine/caffeine ratio in plasma samples obtained 4 h after the caffeine dose.[11] In the Cooperstown cocktail, the metabolic ratio of [5-acetylamino-6-formyamino-3-methyluracil + 1-methylxanthine + 1-methylurate]/[1, 7-dimethylurate] in 12 h urine samples is used instead of the paraxanthine/caffeine ratio in plasma samples.[9] This ratio is also a well-established metabolic index and has the advantage that the activity of *N*-acetyltransferase-2 and xanthine oxidase can also be evaluated using other metabolites in the urine samples. However, the long urine collection time and the large number of metabolites that have to be analyzed are disadvantages. We evaluated all achieved blood samples to determine the appropriate sampling time for the plasma paraxanthine/caffeine concentration ratios. Among them, the 3, 4, 5, and 6 h plasma concentration ratios of paraxanthine/caffeine were highly correlated with the AUC_{last} ratio of paraxanthine/caffeine ($r > 0.96$, $P < 0.001$). In our cocktail,

Table 3 Geometric mean ratio and its 90% CI for phenotyping indices

Enzyme	Phenotypic index	Five-drug cocktail /single drug	90% CI(%)	*P*-value
CYP3A	$[Midazolam]_{4\,h}$	0.98	84–116	0.857
CYP2C9	$[Losartan]/[E\text{-}3174]_{8\,h}$	0.89	71–112	0.583
Enzyme	**Phenotypic index**	**Five-drug cocktail /three drug cocktail**	**90% CI(%)**	***P*-value**
CYP1A2	$[Paraxanthine]/[caffeine]_{4\,h}$	0.95	84–107	0.561
CYP2C19	$[Omeprazole]/[5\text{-}hydroxyomeprazole]_{4\,h}$ (n=11)	1.09	89–130	0.328
CYP2D6	Log $([DMP]/[DP])_{8\,h}$	1.06	98–113	0.136

CI, confidence interval; DP, dextrorphan; DMP, dextromethorphan.

we selected the 4 h plasma paraxanthine/caffeine concentration ratios as a single-point phenotyping index on the basis of both suitability and practicality. Bioequivalence test revealed that 90% CI for AUC of caffeine from three-drug and five-drug cocktail was 89–104% and that for AUC of paraxanthine was 82–103%, indicating that caffeine did not show a significant interaction when caffeine was coadministered with other probe drugs.

Tolbutamide, phenytoin, warfarin, and diclofenac have been used as probe drugs to evaluate CYP2C9 activity.[8] In the Cooperstown 5 + 1 cocktail, warfarin was used to add phenotyping of CYP2C9 activity to the established Cooperstown cocktail.[12] However, as no representative metabolic indices for warfarin were available, many blood samplings were needed to characterize the pharmacokinetic parameters of warfarin, such as AUC and oral clearance (CL/*F*). Recently, losartan has been suggested as a highly specific and sensitive probe for CYP2C9 activity *in vivo* and *in vitro* because E-3174, a carboxylic acid metabolite of losartan, is specifically produced by CYP2C9 metabolism of losartan.[22,23] According to Yasar *et al.*,[22] the plasma $AUC_{12\,h}$ ratio of losartan/E-3174 showed a good correlation with a ratio of losartan/E-3174 in urine collected for 8 or 24 h after drug intake. We tested the 0–4, 0–8, and 0–12 h urine samples and the urinary recovery ratios of losartan/E-3174 in the 0–8 and 0–12 h sample showed a good correlation with the plasma AUC_{last} ratios of losartan/E-3174 ($r > 0.7$, $P < 0.001$). Considering the sampling schedule and convenience, we adopted the 8 h urinary ratios of losartan/E-3174 as an appropriate index reflecting CYP2C9 activity. Although no significant difference between losartan and other cocktail drugs was observed ($P = 0.583$), the 90% CI for the geometric mean ratios of the 0–8 h metabolic index (71–112%) was not entirely within 80–125%, which would not result from possible drug interaction between probe drugs considering that losartan showed relatively high intraindividual variability (%CV, 29%) for phenotyping index and that the 90% CI for the geometric mean ratios of the plasma AUC of losartan and E-3174 fell within 80–125%.

Mephenytoin, omeprazole, and chloroguanide have been used as probe drugs for CYP2C19 and the *S/R* ratio of mephenytoin has been commonly used for phenotyping of CYP2C19 activity.[8] However, the sedative effect of mephenytoin, the low concentrations of urinary *S*-mephenytoin in ultra-rapid metabolizers, and the controversial urinary stability of *S*-mephenytoin conjugates are serious limitations in its use.[8,9,24,25] Recently, omeprazole has been suggested as a probe for phenotyping of CYP2C19 activity because it is widely used and produces few side effects.[24,25] The 3 h plasma concentration of omeprazole/5-hydroxyomeprazole has been used as a phenotypic index of CYP2C19 activity.[9,11,12] This ratio showed a significant correlation with the mephenytoin *S/R* ratio.[24] In this study, we evaluated all time points to determine the appropriate single-point index for phenotyping CYP2C19. The omeprazole concentrations at 0.25, 0.5, 1.5 and 2 h were not quantified in some subjects and this was not unusual in other clinical trials performed in our lab using the same omeprazole preparation (unpublished data). Omeprazole is an enteric coated drug and omeprazole has a long lag time in absorption.[26] So, it may not be detected within 2 h plasma samples. The plasma omeprazole/5-hydroxyomeprazole concentration ratios at 3, 4, 5, and 6 h were all significantly correlated with the plasma omeprazole/5-hydroxyomeprazole $AUC_{12\,h}$ ratio ($r > 0.9$, $P < 0.001$). We selected the 4 h omeprazole/5-hydroxyomeprazole ratio in plasma as an index of metabolic activity of CYP2C19 based on the r^2 and %RMSE and considering blood sampling schedule. The upper boundary of the 90% CI for the ratios of the 4 h metabolic index (89–130%) was slightly over 125%. The 90% CI for the geometric mean of the plasma AUC of omeprazole and 5-hydroxyomeprazole were 88–124% and 87–118%, respectively. When considered together, it is unlikely to be considered as a possible drug interaction between single administration of omeprazole and five-drug cocktail administration. In another study using high-performance liquid chromatography with UV detection system,[11] quantification of 3.5 h omeprazole concentration was not achieved in some subjects. In this study, a liquid chromatography tandem mass spectrometry system was used to detect omeprazole and 5-hydroxyomeprazole in plasma, enabling the detection of low concentrations of both substances (5.0 and 1.25 ng/ml, respectively).

Codeine, metoprolol, desipramine, dextromethorphan, and debrisoquin are substrates of CYP2D6, and dextromethorphan and debrisoquin have been widely used as probe drugs for CYP2D6.[9–12] However, the use of debrisoquin is

limited to certain countries because it is not commercially available everywhere. It also has blood pressure-lowering effects and a possibility exists that debrisoquin interacts with losartan, another antihypertensive drug, resulting in serious side effects.[11] This could be overcome by lowering doses of both drugs.[11] Dextromethorphan has advantages in its wide safety margin and market availability.[12] The 0–8 h urinary recovery ratio of dextromethorphan/dextrorphan has been conventionally used as a phenotyping index.[27] Thus, we adopted the 0–8 h urinary ratio considering the sampling schedule and convenience. Our study presented that there was lack of drug interaction with other probe drugs in the new cocktail regimen. Previous studies showed large intraindividual variability in dextromethorphan metabolic ratio (35–56%),[28] which has been usually explained up to 80% of the observed variability by effect of urinary pH, independent of menstrual cycle phases, sex hormones, time variables, or phenotype.[29] In our cocktail, contrary to other reports, dextromethorphan phenotyping ratio showed lower intraindividual variability (%CV, 13%), which may result from the relatively low difference of urinary pH between the participants. Further evaluation would be needed to confirm the small variability in urinary metabolic ratio of dextromethorphan/dextrorphan. The 0–8 h urine collection time may be relatively short to estimate the true metabolic ratio in poor metabolizers *(CYP2D6*5/*5)*.[30] However, the poor metabolizers were excluded in this cocktail study because they do not contribute to the metabolism of dextromethorphan. In a recent study by Borges *et al.*,[31] the plasma concentration ratio and AUC ratio of dextromethorphan/dextrorphan appeared more predictive than the urinary ratio. However, because of the limitation of analytical method, we did not quantify the dextromethorphan and dextrorphan in the blood samples. The evaluation for the plasma concentration ratio should be considered in further study.

Dapsone, erythromycin, and cortisol were used as a probe drug of CYP3A activity. However, no significant correlations were observed among any of the phenotyping measurements from these probe drugs.[32] A large number of substrates of CYP3A appear to be also substrates of other drug-metabolizing enzymes and P-glycoprotein. N-hydroxylation of dapsone is mediated by multiple CYP enzymes[33] and erythromycin is a substrate of P-glycoprotein.[34] Midazolam is the well-established drug commonly used for phenotyping of CYP3A activity.[8] Intravenous injection of midazolam has been used to evaluate the activity of CYP3A in most studies.[9,10,12,35] However, intravenous injection of midazolam evaluates only hepatic metabolism and does not reflect CYP3A activity in the intestine. The intestinal CYP3A plays an important role in drug interactions and drug metabolism in the gastrointestinal tract.[36,37] CL/*F* of midazolam was generally used to access whole CYP3A enzyme activity.[38] The limitation of this index is that many sampling points are needed to estimate the CL/*F*. This was not compatible with our goal of developing a new cocktail method for high-throughput evaluation of enzyme activity with the least sampling. Some studies suggested that the plasma concentration of midazolam represented a useful index for CL/*F*.[38,39] To determine appropriate phenotyping index reflecting CYP3A enzyme activity, we evaluated the plasma concentration of midazolam at all sampling points. The concentrations of midazolam at the 4, 5, and 6 h were highly correlated with the AUC_{inf} of midazolam ($r > 0.9$, $P < 0.001$). Finally, we adopted the 4 h plasma concentration of midazolam as the best phenotyping index for CYP3A activity considering r^2, %RMSE, and sampling schedule. However, intravenous clearance of midazolam predominantly reflects hepatic CYP3A activity and CL/*F*, the combination of intestinal and hepatic CYP3A enzyme activity, with a lack of correlation between the two. Therefore, to differentiate between the intestinal and hepatic CYP3A activity, midazolam would be needed to be given both oral administration and intravenous injection.[37,40]

Phenotyping for CYP2E1 activity was not included in this cocktail. Chlorzoxazone is the known probe drug for CYP2E1. However, one study reported an interaction between chlorzoxazone and midazolam when midazolam was orally administered with chlorzoxazone.[41] In contrast, intravenous injection of midazolam did not show such interaction.[35]

In conclusion, the new five-drug cocktail regimen, which we named the "Inje cocktail," was validated in terms of drug interaction. The Inje cocktail can be used as a tool to phenotype the *in vivo* enzyme activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A with only 4 h blood sampling and 8 h urine collection after simultaneous oral administration of the five probe drugs (caffeine, losartan, omeprazole, dextromethorphan, and midazolam). This modified cocktail method will be useful in countries where some of the probe drugs in other cocktails are not available. This high-throughput cocktail method can be applied as a tool in drug–drug interaction or food–drug interaction studies involving possible pharmacologic and pathologic effects on enzyme induction or inhibition, including gastrointestinal metabolism.

METHODS

Subjects. This study was approved by the institutional review board of Busan Paik Hospital (Busan, Korea) and written informed consent was obtained from all participants before the performance of any study procedure.

Twelve Korean male subjects were deemed healthy according to their medical histories, physical examinations, and routine laboratory tests, including standard 12-lead electrocardiogram. Subjects were not permitted to take any medications or herbal supplements during the study procedure and were required to abstain from alcohol, caffeine, grapefruit juice, citrus fruits, and cruciferous vegetables during the 72 h before each drug administration and throughout the study.

Study design and collection of plasma and urine samples. A randomized, four-way, Latin-square, crossover study design was used to compare the phenotyping indices determined after oral administration of (a) midazolam alone (2 mg, Bukwang midazolam;

Bukwang Pharmaceutical, Seoul, Korea), (b) losartan alone (50 mg, Cozaar; MSD Korea, Seoul, Korea), (c) the three-drug cocktail regimen of caffeine (93 mg, Kafesoft; Eisai, Tokyo, Japan), dextromethorphan (30 mg, Romilar; Roche Korea, Seoul, Korea), and omeprazole (20 mg, Losec; Yuhan Pharmaceutical, Seoul, Korea), and (d) the five-drug cocktail regimen of caffeine (93 mg), dextromethorphan (30 mg), omeprazole (20 mg), losartan (30 mg), and midazolam (2 mg). To avoid possible carryover effects, each trial was separated by a 1-week washout period. Control blood for genotyping was obtained from all participants before phenotyping.

Serial 10 ml blood samples were drawn via indwelling venous catheters immediately before and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after administration of probe drugs. Plasma samples were stored at −80°C until use. Urine samples were collected during the periods 0–4, 4–8, and 8–12 h after administration of probe drugs. After measurement of urine volume, a 15-ml urine aliquot was stored at −80°C until use.

Genotype analysis of *CYP2D6, CYP2C9,* and *CYP2C19*. To detect CYP2D6*5 alleles, we used LA Taq polymerase (TaKaRa, Shiga, Japan), according to the manufacturer's protocol. Genomic DNA (200–400 ng) was amplified using a 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). The deletion of the CYP2D6*5 allele was identified using primers 5'-2D6*5 and 3'-2D6*5, which are located in CYP2D7 and in the 3'-flanking region of CYP2D6, respectively. The CYP2D6 gene was amplified using primers 5'-2D6 and 3'-2D6.[42] Of the 50 μl LA-polymerase chain reaction (PCR) solution, 5 μl was analyzed by agarose gel electrophoresis for the presence of CYP2D6 and CYP2D6*5. The amplicons of alleles CYP2D6*5 and CYP2D6 were 3.5 and 5.1 kb, respectively.

The CYP2C9*2 and CYP2C9*3 genotypes were determined by PCR and pyrosequencing methods. In brief, PCR was carried out in a final reaction mixture containing 0.2 mM deoxyribonucleotide triphosphate mixture, 1 unit Taq polymerase (TaKaRa), approximately 80 ng human genomic DNA, a pair of primers (10 pmol each), and 1 × PCR buffer. The primers used for the amplification were as follows: 5′-biotin-GTATTTTGGCTTGAAACCCATA-3' and 5′-GGCCTTGGTTTTTCTCAACTC-3′ (for CYP2C9*2) and 5′-biotin-TGCACGAGGTCCAGAGGTAC-3′ and 5′-ACAAACTTACC TTGGAATGAGA-3′ (for CYP2C9*3). After PCR, biotinylated PCR products were immobilized on streptavidin-coated beads and transferred to a filter plate. The DNA strands were separated in denaturation solution (0.5 M NaOH) for 5 s. The immobilized template was washed in 10 mM Tris-acetate (pH 7.6) solution, transferred to a PSQ 96 plate, and resuspended in annealing buffer (20 mM Tris–acetate, pH 7.6), containing a sequencing primer (5′-TGAACACGGTGGTGA-3′ for CYP2C9*2 and 5′-TGGTGGGGAG AAGGTC-3′ for CYP2C9*3). The sequence was analyzed using a PSQ 96 system with the SNP Reagent Kit (Pyrosequencing AB, Uppsala, Sweden).

The CYP2C19*2 and CYP2C19*3 genotypes were determined by PCR and pyrosequencing methods. The primers used for the amplification were as follows: 5′-biotin-AATTACAACCAGAGC TTGGC-3′ and 5′-TATCACTTTCCATAAAAGCAAG-3′ (for CYP2 C19*2) and 5′-biotin-CCAATCATTTAGCTT CACCC-3′ and 5′-ACTTCAGGGCTTGGTCAATA-3′ (for CYP2C19*3).

Assays of probe drugs and their metabolites. After completion of the four-phase crossover trial, the plasma and urine concentration of the probe drugs and their metabolites were determined simultaneously for all samples from the subjects.

Dextromethorphan and dextrorphan in urine were analyzed by reversed-phase high-performance liquid chromatography with fluorescence detection, as described previously,[43] with some modifications. The lower limits of quantitation for dextromethorphan and dextrorphan were 5.0 ng/ml. The interassay precision values for all samples were less than 9.3%.

Losartan and its metabolite (E-3174) in urine were analyzed by reversed-phase high-performance liquid chromatography, with fluorescence detection, as described by Gonzalez *et al.*,[44] with some modifications. The lower limits of quantitation for losartan and its metabolite (E-3174) were 5.0 ng/ml. The interassay precision values for all samples were less than 15.9%.

Plasma concentrations of midazolam were determined by liquid chromatography tandem mass spectrometry as described previously,[45] with some modifications. Briefly, plasma samples (0.5 ml) were spiked with an internal standard (10 μl of 1 μM phenacetin), alkalinized with 0.6 ml of 2 N NaOH, and extracted with 5 ml of diethylether/methylene chloride (6:4, v/v). Aliquot of reconstituted residue of the organic phase was analyzed in an API 3000 liquid chromatography–tandem mass spectrometry system (Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series high-performance liquid chromatography system. Chromatographic separation of the compounds was accomplished using a Luna C18 column (2.0 × 50 mm, 3 μm; Phenomenex, Torrance, CA), with water and acetonitrile (7:3, v/v) containing 0.1% formic acid as the mobile phase at a flow rate of 0.2 ml/min. The Tandem mass spectrometry system was operated using an electrospray in positive ionization mode. For midazolam and phenacetin, the precursor-to-product ion reactions monitored were as follows: mass-to-charge ratios of 326 → 291 and 180 → 110, respectively. The lower limit of quantitation for midazolam was 0.1 ng/ml. The interassay precision values for all samples were less than 5.3%.

Plasma concentrations of caffeine, paraxanthine, omeprazole, and 5-hydroxyomeprazole were determined by liquid chromatography tandem mass spectrometry, as described previously,[46] with some modifications. Briefly, plasma samples (0.5 ml) were spiked with an internal standard (10 μl of 0.5 μM phenacetin), mixed with 1.0 ml of 0.5 M NaH_2PO_4 solution (pH 8.77), and extracted with 5 ml of diethylether/methylene chloride (6:4, v/v). For caffeine, paraxanthine, omeprazole, 5-hydroxyomeprazole, and phenacetin, the precursor-to-product ion reactions monitored were as follows: mass-to-charge ratios of 194 → 138, 181 → 124, 346 → 198, 362 → 214 and 180 → 110, respectively. The lower limits of quantitation for caffeine, paraxanthine, omeprazole, and 5-hydroxyomeprazole were 5.0, 5.0, 5.0, and 1.25 ng/ml, respectively. The interassay precision values for all samples were less than 14.2%.

Phenotype assessment and statistical analysis. The $AUC_{0-\infty}$ was calculated by use of the liner trapezoidal rule. The elimination constant (k_e) was estimated from the least-squares regression slop of terminal plasma concentration and the AUC from time 0 to infinity ($AUC_{0-\infty}$) was calculated by the equation $AUC_{(0-\infty)} = AUC + C_t/k_e$ in which C_t is the last plasma concentration measured.

For midazolam, losartan, caffeine, and omeprazole, the 90% CI for the geometric mean ratio of the plasma AUC of drug or metabolite with single or three- and five-drug cocktail administration were used to evaluate the equivalence of enzyme activities between probe drug administrations. In case of dextromethorphan, the drug interaction was assessed by the mean ratio of the 0–8 h urinary metabolic ratio. If the 90% CI fell within the range of 80–125%, we were to conclude that a lack of possible interaction between probe drugs was present.[47]

Determination of appropriate single-point phenotyping indices for CYP3A, CYP2C9, CYP1A2, and CYP2C19 was undertaken by comparing the plasma AUC or the plasma AUC ratio of drug/metabolite of probe drugs with the drug concentration or the concentration ratio of drug/metabolite in plasma and urine samples of various sampling time. The phenotyping indices used in this study were as follows. Plasma concentration of midazolam was used to assess CYP3A activity. Plasma concentration ratios of paraxanthine/caffeine and omeprazole/5-hydroxyometrazole were used to assess CYP1A2 and CYP2C19 activity, respectively. Losartan

oxidation to the active metabolite E-3174 was estimated by the urinary ratios to assess CYP2C9 activity.

Unlike other CYP enzymes, we adopted the 0–8 h urinary molar ratios of dextromethorphan/dextrorphan to assess CYP2D6 activity, which has been conventionally used as a phenotyping index.[27]

Linear regression analysis was performed to assess the linear relation between the phenotyping indices. r^2 and %RMSE were used to determine proper single-point phenotyping index among all time points.[48] Acceptable percentage limits for RMSE was below 15%. Ninety percent CI for the geometric mean ratio of the adopted phenotyping indices with single or three- and five-drug cocktail administration were calculated using equivalence test. Wilcoxon's signed-ranks test was also used for comparisons of phenotyping results for each enzyme after administration of the single or three-drug and the new five-drug cocktail. Analyses were performed using SAS software system version 8.01 (SAS Institute, Cary, NC). For all statistical procedures, P values of $\leqslant 0.05$ were deemed statistically significant.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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