Comparison of the Effects of Omeprazole and Rabeprazole on Ticlopidine Metabolism In Vitro

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Abstract. The thienopyridine derivative ticlopidine (TCL) is an inhibitor of adenosine diphosphate-induced platelet aggregation. Combination therapy with a thienopyridine derivative and aspirin is standard after coronary stenting, although more hemorrhagic complications occur with the combination therapy than with aspirin alone. A proton pump inhibitor (PPI) is required for prevention or treatment of upper gastrointestinal bleeding in such cases. We examined the effects of PPIs [omeprazole (OPZ) and rabeprazole (RPZ)] on TCL metabolism using pooled human liver microsomes prepared from various human liver blocks and 12 individual human liver microsomes. We calculated the \( K_i \) values of each PPI for TCL metabolic activity and compared the inhibitory effect of each PPI on TCL metabolism. The \( K_i \) values of OPZ and RPZ were 1.4 and 12.7 μM, respectively. The inhibitory effect of OPZ (78.6 ± 0.05%) was significantly greater than that of RPZ (24.2 ± 0.05%) (\( P < 0.001 \)). Interestingly, a negative correlation existed between the inhibitory effect of OPZ and CYP2C19 activity (\( r = -0.909, P < 0.001 \)). These results suggest that the inhibitory effect of OPZ is more potent than that of RPZ in vitro. In conclusion, RPZ appears preferable when administering TCL, aspirin, and a PPI in combination.

Keywords: ticlopidine, proton-pump inhibitor, drug–drug interaction, clopidogrel, cytochrome P450 (CYP) 2C19
including proton-pump inhibitors (PPIs), via CYP2C19 (7), although it has not been reported that other drugs have inhibitory effects on TCL metabolism. It appears that the metabolic study of TCL is difficult since its metabolic pathway is complicated and the active metabolite has not been detected in humans.

Combination therapy with thienopyridine derivatives and low-dose aspirin has been the clinical gold standard to prevent stent thrombosis in patients with ischemic coronary syndromes (8, 9). It is well known that the combination of a thienopyridine derivative and low-dose aspirin increases the risk of bleeding complications post-stenting in ischemic coronary syndromes (8, 9). Even the combination of CL and low-dose aspirin significantly increases the risk (OR = 7.4; 95% CI, 3.5 – 15) of serious upper gastrointestinal bleeding (UGIB), compared with no treatment (10). Thus, clinicians prescribe H₂-receptor antagonists or PPIs to prevent or treat UGIB. The risk of UGIB is slightly reduced with the addition of an H₂-receptor antagonist (OR = 0.43; 95% CI, 0.18 – 0.91) and significantly reduced by the addition of a PPI (OR = 0.04; 95% CI, 0.002 – 0.21), compared with no treatment (11). Prescriptions of PPIs have increased to reduce the risk of UGIB.

Recently, some studies have suggested that omeprazole (OPZ) decreases the antiplatelet effect of CL and increases the risk of recurrence of acute coronary syndrome, due to an interaction between OPZ and CL (12, 13). It is assumed that OPZ inhibits CYP2C19, the main pathway of CL activation (14), although this assumption has not been confirmed in vitro. In addition, it has not been confirmed whether PPIs reduce the metabolism of TCL and its antiplatelet effects, in vivo or in vitro. However, CL is structurally similar to TCL, except that it has a carboxymethyl group. The intermediate, active metabolite, and activation pathway of TCL are also similar to those of CL (3). We hypothesized that the addition of a PPI to combined TCL and low-dose aspirin therapy decreases the antiplatelet effect not only of CL but also of TCL, since PPIs reduce TCL metabolic activity.

In this study, we investigated the metabolic activity of TCL, which has a complicated pathway, by measuring the disappearance rate of TCL using high-performance liquid chromatography (HPLC). Then we compared TCL metabolic activity with the activities of CYP2C19, CYP2C9, and CYP3A4 in individual human liver microsomes (HLMs). We also investigated the inhibitory effect of PPIs on the metabolic activity of TCL to determine which PPI inhibited the metabolism of TCL less potently, since it is known that OPZ has greater inhibitory potency against CYP2C19 activity than other PPIs, especially rabeprazole (RPZ) (15).

### Materials and Methods

#### Chemicals

TCL was purchased from Cosmo Bio Co. (Tokyo). OPZ, nitrendpine (NIT), n-hexane, and acetonitrile (ACN) were purchased from Wako Pure Chemical Industries (Osaka). The n-hexane and ACN were of HPLC grade. RPZ was provided by Eisai Co. (Tokyo). Glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide (NADP⁺), and glucose-6-phosphate dehydrogenase (G6P-DH) were purchased from Oriental Yeast Co., Ltd. (Tokyo). All other chemicals were used of the highest quality commercially available.

#### Preparation of HLMs

Pooled human liver microsomes (PHLMs) were prepared from various human liver blocks provided by the Human and Animal Bridging (HAB) Research Organization (Tokyo) according to the previously reported method (16). In brief, each liver block was homogenized with 3 volumes of 1.15% KCL. The hepatic microsomes were prepared by sequential centrifugation of the homogenate at 9000 × g for 20 min and of the resultant supernatant at 105,000 × g for 60 min, both at 4°C. The precipitates were mixed and diluted with distilled water. The PHLMs were immediately frozen in liquid nitrogen and stored at −80°C until use. The amount of microsomes was measured using a protein assay kit (Bio-Rad, Hercules, CA, USA) (17). Twelve human liver microsomes were obtained from the HAB Research Organization, for which CYP2C19, CYP3A4, and CYP2C9 activity data were published on its homepage (18, 19). This study was approved by the Ethics Committee of St. Marianna University School of Medicine (Kawasaki).

#### Incubation conditions and extraction substrate

All reactions were performed according to the previously reported methods as outlined below (20, 21). PHLMs (0.1 – 1.0 mg) were mixed with TCL (1.0 – 10.0 μM), 0.5 mM EDTA, 100 mM Na-K phosphate buffer (pH 7.4), and distilled water. After preincubation for 5 min at 37°C, the reaction was initiated by the addition of an NADPH-generating system (5 mM magnesium chloride, 0.5 mM NADP⁺, 5 mM G6P, and G6P-DH, 1 unit in final volume). After incubation for 0 – 15 min at 37°C, the reaction was terminated by icing. Incubation mixtures without the NADPH-generating system (G-system) served as negative controls. These incubation studies were performed in triplicate.

The mixtures were centrifuged at 15,000 × g for 10 min at 4°C. A 100-μl aliquot of NIT (20 μg/ml, final volume 5.5 μM) was added to a 900-μl aliquot of the supernatant layer as an internal standard. The extraction
of TCL was performed as previously described and outlined below (22). These mixtures of 1 ml were alkalized with 6 N sodium hydroxide solution. The extraction mixtures with 4 ml n-hexane added were shaken vigorously for 10 min and then centrifuged at 3000 × g for 20 min at 35°C. Then the n-hexane upper layer of 3 ml was transferred to glass tubes and evaporated. These residues were stored at −20°C.

HPLC conditions

The samples were brought to room temperature and dissolved in the mobile phase (10 mM phosphate buffer, pH 5.0 : ACN = 4 : 6) by vortex mixing for 60 s, following the previous method with slight modification (23, 24). After filtration with a 0.22-μm Ultrafree®-MC filter (Millipore Co., Bedford, MA, USA), 100-μl aliquots were injected onto an HPLC apparatus. The HPLC system was comprised of a CCPM-II solvent pump (Tosoh Co., Tokyo) and an MCPD-3600 detector (Otsuka Electronics Co., Osaka) set for measurement at a wavelength of 235 nm. Separation was achieved on a COSMOSIL Packed Column 5C18 (4.6 mm i.d. × 15 cm; Nacalai Tesque Inc., Kyoto). The flow rate was 1.0 ml/min with column temperature maintained at 25°C.

Dixon plot analysis and inhibition study

In Dixon plot analysis, OPZ or RPZ (0 – 60 μM) as an inhibitor of CYP2C19 and TCL at four different concentrations (1.0, 2.0, 5.0, 10.0 μM) were added to 0.4 mg PHLMs. All samples were incubated for 4 min at 37°C. The Ki values of OPZ and RPZ were calculated with Dixon plot analysis using the equation assuming competitive inhibition.

In the inhibition study using 12 HLMs, 1.2 μM OPZ or RPZ and 5.0 μM TCL were added to each HLM (0.4 mg). All samples were incubated at 37°C for 4 min. The extraction methods and the HPLC conditions were the same as those described above.

Calculation of TCL metabolic activity

TCL metabolic activity [v (nmol·min⁻¹·mg⁻¹ protein)] was calculated following the previous method (25). In brief, TCL of a known quantity [q (nM)] was incubated for a period of time [t (min)]. The protein content was p (mg). At the end of the incubation, TCL remaining unchanged in this mixture was analyzed with HPLC. A chromatographic peak area (a') was obtained from the sample, which was presented as the G-systems equivalent. The area (a) was obtained from the reference in which the G-system was absent. Metabolic activity was expressed as v = q(1−a'/a)t/p (nmol·min⁻¹·mg⁻¹ protein).

Statistical analyses

Data are presented as the mean ± S.E.M. and were analyzed using KyPlot ver. 5.0 software (KyensLab Co., Tokyo). Correlations were estimated by regression analysis. Analysis of significant differences was performed by the Wilcoxon signed-rank test and Student’s t-test. A P-value of < 0.05 was considered to represent a statistically significant difference.

Results

Determination of experimental conditions

When the incubation time and TCL concentration were set at 4 min and 5.0 μM, respectively, as shown in Fig. 1A, the TCL disappearance rate increased linearly (93.4 – 492.8 nmol/min) with the increase in protein content (0.1 – 1.0 mg). When the protein content and TCL concentration were set at 0.4 mg and 5.0 μM, respectively, as shown in Fig. 1B, the disappearance of TCL increased (0.0 – 6793.2 nmol/mg) with increasing incubation time (0 – 15 min). In addition, there was a linear relationship between the incubation time and the disappearance of TCL when the incubation time was short (0 – 4 min).

When the incubation time and protein content were set at 4 min and 0.4 mg, respectively, the metabolic activity of TCL increased with increasing TCL concentration (2.0 – 10.0 μM) (Fig. 1C). The Cmax and Km values were calculated by using Lineweaver-Burk plots to be 1597.2 (nmol·min⁻¹·mg⁻¹ protein) and 6.5 μM, respectively (Fig. 1D). In addition, the Cmax value of TCL following multiple-dose administration was approximately 5.0 μM in vivo (21). We then used the experimental conditions described below when the protein content, incubation time, and TCL concentration were 0.4 mg, 4 min, and 5.0 μM, respectively.

Dixon plot analysis

OPZ or RPZ (0 – 60 μM) was incubated with TCL at concentrations of 2.5, 5.0, and 10.0 μM. The metabolic activities of TCL decreased with the increase in PPI concentrations (Fig. 2: A, B). From these Dixon plot analyses, the mechanism of the inhibition by OPZ and RPZ appeared to be competitive inhibition, and the respective Ki values were 1.4 and 12.7 μM for OPZ and RPZ, respectively (Fig. 2: C, D).

TCL metabolic activities and inhibitory effects of PPIs in 12 HLMs

We measured the metabolic activities of TCL and investigated the inhibitory effects of OPZ and RPZ on TCL metabolic activities in each of 12 HLMs. The concentration of TCL, OPZ, and RPZ was 5.0, 1.2, and 1.2 μM,
respectively. Individual differences were observed in the metabolic activity of TCL, and the greatest metabolic activity was 6-fold higher than the least (Fig. 3A). Additionally, a significant correlation was observed between TCL metabolic activity and CYP2C19 activity ($r = 0.979, P < 0.001$) (Fig. 3B), although there was no significant correlation between TCL metabolic activity and CYP2C9 activity or CYP3A4 activity (Fig. 3: C, D).

TCL metabolic activity was significantly reduced in the presence of OPZ (119.9 ± 47.6 nmol·min$^{-1}$·mg$^{-1}$ protein) ($P < 0.01$). Although no significant reduction was seen in the presence of RPZ, the metabolic activity of TCL tended to be reduced (314.8 ± 65.5 nmol·min$^{-1}$·mg$^{-1}$ protein), compared with the activity in the absence of PPIs (399.4 ± 68.2 nmol·min$^{-1}$·mg$^{-1}$ protein) (Fig. 4: A, B). TCL metabolic activity was thus decreased due to the inhibitory effect of PPIs. The inhibitory effect of OPZ (78.6 ± 0.05%) on TCL metabolic activity was significantly greater than that of RPZ (24.2 ± 0.05%) ($P < 0.001$). In addition, there was a significant negative correlation between the inhibitory effect of OPZ on TCL metabolic activity and CYP2C19 activity ($r = -0.909, P < 0.001$) in the 12 individual HLMs, although there was no significant correlation between the inhibitory effect of RPZ and CYP2C19 activity in the 12 HLMs ($r = 0.179$, N.S.) (Fig. 4C).

**Discussion**

To the best of our knowledge, this study was the first to investigate the metabolic activity of TCL, which has a complicated metabolic pathway and an active metabolite that is difficult to detect, by measuring the remaining substrate (TCL) with HPLC after reaction. In addition, this study was the first to investigate the inhibitory potency of PPIs against TCL metabolism in vitro. This HPLC analytical method is relatively easy to use and allows simultaneous investigation of the metabolic activity of other substrates. We expect this method to have wide application in the field of drug–drug interactions. Our results showed that 1) the contribution rate of CYP2C19 to TCL metabolism was greater than that of other CYP enzymes and 2) OPZ inhibited TCL metabolism more potently than RPZ in vitro.

The disappearance rate of TCL increased linearly with the increase in protein content (0.1 – 1.0 mg) (Fig. 1A).
This result shows that the rate of CYP-related TCL metabolism is proportional to enzyme volume (25). The disappearance amount of TCL showed a brief linear increase during the incubation period from 0 to 4 min (Fig. 1B), indicating that the disappearance of TCL through pathways other than NADPH-related metabolism is negligible during this short incubation period. This linear correlation immediately disappeared after 4 min, probably because TCL is a suicide substrate that exerts mechanism-based inhibition of CYP2C19 (5). Although we did not investigate the mechanism-based inhibition of CYP2C19 in detail, our assumption is consistent with the results of a previous report showing that the formation of the metabolite of TCL occurred during a short linear period (5 min), followed by a rapid decrease in metabolite formation (5).

Based on the results of protein content (Fig. 1A), incubation time (Fig. 1B), and TCL concentration (Fig. 1: C, D) and because the $C_{\text{max}}$ value of TCL following multiple-dose administration is approximately 5.0 μM (21), the protein content, incubation time, and concentration of TCL were 0.4 mg, 4 min, and 5.0 μM, respectively, in the present study. Both OPZ and RPZ reduced the metabolic activity of TCL (Fig. 2: A, B). Dixon plot analyses (Fig. 2: C, D) indicated that the mechanism of inhibition by OPZ and RPZ was competitive, with $K_i$ values of 1.4 and 12.7 μM, respectively. These results show that the inhibitory potency of OPZ is greater than that of RPZ, consistent with a previous report on the inhibitory potency of several PPIs against the metabolism of S-mephenytoin, a well-known substrate of CYP2C19 (15). Sakai et al. have reported that the clinical dosing of OPZ is 20 mg/day, the $C_{\text{max}}$ value of OPZ ranging between 0.7 – 3.0 μM (26) in Japanese. In our study, the $K_i$ value of OPZ was 1.4 μM, ranging within the reported $C_{\text{max}}$ range of 0.7 to 3.0 μM. In this clinical dosing, OPZ may inhibit TCL metabolism by CYP2C19. In addition, the reported clinical dosing of RPZ is 20 mg/day (26). The $C_{\text{max}}$ values of RPZ have ranged between 1.1 – 1.4 μM (26). The $K_i$ value of RPZ was 12.7 μM in our study, and this $K_i$ value was much higher than the $C_{\text{max}}$ value in the clinical dosing. From the above reason, we thought the inhibition of CYP2C19 by
RPZ might be negligible in clinical use.

TCL, OPZ, and RPZ concentration were 5.0, 1.2, and 1.2 μM, respectively, in experiments to investigate TCL metabolic activity and the inhibitory effects of OPZ or RPZ on that activity in 12 HLMs. TCL at 5.0 μM was assumed to be the C_{max} value following multiple-dose administration (21). OPZ at 1.2 μM and RPZ at 1.2 μM were assumed to be near the C_{max} values following single-dose administration when comparing their inhibitory effects (26). These conditions may occur in the clinical setting when a PPI is added to combination therapy with TCL and low-dose aspirin.

Under our experimental conditions, a significant correlation between TCL metabolic activity and CYP2C19 activity (r = 0.979, P < 0.001) was seen in 12 individual HLMs (Fig. 3B). This result indicates the contribution of CYP2C19 to TCL metabolism. A previous study found that not only CYP2C19 but also CYP2C9, CYP3A4, and peroxidase participate in TCL metabolism (21), although the relative contributions of these enzymes were not reported. In our study, the contribution of CYP2C19 was greater than that of CYP2C9 or CYP3A4, since there were no significant correlations between TCL metabolic activity and CYP2C9 or CYP3A4 activity. Liu et al reported that peroxidase was involved in active neutrophils and the metabolite of TCL by activate neutrophils was significantly different from that by liver (27). We thought that the contribution of peroxidase was negligible in this experiment, since we used HLMs, not activated neutrophils, as the source of enzyme for TCL metabolism.

Both OPZ and RPZ reduced the metabolic activities of TCL in the 12 HLMs, although the reduction by RPZ was not significant. The reason for the weak inhibition by RPZ was reported to be its nonenzymatic degradation, which forms the RPZ thioether that inhibits CYP2C19 (K_{i} = 2 – 8 μM) and CYP2C9 (K_{i} = 6 μM) (15). However, the decrease in TCL metabolism by OPZ was significant (P < 0.01), and its inhibitory effect was significantly greater than that of RPZ (P < 0.001) in individual HLMs, as reflected by the K_{i} values. These results are consistent with a previous report showing that CYP2C19 plays a major role in OPZ metabolism, but only a minor role in

![](image)
RPZ metabolism (28).

Interestingly, a significant negative correlation was found between the inhibitory effect of OPZ on TCL metabolism and CYP2C19 activity ($r = -0.909$, $P < 0.001$). This suggests that the inhibitory potency of OPZ is greater in cell populations with weak CYP2C19 activity. However, the inhibitory effect of RPZ does not correlate with CYP2C19 activity, indicating that the inhibitory potency of RPZ is not affected by CYP2C19 activity.

In addition, there are interethnic differences in the frequencies of poor metabolizers (PMs) of CYP2C19. The population of PMs among Japanese (19%) is larger than that among Caucasians (3%) and Africans (4%) (29). Therefore, the inhibitory effect of OPZ on TCL metabolism is likely to occur more frequently among Japanese than among other ethnic groups.

TCL is well known to be a suicide substrate and selective mechanism-based inhibitor of CYP2C19 (3). These mean that CYP2C19 under goes the inactivation process during the CYP2C19-catalyzed oxidation of TCL. In the patients during the combination therapy with TCL and low-dose aspirin, their CYP2C19 activities may be reduced by TCL. And the population of CYP2C19 poor metabolizer among Japanese (19%) is larger than that among Caucasians (3%). This study suggests that the inhibition of TCL metabolism by OPZ is significantly greater than that of RPZ and this inhibition is significant in cell populations with weak CYP2C19 activity. However, the inhibition of TCL metabolism by RPZ is not significant and does not correlate with CYP2C19 activity. In conclusion, RPZ appears preferable when administering TCL, low-dose aspirin, and a PPI in combination to such a patient.
References