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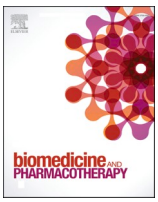

Contribution of human organic anion transporter 3-mediated transport of a major linezolid metabolite, PNU-142586, in linezolid-induced thrombocytopenia

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ABSTRACT

Thrombocytopenia, a common adverse effect of linezolid, often occurs in patients lacking typical risk factors. In this study, we investigated the key risk factors for linezolid-induced thrombocytopenia using two real-world clinical databases and explored its underlying mechanism through *in vitro* and *in vivo* experiments. In a retrospective analysis of 150 linezolid-treated patients, multivariate analysis identified coadministration of lansoprazole, a proton pump inhibitor, as a significant independent risk factor for thrombocytopenia (odds ratio: 2.33, $p = 0.034$). Additionally, analysis of the Food and Drug Administration Adverse Event Reporting System database revealed a reporting odds ratio of thrombocytopenia for lansoprazole of 1.64 (95% CI: 1.25–2.16). *In vitro* studies showed that the uptake of PNU-142586, a major linezolid metabolite, was significantly higher in human organic anion transporter 3-expressing HEK293 (HEK-hOAT3) cells compared to HEK-pBK cells. The apparent IC_{50} value of lansoprazole against hOAT3-mediated transport of PNU-142586 was 0.59 ± 0.38 μM. In a pharmacokinetic study using rats, coadministration of linezolid with lansoprazole intravenously resulted in approximately a 1.7-fold increase in the area under the plasma concentration-time curve of PNU-142586, but not linezolid and PNU-142300. Moreover, PNU-142586, but not linezolid, exhibited concentration-dependent cytotoxicity in a human megakaryocytic cell line. These findings suggest that linezolid-induced thrombocytopenia should be due to delayed elimination of PNU-142586. Furthermore, delayed elimination of PNU-142586 due to renal failure and hOAT3-mediated transport inhibition by lansoprazole should exacerbate linezolid-induced thrombocytopenia.

1. Introduction

Linezolid, an oxazolidinone antibiotic, exhibits potent broad-spectrum activity against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci [1]. However, thrombocytopenia, a condition characterized by a low platelet count, is a significant adverse effect of linezolid treatment and often imposes limitations on its clinical use [2]. Although previous studies have identified the duration of linezolid treatment and renal impairment as recognized risk factors for linezolid-induced thrombocytopenia [3,4], the occurrence of this adverse effect in patients without these risk factors necessitates exploration into additional significant risk factors and elucidation of the underlying mechanism.

Linezolid undergoes metabolism through both lactam and lactone pathways, resulting in the formation of major metabolites, PNU-142300 and PNU-142586, respectively [5]. In patients with normal renal function, linezolid primarily circulates as the parent drug in plasma [6]. Approximately 35% of the dose is excreted in urine as the parent drug, while approximately 50% is excreted as metabolites, with approximately 40% as PNU-142586 and 10% as PNU-142300 [7]. A study by Souza et al. [6] has reported significantly elevated plasma

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concentrations of PNU-142300 and PNU-142586 compared to linezolid in patients with renal failure. These findings suggest that the delayed elimination of PNU-142586 and/or PNU-142300, rather than linezolid, may contribute to the development of linezolid-induced thrombocytopenia.

Recent research demonstrated that various drug metabolites serve as substrates and/or inhibitors of human organic anion transporter 1 and 3 (hOAT1 and hOAT3) at the basolateral membrane of the proximal tubules in the kidney [8]. It is hypothesized that drug metabolites may modulate therapeutic and adverse effects through drug interactions via renal drug transporters. However, information regarding drug transporters associated with renal tubular secretion of linezolid, PNU-142300, and PNU-142586 remains scarce. Considering that the development of linezolid-induced thrombocytopenia is associated with the delayed elimination of PNU-142300 and PNU-142586 in patients with renal failure [6], as well as the findings of Komazawa et al. [9] demonstrating significant decreases in protein and mRNA levels of rOAT1 and rOAT3 in rats with chronic renal failure, we hypothesized that altered renal elimination of PNU-142300 and PNU-142586 via hOAT1 and/or hOAT3 may influence linezolid-associated thrombocytopenia.

The aim of this study was to investigate the significant risk factors associated with linezolid-induced thrombocytopenia using two real-world clinical databases: electronic medical records and the Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS). Additionally, the study aimed to elucidate the underlying mechanism of linezolid-induced thrombocytopenia through *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Study flow

First, the significant risk factors associated with linezolid-induced thrombocytopenia were investigated in retrospective study using electronic medical records. Second, the effects of proton pump inhibitors (PPIs) on linezolid-induced thrombocytopenia were validated by the FAERS database analyses. Third, drug interaction mediated by drug transporter between linezolid, PNU-142300, PNU-142586, and PPIs were evaluated through *in vitro* and *in vivo* experiments. Finally, the cytotoxicity of linezolid, PNU-142300, and PNU-142586 for platelets was assessed using the human megakaryocytic cell line (MEG-01 cell).

2.2. Materials

Linezolid and rabeprazole were sourced from LKT Laboratories, Inc. (St. Paul, MN). Benzyl (3-fluoro-4-morpholinophenyl) carbamate and 6-carboxyfluorescin (6-CF) were procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PNU-142300 sodium salt and PNU-142586 sodium salt were obtained from Toronto Research Chemicals (ON, Canada). Probenecid, lansoprazole, and omeprazole were acquired from FUJIFILM WAKO Pure Chemical (Osaka, Japan). Esomeprazole was purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals utilized were of the highest available purity.

2.3. Retrospective study in patients receiving linezolid

Clinical data were retrieved from electronic medical records of 225 hospitalized patients who underwent 1200 mg/day linezolid (ZYVOX® Injection 600 mg) therapy in the Department of Intensive Care Unit at Osaka University Hospital from January 2000 to June 2020. Patients were excluded if they had missing data, baseline platelet counts (PLTs) < 100 ($\times 10^9$/L) before linezolid therapy, received platelet infusion treatment, or had a linezolid dosing period < 3 days. Thrombocytopenia was defined as a reduction in platelet counts < 100 ($\times 10^9$/L) or a reduction of more than 30% from the baseline value according to previous criteria [10]. Given the inhibitory effect of PPIs on hOAT3 [11,12], the impact of concomitant PPIs on the development of linezolid-induced thrombocytopenia was assessed. Patients on PPIs were those who continued PPI treatment during linezolid therapy. This study was conducted in compliance with the Declaration of Helsinki and approved by the Ethics Committee of Osaka University Hospital (No.16002–8).

2.4. Analyses on the effect of PPIs on linezolid-associated thrombocytopenia using the FAERS database

Data on patient demographics and administration information (DEMO), drug/biologic information (DRUG), and adverse events (REAC) from July 2014 to December 2019 were obtained from the FAERS database released by the FDA. Duplicate reports were excluded following FDA recommendations. Data analyses were conducted using ACCESS® 2019 software (Microsoft, Redmond, WA). Information related to linezolid administration was extracted, and disease names were defined using the Medical Dictionary for Regulatory Activities (MedDRA/J) version 24.0. The standardized MedDRA Query (SMQ) was employed to search for thrombocytopenia (SMQ code: 20000027). The effect of PPIs (lansoprazole, rabeprazole, omeprazole, and esomeprazole) on linezolid-associated thrombocytopenia was evaluated using the reporting odds ratio (ROR). To calculate the ROR, linezolid-associated thrombocytopenia and all other reported adverse events linked to linezolid were categorized as "cases" and "non-cases," respectively. RORs were computed from two-by-two contingency tables of counts with or without PPIs. RORs were expressed as point estimates with a 95% confidence interval (CI). A positive signal was defined as the lower limit of the 95% CI for the ROR > 1 [13].

2.5. Cell culture

The hOAT1 and hOAT3-expressing human embryonic kidney cell line HEK293 (HEK-hOAT1 and HEK-hOAT3) and mock-transfectants obtained by transfecting pBKCMV vector into HEK293 cells (HEK-pBK) were generously provided by Dr. Atsushi Yonezawa (Department of Pharmacy, Kyoto University Hospital, Japan) and cultured as previously described [12]. MEG-01 cell was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). MEG-01 cells have the capability to produce platelet-like particles and are considered the most suitable cell line for analyzing human megakaryocytic maturation and differentiation [14]. MEG-01 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. HEK-hOAT1, HEK-hOAT3, and HEK-pBK cells were used between passage numbers 90 and 100, while MEG-01 cells were used between passage numbers 10 and 16. All cell lines were maintained at 37°C under 5% CO_2 in a humidified atmosphere.

2.6. Uptake study using HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells

The cells (12×10^5 cells/dish) were seeded in 3.5 cm dishes with culture medium in the absence of G418. After 48 h of culture, the cell monolayers were utilized for the uptake study. The cellular uptake of 6-CF (a well-established substrate of hOAT1 and hOAT3), linezolid, PNU-142300, and PNU-142586 was determined using monolayer cultures of HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM HEPES (pH 7.4). After preincubation with the incubation medium for 10 min at 37°C, the cells were incubated with 5 μM 6-CF for 2 min or with 1 μM linezolid, PNU-142300, and PNU-142586 for a specified duration at 37°C. For inhibition experiments of PNU-142586 in HEK-hOAT3 cells, the cells were incubated for 5 min with 1 μM PNU-142586 in the absence or presence of 100 μM probenecid (a typical inhibitor of hOAT3) or various concentrations of PPIs (lansoprazole, rabeprazole, omeprazole, and

esomeprazole). To evaluate the accumulation of linezolid, PNU-142300, and PNU-142586 into the cells, these drugs were eluted with 0.5 mL of 50% MeOH and then subjected to ultra-performance liquid chromatography equipped with tandem mass spectrometry (UPLC-MS/MS). To assess intracellular 6-CF accumulation, the cells were solubilized in 1 M NaOH, and fluorescence was measured with a fluorescence spectrophotometer (SH-9000lab, CORONA, Ibaraki, Japan) at 495 nm excitation/517 nm emission. The protein contents of the solubilized cells were measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). The apparent IC_{50} values were generated from curve fits using GraphPad Prism version 8.4.3 (GraphPad Software Inc., San Diego, CA).

2.7. Cell viability in MEG-01 cells

MEG-01 cells were seeded on a 96-well plate at a density of 5.0×10^3 cells/well and were incubated with the culture medium containing various concentrations of linezolid, PNU-142300, and PNU-142586 for 72 h. After incubation, the cell viability was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. The absorbance was measured at 450 nm with the MultiskanTM FC Microplate Absorbance Reader (ThermoFisher Scientific, Waltham, MA). The cell viability after treatment with the vehicle (control) was set at 100%.

2.8. Effect of concomitant lansoprazole on the pharmacokinetics of linezolid, PNU-142300, and PNU-142586 after intravenous administration of linezolid in rats

Eight-week-old male Wistar rats (SLC Japan Co., Shizuoka, Japan) were utilized for the pharmacokinetics study. All animal procedures were conducted following guidelines published by the Ministry of Education, Culture, Sports, Science, and Technology in Japan, and were approved by the ethics boards of Osaka University (No. 03–018–000). The rats were anesthetized with an intraperitoneal injection of a mixture of medetomidine, midazolam, and butorphanol at doses of 0.38, 2.0, and 2.5 mg/kg, respectively. Polyethylene catheters were implanted into the femoral vein and femoral artery to administer the drug and facilitate frequent blood collection. Subsequently, the rats received intravenous injection (i.v.) of linezolid (10 mg/kg) through the femoral vein with and without lansoprazole (4 mg/kg, i.v.). Blood samples were obtained from the femoral artery at 0, 1, 5, 10, 15, 30, 60, 90, 120, 150, 180, 210, and 240 min after linezolid administration. The concentrations of linezolid, PNU-142300, and PNU-142586 in plasma were determined by UPLC-MS/MS. The area under the plasma concentration-time curve from 0 to 240 min ($AUC_{0–240}$) was calculated using the trapezoidal rule. Moreover, the systemic clearance (CL_{tot}), elimination rate constant from the central compartment (K_{el}), distribution volume of the central compartment (V_d), and half-life ($T_{1/2}$) were calculated according to the procedures for 2-compartmental analysis.

2.9. Determination of linezolid, PNU-142300, and PNU-142586 in cells and plasma

Based on a previous report [15], UPLC-MS/MS was employed for the determination of linezolid, PNU-142300, and PNU-142586 in cells and plasma. The LC-MS/MS system was applied with the ACQUITY HPLC H-class/ACQUITY TQD with electrospray ionization (Waters, Milford, MA). First, 10 μL of 10 ng/mL benzyl (3-fluoro-4-morpholinophenyl) carbamate, utilized as an internal standard (IS), was added to the samples (100 μL). The samples (10 μL) were then subjected to LC-MS/MS. LC separations were performed on an InterSustainSwift C18 HP column (2.1 × 150 mm, 3 μm, GL Sciences, Tokyo, Japan) maintained at 40°C with a flow rate of 0.2 mL/min. Solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The entire LC gradient was 16 min. Mobile phase B was initially at 10%, ramped to 95% from 1 to 12 min, and then back to 10% from 12 to 16 min. Linezolid, PNU-142300, PNU-142586, and IS were detected by multiple reaction monitoring mode. MS/MS conditions involved cone voltages and collision energies of 40 V/20 eV (linezolid, PNU-142586, and IS) and 60 V/10 eV (PNU-142300) in positive mode, respectively. MS/MS monitoring ions were as follows: linezolid (*m/z* 338.45 $[M + H]^+ \rightarrow$ *m/z* 296.37), PNU-142300 (*m/z* 370.15 $[M + H]^+ \rightarrow$ *m/z* 340.24), PNU-142586 (*m/z* 370.15 $[M + H]^+ \rightarrow$ *m/z* 324.38), and IS (*m/z* 331.44 $[M + H]^+ \rightarrow$ *m/z* 91.19). The desolvation temperature was 350°C, cone gas flow was 50 L/h, and desolvation gas flow was 600 L/h. All LC-MS/MS data were collected and processed by Masslynx 4.1 software (Waters, Milford, MA).

Table 1
Patients' characteristics.

	No thrombocytopenia (n = 85)	Thrombocytopenia (n = 65)	*p* value
Age (years)	63 [24–90]	67 [29–95]	0.208
Male	66 (78)	48 (74)	0.700
Linezolid dose (mg/kg/day)	19.5 [9.4–59.1]	20.1 [7.2–31.7]	0.934
Duration of linezolid treatment (days)	7 [3–42]	8 [3–81]	0.009
Baseline biological parameters			
AST (U/L)	38 [13–235]	42 [11–3556]	0.134
ALT (U/L)	29 [6–323]	33 [5–1921]	0.866
T-Bil (mg/dL)	0.6 [0.2–14.6]	0.8 [0.2–23.0]	0.080
CCr (mL/min)	87.5 [5.6–285.1]	61.6 [7.1–182.9]	0.011
WBC ($\times 10^9$/L)	11.22 [0.10–30.84]	11.60 [3.27–27.85]	0.833
PLT ($\times 10^9$/L)	227 [111–667]	225 [106–564]	0.935
Hb (g/dL)	9.7 [6.2–14.0]	10.0 [6.0–15.8]	0.143
Alb (g/dL)	2.3 [0.4–4.4]	2.4 [0.8–4.4]	0.784
CRP (mg/dL)	10.99 [0.18–27.02]	12.20 [0.06–35.64]	0.416
Co-administrated PPIs			
Lansoprazole	35 (41)	40 (62)	0.021
Other PPIs	12 (14)	8 (12)	0.812
Type of infection			
Sepsis	20 (24)	19 (29)	0.457
Pneumonia	39 (46)	23 (35)	0.242
Others	26 (31)	23 (35)	0.600

Values are presented as median [range] or number (%). These results are based on a review of electronic medical records at Osaka University Hospital. Statistical analyses were performed using Fisher's exact test or Mann–Whitney U test. "Other PPIs" represents esomeprazole (n = 6), omeprazole (n = 13), and rabeprazole (n = 1).
CCr was estimated by the Cockcroft–Gault equation.
Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCr, creatinine clearance; CRP, c-reactive protein; Hb, hemoglobin; PLT, platelet; T-bil, total bilirubin; WBC, white blood cell.

2.10. Statistical analyses

The results of the *in vitro* and *in vivo* experimental data are expressed as the mean ± standard deviation (S.D.). Statistical analyses for multiple groups were carried out using one-way analysis of variance followed by Dunnett's test. Differences between two groups in the *in vitro* study were determined by the unpaired t-test. For the clinical study, statistical comparisons between two groups were performed using the Mann–Whitney U test and Fisher's exact test for continuous and categorical variables, respectively. The incidence of thrombocytopenia was analyzed using the Kaplan–Meier curve method and assessed with the Gehan–Breslow–Wilcoxon test. Multivariate logistic regression analysis was performed to identify the risk factors of thrombocytopenia following linezolid administration with JMP® Pro 14.3.0 (SAS Institute Inc., Cary, NC, USA). The logistic regression model was adjusted for the following potential confounding factors: age, sex, linezolid dose, duration of linezolid treatment, PLT, creatinine clearance (CCr), total

Table 2
Multivariate analysis for risk factors of thrombocytopenia following linezolid administration.

Variable	Odds ratio	95% CI	*p* value
Age (years)	1.01	0.98–1.03	0.595
Male	0.71	0.30–1.72	0.454
Linezolid dose (mg/kg/day)	0.96	0.88–1.04	0.258
Duration of linezolid treatment (days)	1.07	1.01–1.13	0.007
Baseline PLT ($\times 10^9$/L)	1.00	0.99–1.01	0.067
Baseline CCr (mL/min)	0.99	0.98–0.99	0.008
Baseline T-Bil (mg/dL)	1.05	0.89–1.21	0.556
Lansoprazole	2.33	1.06–5.16	0.034
Other PPIs	1.84	0.60–5.66	0.292

"Other PPIs" represents esomeprazole, omeprazole, and rabeprazole. These results are based on a review of electronic medical records at Osaka University Hospital.
CCr, creatinine clearance; CI, confidence interval; PLT, platelet; PPI, proton pump inhibitor; T-bil, total bilirubin.

bilirubin (T-Bil), co-administration of lansoprazole, and other PPIs. Statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad Software Inc., San Diego, CA). A two-tailed *p*-value < 0.05 was considered statistically significant, and the CI was set to 95%.

3. Results

3.1. Patients' characteristics

According to the inclusion and exclusion criteria, 150 patients were enrolled in the present study. Table 1 compares the characteristics of patients with and without thrombocytopenia. Among the enrolled patients, 65 (43%) developed thrombocytopenia, while 85 (57%) did not. Patients who developed thrombocytopenia had a significantly longer duration of linezolid treatment compared to those who did not ($p = 0.009$). Additionally, patients with thrombocytopenia had significantly lower CCr values than those without thrombocytopenia ($p = 0.011$). Notably, the rate of concomitant lansoprazole use, but not other PPIs, was significantly higher in patients with thrombocytopenia (62%) compared to those without (41%, $p = 0.021$). However, other patient characteristics did not show significant differences.

3.2. Multivariate analysis for thrombocytopenia associated with linezolid

A multivariate logistic regression analysis was conducted to assess the risk factors for thrombocytopenia in patients receiving linezolid (Table 2). The results revealed that the duration of linezolid treatment (OR: 1.07, $p = 0.007$), CCr value (OR: 0.99, $p = 0.008$), and co-administration of lansoprazole (OR: 2.33, $p = 0.034$) were significant risk factors for thrombocytopenia associated with linezolid. Conversely, the co-administration of other PPIs was not found to be a significant risk factor for thrombocytopenia.

3.3. Comparison of minimum PLT count and onset time of thrombocytopenia following linezolid between patients with and without lansoprazole

Based on the results of univariate and multivariate analyses (Tables 1 and 2), we investigated the impact of concomitant lansoprazole on the severity and onset time of thrombocytopenia following linezolid. Fig. 1A illustrates the comparison of the minimum platelet count following linezolid administration between patients with and without lansoprazole. Patients receiving lansoprazole exhibited a significantly lower minimum platelet count compared to those not receiving lansoprazole ($p = 0.003$). Furthermore, we assessed the effect of lansoprazole co-administration on the time to develop thrombocytopenia following linezolid administration using Kaplan–Meier analysis (Fig. 1B). The analysis demonstrated that the time to thrombocytopenia following linezolid administration was significantly shorter in patients receiving lansoprazole ($p = 0.020$, Wilcoxon test).

Table 3
Analyses of the impact of PPIs on thrombocytopenia following linezolid therapy using the FAERS database.

PPIs	Linezolid-induced thrombocytopenia (%)		ROR (95% CI)	*p* value
	Without drug	With drug		
Lansoprazole	1060 / 11,715 (9)	63 / 449 (14)	1.64 (1.25–2.16)	< 0.001
Other PPIs	1003 / 10,832 (10)	120 / 1332 (10)	0.97 (0.80–1.18)	0.802

Linezolid-induced thrombocytopenia presented as cases / (cases + non-cases) (%).
Fisher's exact test was performed.
"Other PPIs" represents esomeprazole, omeprazole, and rabeprazole.
CI, confidence interval; FAERS, FDA adverse event reporting system; PPI, proton pump inhibitor; ROR, reporting odds ratio.

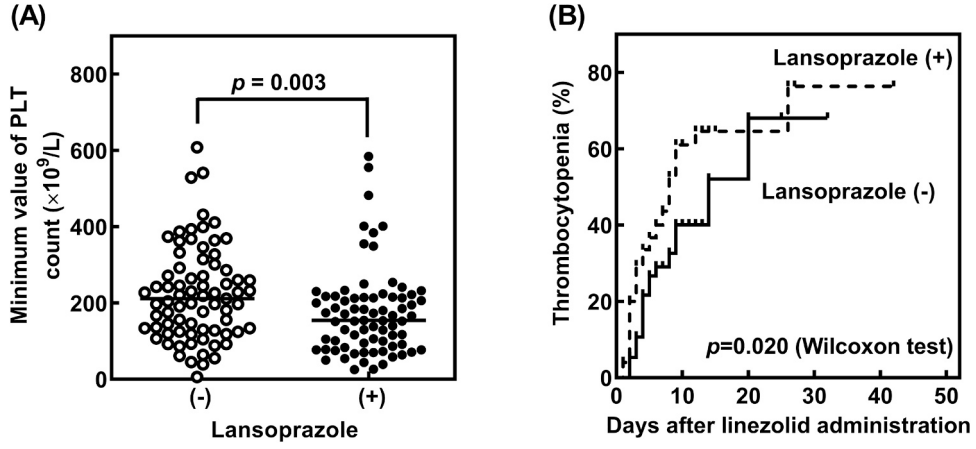

Fig. 1. Comparison of minimum value of PLT counts (A) and Kaplan–Meier analysis for thrombocytopenia (B) following linezolid administration in patients without lansoprazole (n = 75) and with lansoprazole (n = 75). (A) Each point represents a patient, and medians are indicated by horizontal lines. Statistical analyses were performed using the Mann–Whitney U test. (B) Statistical analyses were performed using the Gehan–Breslow–Wilcoxon test. These results are based on a review of medical records at Osaka University Hospital.

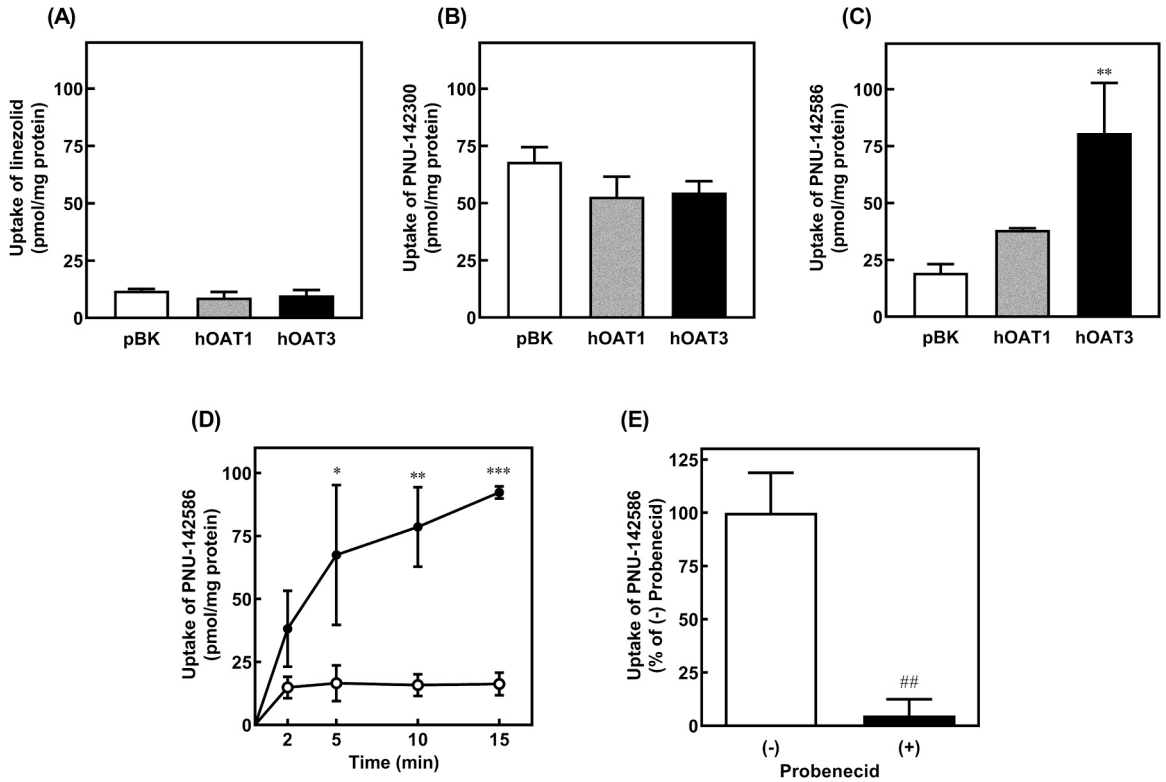

Fig. 2. Uptake of linezolid, PNU-142300, and PNU-142586. (A, B, C) HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells were incubated for 5 min at 37°C with 1 μM linezolid, PNU-142300, and PNU-142586 (pH 7.4). (D) HEK-hOAT3 (closed circles) or HEK-pBK (open circles) cells were incubated with PNU-142586 (1 μM, pH 7.4) for specified durations (2, 5, 10, and 15 min) at 37°C. (E) HEK-hOAT3 cells were incubated at 37°C for 5 min with PNU-142586 (1 μM) in the absence or presence of probenecid (100 μM). Each column and point represent the mean ± S.D. of three separate experiments using three monolayers. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with HEK-pBK cells. $^{\#\#}p < 0.01$ compared with probenecid (–).

3.4. Analyses of the impact of PPIs on thrombocytopenia following linezolid therapy using the FAERS database

From the FAERS database, we extracted data from 12,164 patients treated with linezolid. We analyzed the reporting ratio of linezolid-induced thrombocytopenia, ROR, and 95% CI (Table 3). The reporting ratio of thrombocytopenia in patients receiving lansoprazole (14%) was significantly higher than that in patients not receiving lansoprazole (9%, $p < 0.001$). Additionally, a positive signal was observed with co-administered lansoprazole (ROR: 1.64, 95% CI: 1.25–2.16), whereas no significant signals were found with co-administered other PPIs (ROR: 0.97, 95% CI: 0.80–1.18).

3.5. Uptake of 6-CF in HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells

To confirm the activity of hOAT1 and hOAT3 in HEK-hOAT1 and HEK-hOAT3 cells, respectively, we conducted an uptake study of 6-CF (5 μM) (Supplementary Figure 1). The uptake of 6-CF in HEK-hOAT1 and HEK-hOAT3 cells was approximately 10.2- and 3.9-fold higher, respectively, than in HEK-pBK cells, the corresponding controls. These results confirmed the activities of each transporter in HEK-hOAT1 and HEK-hOAT3 cells.

3.6. Uptake of linezolid, PNU-142300, and PNU-142586 in HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells

We evaluated the uptake of linezolid, PNU-142300, and PNU-142586 (Figs. 2A, 2B, and 2C, respectively) for 5 min in HEK-pBK, HEK-hOAT1, and HEK-hOAT3 cells. The uptake of PNU-142586, but not linezolid and PNU-142300, was significantly higher in HEK-hOAT3 cells compared to HEK-pBK and HEK-hOAT1 cells ($p < 0.01$). Furthermore, the uptake of PNU-142586 in HEK-hOAT3 cells increased in a time-dependent manner (Fig. 2D). To verify whether PNU-142586 is specifically transported by hOAT3, we measured the cellular uptake of PNU-142586 (1 μM) for 5 min in the absence or presence of 100 μM probenecid, a typical inhibitor of hOAT3 (Fig. 2E). Probenecid potently inhibited the hOAT3-mediated transport of PNU-142586 (Fig. 2E).

3.7. Inhibition of PNU-142586 uptake by lansoprazole and other PPIs in HEK-hOAT3 cells

To assess whether lansoprazole and other PPIs inhibit hOAT3-mediated transport of PNU-142586, we measured the cellular uptake of PNU-142586 (1 μM) for 5 min in the absence or presence of various concentrations of PPIs (Fig. 3). All investigated PPIs inhibited hOAT3-mediated uptake of PNU-142586 in a concentration-dependent manner. Particularly, lansoprazole demonstrated a potent inhibitory effect (IC_{50} = 0.59 ± 0.38 μM) against PNU-142586 transport via hOAT3. The rank order of inhibitory effect on hOAT3-mediated transport of PNU-142586 was as follows: lansoprazole ≫ esomeprazole ≈ omeprazole ≈ rabeprazole.

3.8. Effect of concomitant lansoprazole on the pharmacokinetics of linezolid, PNU-142300, and PNU-142586 after intravenous administration of linezolid in rats

To verify whether the plasma concentration of PNU-142586 is increased by concomitant lansoprazole, we conducted a pharmacokinetic study in rats after intravenous administration of linezolid. When we preliminarily examined the plasma concentration of lansoprazole after intravenous administration of lansoprazole (4 mg/kg) in rats, the maximum plasma concentration (C_{max}) of lansoprazole was

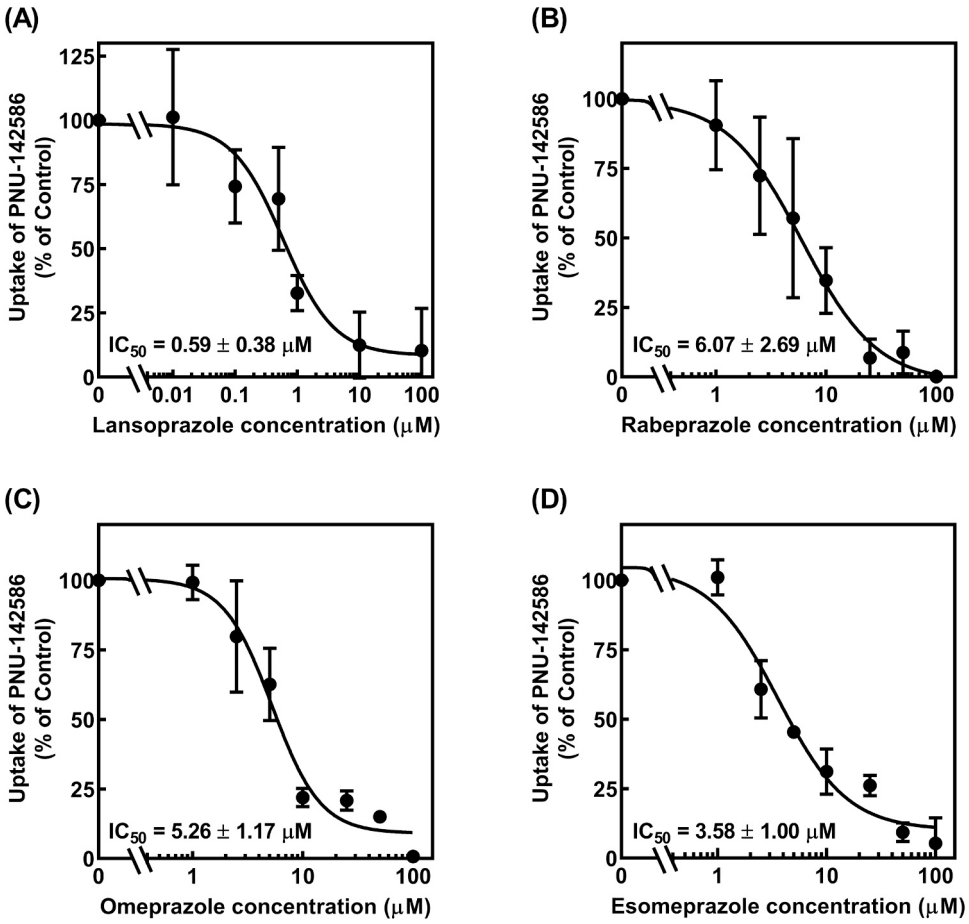

Fig. 3. Inhibition of PNU-142586 uptake by PPIs in HEK-hOAT3 cells. HEK-hOAT3 cells were incubated at 37°C for 5 min with 1 μM PNU-142586 (pH 7.4) in the absence or presence of various concentrations of lansoprazole (A), rabeprazole (B), omeprazole (C), and esomeprazole (D). Each point represents mean ± S.D. of three separate experiments using three monolayers. When the standard errors of the means are small, they are contained within the symbols. The apparent IC_{50} values were calculated by fitting the data to a sigmoidal dose-response regression curve.

approximately 3.8 μg/mL, comparable to clinical concentration (2.3–3.1 μg/mL). Therefore, the dose of lansoprazole was set at 4 mg/kg. The plasma concentration-time profiles of linezolid, PNU-142586, and PNU-142300 after linezolid (10 mg/kg) with or without lansoprazole (4 mg/kg) are shown in Fig. 4A, C, and E, respectively. The pharmacokinetic parameters of linezolid are summarized in Supplementary Table 2. Although there were no significant differences in pharmacokinetic parameters of linezolid (AUC, CL_{tot}, K_{el}, V_d, and $T_{1/2}$) between rats with and without lansoprazole, the plasma concentration of PNU-142586 was significantly increased at 90, 180, and 240 min by concomitant lansoprazole. Additionally, the AUC_{0-240} of PNU-142586, but not linezolid and PNU-142300, was increased approximately 1.7-fold when linezolid was administered intravenously with lansoprazole (Fig. 4B, D, and F).

3.9. Effect of linezolid, PNU-142300, and PNU-142586 on the viability in MEG-01 cells

To assess whether linezolid, PNU-142300, and PNU-142586 have cytotoxic effects on platelets, we investigated viability in MEG-01 cells after exposure to various concentrations of linezolid, PNU-142300, and PNU-142586 for 72 h (Fig. 5). The cell viability was not affected by exposure to linezolid. However, cell viability decreased after exposure to PNU-142300 and PNU-142586 in a concentration-dependent manner. Exposure to PNU-142300 and PNU-142586 at a concentration of 25 μM significantly decreased viability in MEG-01 cells compared to the control (vehicle).

4. Discussion

The underlying mechanism of linezolid-induced thrombocytopenia remains to be fully clarified. Our study demonstrated for the first time, to our knowledge, that linezolid-induced thrombocytopenia should be caused by the delayed elimination of PNU-142586, and that this delayed elimination due to renal failure and hOAT3-mediated transport inhibition by lansoprazole should exacerbate linezolid-induced thrombocytopenia. Moreover, we clarified that PNU-142586, but not linezolid, has cytotoxic effects on platelets.

The analyses of two clinical databases demonstrated that concomitant lansoprazole, but not other PPIs, was a significant risk factor for thrombocytopenia associated with linezolid (Tables 2 and 3). A previous retrospective study showed that concomitant PPIs did not affect the development of linezolid-induced thrombocytopenia [16]. However, in a pharmacokinetics/pharmacodynamics analysis of linezolid in pediatric patients treated with linezolid, concomitant PPI use was found to significantly contribute to increased trough concentrations of linezolid [17]. Therefore, the potential drug interaction between linezolid and PPIs remains controversial. Our previous study demonstrated that lansoprazole is a more potent inhibitor of hOAT3 compared to other PPIs [12]. Despite this, the contribution of hOAT3 to the pharmacokinetics of linezolid, PNU-142300, and PNU-142586 remained to be elucidated. Therefore, we first examined whether linezolid, PNU-142300, and PNU-142586 are transported by hOAT1 and/or hOAT3. As shown in Fig. 2, our present study demonstrates for the first time, to our knowledge, that PNU-142586, but not linezolid and PNU-142300, is

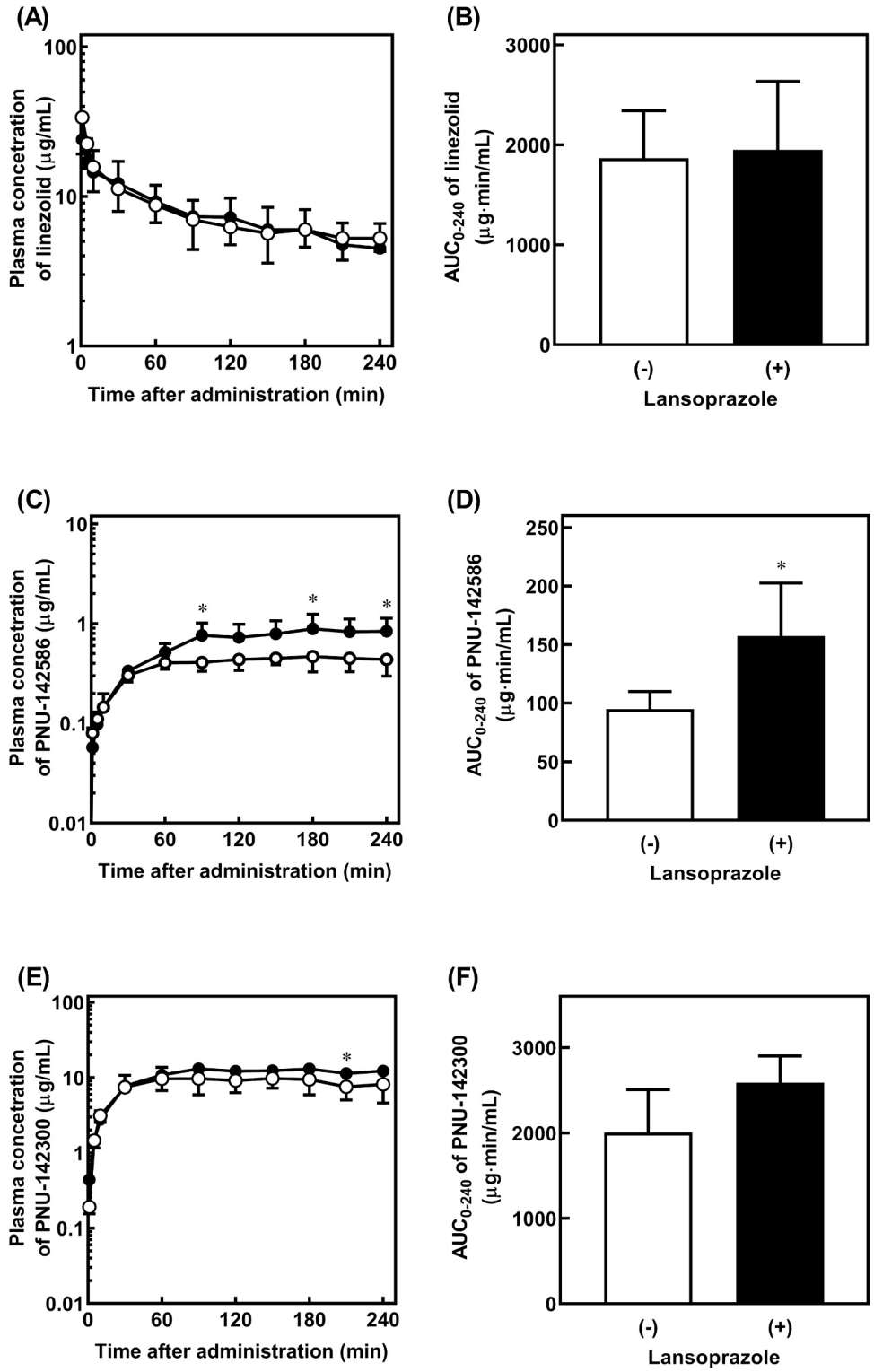

Fig. 4. Plasma concentration of linezolid (A), PNU-142586 (C), and PNU-142300 (E) after intravenous administration of linezolid (10 mg/kg) without (open circles) or with (closed circles) lansoprazole (4 mg/kg) in rats. The area under the plasma concentration (AUC) of linezolid (B), PNU-142586 (D), and PNU-142300 (F) from 0 to 240 min after linezolid administration. The AUC was calculated using the trapezoidal rule. Each point represents the mean ± S.D. of four rats. These results are based on *in vivo* experiments using rats. *$p < 0.05$ compared to rats without lansoprazole.

transported by hOAT3. These findings strongly suggest that hOAT3 should be involved in the clearance of PNU-142586.

The guidance regarding transporter-mediated drug interactions published by the U.S. FDA in 2020 indicates that a ratio of unbound C_{max} to IC_{50} value ≥ 0.1 indicates recommendation for the evaluation of clinical drug interaction. As shown in Supplementary Table 1, the ratio of unbound C_{max} to IC_{50} value of lansoprazole was 0.19–0.37 (≥ 0.1), suggesting that clinical drug interaction between PNU-142586 and lansoprazole should be investigated. Conversely, the ratios of unbound C_{max} to IC_{50} values of other PPIs, excluding lansoprazole, were much

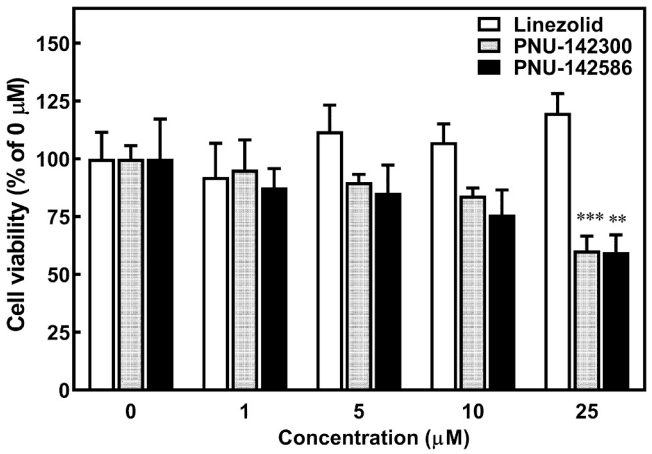

Fig. 5. Effect of linezolid, PNU-142300, and PNU-142586 on cell viability in MEG-01 cells. Cell viability after exposure to various concentrations of linezolid, PNU-142300, and PNU-142586 in MEG-01 cells for 72 h. The cell viability after treatment of control (vehicle) was set at 100%. Each column represents the mean ± S.D. of three separate experiments (n = 3). Statistical analyses were performed using Dunnett's test. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control (vehicle) in each drug.

lower than 0.1 (Supplementary Table 1). Our previous studies reported that lansoprazole exhibited a potent inhibitory effect against hOAT3 compared to other PPIs, such as pantoprazole, omeprazole, rabeprazole, esomeprazole, and vonoprazan [12,18], which is consistent with the present results. Therefore, co-administration of lansoprazole, but not other PPIs, could potentially lead to clinical drug interactions with PNU-142586.

Based on these findings, we investigated the effect of concomitant lansoprazole at clinical concentrations on the pharmacokinetics of linezolid, PNU-142300, and PNU-142586 after intravenous administration of linezolid in rats. Interestingly, we confirmed an increased AUC of PNU-142586, but not linezolid and PNU-142300, with concomitant lansoprazole (Fig. 4). In addition, concomitant lansoprazole did not affect the elimination and distribution of linezolid (Supplementary Table 2). Thus, these results suggest that the delayed elimination of PNU-142586 after linezolid administration would be caused when combined with lansoprazole. However, it is worth noting that the plasma metabolite profile differs slightly between humans and rats. In humans, PNU-142586 is the most abundant in plasma, unlike in rats [19]. Therefore, it is presumed that the impact of delayed elimination of PNU-142586 due to concomitant lansoprazole should be more pronounced in humans than in rats.

Although the mechanism underlying the development of linezolid-induced thrombocytopenia has not been fully clarified, Tajima et al. [20] suggested that it might be caused by enhanced consumption/destruction or reduced production of platelets after treatment with linezolid. However, they did not observe cytotoxicity of linezolid in rat platelet-rich plasma or the human platelet precursor cell line (MEG-01 cells) [20], and the cytotoxicity of PNU-142586 and PNU-142300 against platelets remains unclear. Therefore, we hypothesized that PNU-142586 and/or PNU-142300, but not linezolid, may have cytotoxic effects on platelets. As shown in Fig. 5, we observed cytotoxicity of PNU-142586 and PNU-142300 (25 μM), but not linezolid, in MEG-01 cells. When linezolid was administered at 1200 mg/day to patients with normal renal function, the maximum unbound plasma concentrations of PNU-142586 and PNU-142300 were reported to be approximately 9 and 3 μM, respectively. In contrast, in patients with renal dysfunction, these concentrations reached approximately 26 and 14 μM, respectively [6]. Additionally, the AUC of PNU-142586, but not linezolid and PNU-142300, increased approximately 1.7-fold when linezolid was administered intravenously with lansoprazole in rats (Fig. 4). Therefore, these findings suggest that the increased plasma concentration of PNU-142586, and its consequent cytotoxicity for platelets, could be more relevant to the development of thrombocytopenia associated with linezolid.

As shown in Table 2, a multivariate logistic regression analysis revealed that the duration of linezolid treatment and CCr were significant risk factors associated with linezolid-induced thrombocytopenia. Given that linezolid induces reversible and time-dependent myelosuppression, prolonged exposure to linezolid could potentially increase the incidence of thrombocytopenia [21]. Previous studies have also reported that long-term use of linezolid and reduced renal function are risk factors for increased thrombocytopenia development [3,4]. Our findings align with these previous reports, highlighting the need for vigilance regarding thrombocytopenia development in patients with renal failure and prolonged linezolid use, as well as those receiving lansoprazole. Moreover, when patients were divided to two groups based on CCr (< 60 mL/min, n = 62; ≥ 60 mL/min, n = 88), exacerbation of thrombocytopenia by lansoprazole was observed in both groups. Therefore, concomitant lansoprazole could exacerbate linezolid-induced thrombocytopenia regardless of renal function.

However, our study had some limitations that need to be considered. First, it remains unclear whether the inhibition of lansoprazole on renal hOAT3-mediated excretion of PNU-142586 primarily contributes to the exacerbation of linezolid-induced thrombocytopenia, as we could not assess increased plasma concentrations of linezolid, PNU-142300, and PNU-142586 in patients with thrombocytopenia and in patients receiving lansoprazole. Second, this study involved patients from a single institution, raising the possibility of selection bias. Additionally, due to the retrospective nature of the study, examining potential confounders was challenging. Therefore, future large-scale and multicenter prospective studies are conducted to evaluate the impact of PPIs on thrombocytopenia and the pharmacokinetics of linezolid, PNU-142300, and PNU-142586.

5. Conclusions

Our study demonstrated for the first time, to our knowledge, that linezolid-induced thrombocytopenia should result from delayed elimination of PNU-142586, exacerbated by renal failure and hOAT3-mediated transport inhibition by lansoprazole. Therefore, careful monitoring for thrombocytopenia development is essential in patients receiving both lansoprazole and linezolid. Alternatively, consideration should be given to discontinuing lansoprazole or switching to other PPIs during linezolid therapy. These findings offer valuable insights into the prudent use of linezolid in antimicrobial treatment strategies.

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CRediT authorship contribution statement

Danni Wang: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis. **Fumihiro Yamane:** Investigation. **Masahiro Okuda:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Kenji Ikemura:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Tsubasa Hasegawa:** Investigation, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116801.

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