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ORIGINAL ARTICLE

Real-time platelet $P2Y_{12}$ receptor occupancy as a promising pharmacodynamics biomarker for bridging the gap between PK/PD of clopidogrel therapy

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Abstract Clopidogrel effectively inhibits platelet aggregation in response to ADP by irreversibly binding to the platelet $P2Y_{12}$ receptor through its active metabolite. However, the observed discrepancies between the pharmacokinetics (PK) and pharmacodynamics (PD) of clopidogrel present substantial challenges in individualizing of antiplatelet therapy. To address these challenges, a robust liquid chromatography–tandem mass spectrometry method has been developed to facilitate the real-time assessment of platelet $P2Y_{12}$ receptor occupancy. This method has been validated in animal models, providing a reliable link between individual PK profiles and PD effects. Target receptor occupancy offers a comprehensive overview of interindividual variations in clopidogrel metabolism, regulation of $P2Y_{12}$ receptor expression, and platelet turnover. Moreover, it directly correlates with the inhibitory effect on platelet aggregation. The levels of platelet $P2Y_{12}$ occupancy accurately reflect the extent of clinical factors influencing the PD of clopidogrel, including dosage, drug–drug interactions (DDI), and type 2 diabetes mellitus (T2DM). As a normalized metric, platelet $P2Y_{12}$ occupancy not only serves potential as a diagnostic tool for personalized clopidogrel therapy but also aids in elucidating the role of the $P2Y_{12}$ signaling pathway in cases of abnormal on-treatment platelet reactivity.

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1. Introduction

Significant inter-individual variability (IIV) in response to clopidogrel-therapy is associated with adverse clinical outcomes. Both high and low platelet reactivities during treatment correlate with increased risks of ischemic and bleeding events, respectively[1]. Studies have shown that the etiology of IIV is multifaceted, encompassing genetic, clinical, and cellular factors that contribute to resistance to clopidogrel-therapy[2]. The current expert consensus statement does not endorse routine genotype testing to evaluate the impact of IIV caused by metabolic dysfunction on individual clinical outcomes[3,4]. Guidelines recommend considering specific clinical characteristics to guide individualized antiplatelet therapy[5].

In the established framework of receptor theory, the efficacy of an antagonist is generally linked to factors such as exposure at the site of action, target engagement, and the expression of functional pharmacological activity[6]. Traditional PK studies usually relied on drug-plasma concentrations as proxies to estimate drug exposure at the target site. However, clopidogrel (Clop) is a typical prodrug, and its parent form lacks biological activity. The conversion of Clop into its active form (AM) is facilitated by various cytochrome P450 (CYP) enzymes in the liver, notably CYP2C19, 1A2, 2B6, 2C9, and 3A4[7]. The AM features a highly reactive free thiol group that interacts with the cysteine 97 in the extracellular domain of $P2Y_{12}$, resulting in the formation of a covalent disulfide bond. By inhibiting the platelet $P2Y_{12}$ receptor, AM suppresses adenylate cyclase activity, which reduces intracellular cyclic adenosine monophosphate levels and ultimately inhibits platelet aggregation. Since the inhibition of $P2Y_{12}$ by AM is irreversible, there is no straightforward correlation between plasma levels of the AM and its antiaggregant activity, nor is there a conventional therapeutic window.

Several platelet function testing (PFT) methods are currently employed to assess the biological impacts of antiplatelet medications[8]. However, platelets can be activated through multiple signaling pathways, including those mediated by purinergic receptors $P2X_1$, $P2Y_1$, $P2Y_{12}$, thromboxane, and collagen receptors[9]. Consequently, the outcomes of most PFTs do not solely reflect the contribution of the $P2Y_{12}$ receptor on platelets. Therefore, most clinical guidelines do not advocate for routine PFT-guided clopidogrel-therapy as a strategy to optimize clinical results[10].

The advancement of the radioactive tracer technique has enabled the implementation of non-invasive target engagement assays for both reversible and irreversible ligands in clinical studies. This approach has been leveraged to investigate the potential of target engagement as a PD biomarker for Clop[11]. Early studies, conducted two decades ago, utilized ^{33}P-labeled 2MeS-ADP to measure $P2Y_{12}$ receptor occupancy by Clop[12,13]. These studies demonstrated variable levels of $P2Y_{12}$ receptor engagement, which were associated with the classification of individuals as low, average, or high responders to Clop[14,15]. However, the specificity of ^{33}P-2MeS-ADP was found to be inadequate. The quantification of ADP receptors using this tracer suggested a range between 500 and 1000 receptors per platelet[16], figures significantly higher than the typical count of approximately 400 $P2Y_{12}$ receptors per platelet[17]. This discrepancy highlights the need for more specific assays in accurately determining receptor occupancy.

Receptor occupancy assays utilizing flow cytometry have become prevalent for quantifying the interaction between therapeutic antibodies and their target receptors. These assays measure the receptors that are either occupied by therapeutics or remain unbound, employing one or more specific antibodies designed for flow cytometry. The data on receptor occupancy thus obtained serve as PD biomarkers, crucial for assessing biopharmaceuticals' dose-response relationship in both nonclinical and clinical studies[18]. However, these assays are limited in their ability to differentiate between unbound receptors and those occupied by small molecular ligands. Additionally, flow cytometry assays require a separate baseline measurement, akin to the methodology used in target engagement assays with radiotracers. Both approaches encounter challenges due to their inability to provide real-time data and the complexities involved in data standardization.

Liquid chromatography-tandem mass spectrometry (LC−MS/MS) is a robust analytical technique that has gained widespread acceptance in pharmacokinetic studies due to its exceptional sensitivity, selectivity, and efficiency[19,20]. Zheng et al.[21] developed a novel strategy for the real-time assessment of receptor occupancy for branebrutinib, a covalent inhibitor of Bruton's tyrosine kinase (BTK). Their strategy employs a structural analog to quench any unoccupied BTK post-administration. The BTKs are subsequently concentrated *via* immunocapture and digested with trypsin. Analysis of the resulting peptide segments, which are bound either to the drug or the quencher, is performed using LC−MS/MS. These peptide segments act as surrogate analytes for BTKs that are either occupied or unoccupied.

Given the soluble nature of BTK, employing the same analytical strategy for $P2Y_{12}$ is unsuitable. $P2Y_{12}$ is a G-protein-coupled receptor (GPCR) embedded in the platelet membrane and lacks solubility in water. A high concentration of surfactant is required to dissolve $P2Y_{12}$ when detached from membrane lipids, yet surfactants substantially impair MS response. Additionally, the reduction of disulfide bonds in the protein's structure for enzymatic digestion requires a reducing agent such as dithiothreitol (DTT). However, DTT not only disrupts the disulfide conjugate in the $P2Y_{12}$−AM complex but also reacts with AM, thereby compromising the accuracy of the results. Consequently, MS analysis of either whole proteins or peptide segments of $P2Y_{12}$−AM presents significant challenges.

Regarding the chemical properties of the disulfide bond, we identify an opportunity to address platelet $P2Y_{12}$ receptor occupancy. AM binds to the $P2Y_{12}$ through a disulfide bond with the cysteine residue. This specific interaction enables the targeted labeling of unoccupied $P2Y_{12}$ receptors with an MS tag and facilitates their subsequent purification. The reactivity of the disulfide bond allows for the conditional release of both AM and the MS tag from the $P2Y_{12}$-complexes, serving as small molecular surrogate analytes for occupied and unoccupied platelet $P2Y_{12}$ receptors, respectively. In our approach, the proportion of occupied versus unoccupied target receptors can be precisely quantified *via* LC−MS/MS, providing an accurate real-time assessment

of receptor occupancy. The correlation between receptor occupancy and the antiaggregant activity of Clop has been corroborated in a rat model. Therefore, we suggest the adoption of normalized platelet $P2Y_{12}$ receptor occupancy as a potential PD biomarker to address the issue of IIV.

2. Materials and methods

2.1. Chemicals and animals

2.1.1. Reagents

Chemicals and reagents were utilized without additional purification. 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES, (CAS No.: 7365-45-9), phenylmethyl sulfonyl fluoride (PMSF, CAS No.: 329-98-6), tris(2-carboxyethyl)phosphine (TCEP, CAS No.: 5961-85-3) were procured from Macklin Biochemical Technology (Shanghai, China). Octyl-β-D-glucopyranoside (β-OG, CAS No.: 29836-26-8), 3′-methoxybenzoyl methyl bromide (MPBr, CAS No.: 874-98-6) were obtained from Sigma−Aldrich (St. Louis, USA). Acetonitrile and methanol of HPLC-grade were sourced from Fisher Scientific (Fair Lawn, USA). Rat $P2Y_{12}$ antibody was acquired from Abcam (Lot No.: EPR18611, Cambridge, UK). Horseradish peroxidase-coupled rabbit anti-mouse IgG was purchased from Proteintech (Lot No.: SA00001-19, Wuhan, China). Rat liver microsomes (RLM) were sourced from IPHASE (Lot No.: 21J001, Beijing, China). The standards of MP-derivatives AM-MP (99.2%) (CAS No.: 1430373-14-0), D-AM-MP (98.3%) (CAS No.: 2982676-68-4), deuterated 2-oxo-clopidogrel (97.6%) (CAS No.: 1557999-92-4), and the internal standard (97.4%) (CAS No.: 2982676-62-8) were provided by Beijing Institute of Drug Metabolism (Beijing, China). Clopidogrel (CAS No.: 113665-84-2) was purchased from Lixin Pharmaceutical Co, Ltd. (Suzhou, China).

2.1.2. Animals

Male Sprague−Dawley (SD) rats, aged 8 weeks and weighing 250−300 g, were sourced from Beijing Vital River Laboratory Animal Technology. Male SD rats were exclusively selected to ensure a relatively consistent baseline. A total of 176 male SD rats were randomly assigned to the single-dose group ($n = 54$), multiple-dose group ($n = 90$), T2DM group ($n = 20$), and DDIs group ($n = 12$). The animal facility was maintained at 22 ± 2 °C and $55 \pm 10\%$ relative humidity with a 12-h light/dark cycle (7:00 AM to 7:00 PM). All animal procedures were conducted according to the procedures approved (No. 2023-YNPZSY-0915) by the Institutional Animal Careened Use Committee of Jilin University. Blood samples (4 mL) were collected from the abdominal aorta of anesthetized rats into tubes pretreated with 3.8% trisodium citrate solution (sodium citrate: blood = 1:9, *v*/*v*).

2.1.3. T2DM model rats

A combination of low-dose streptozotocin injection and high-fat food was used to induce T2DM in rats[22]. The control group rats were fed a standard normal diet (ND), while the diabetic group rats were fed a high-fat diet (HFD) composed of powdered normal pellet food, 588 g/kg; lard, 200 g/kg; sucrose, 200 g/kg; cholesterol, 10 g/kg; protein, 50 g/kg; and sodium cholate, 2 g/kg. After 6 weeks, the rats in the HFD group received an intraperitoneal injection of streptozotocin, while those in the ND group received citrate buffer. Blood glucose levels were monitored after an overnight fast, and rats with fasting blood glucose above 11.1 mmol/L were considered successful diabetic models. Throughout the experiment, the body weight and blood glucose levels of rats were monitored every three days.

2.1.4. Single- and multiple-dose studies

Rats in the single-dose group underwent overnight fasting before receiving oral administration of Clop (10 mg/kg). Blood samples were collected before administration and at 1, 2, 4, 6, 8, 12, 24, and 48 h post-administration ($n = 6$ for each time point). The multiple-dose groups comprised a low-dose group (3 mg/kg, $n = 30$), a medium-dose group (10 mg/kg, $n = 30$), and a high-dose group (30 mg/kg, $n = 30$). Each dose group was administered for 7 consecutive days. Blood samples were collected 4 h post-administration on Days 1 (D1H4), 3 (D3H4), and 7 (D7H4). After the cessation of administration, rats were monitored for an additional 5 consecutive days, with blood samples collected at the same time on Days 9 (D9H4), and 12 (D12H4).

2.1.5. Clinical settings of T2DM and DDIs with omeprazole

In the T2DM group, model rats ($n = 10$) and control rats ($n = 10$) received a single oral dose of 10 mg/kg of Clop. Blood samples were collected at 1 h (D0H1) and 4 h (D0H4) post-administration. The DDI group comprised a co-administration group ($n = 6$) and a control group ($n = 6$). Rats in the control group received Clop (10 mg/kg) orally for 7 consecutive days, while rats in the co-administered group were orally administered Clop (10 mg/kg) along with omeprazole (10 mg/kg) for 7 consecutive days. Blood samples were collected 4 h post-administration on Day 7 (D7H4).

2.2. Experimental methods

To assess real-time quantification of $P2Y_{12}$ receptor occupancy, we developed a robust LC−MS/MS-based assay, as depicted in Fig. 1. This assay utilized deuterium-labeled clopidogrel AM (D-AM) as a labeling agent to measure the proportion of unoccupied $P2Y_{12}$ receptors following drug administration. Due to the high reactivity of the free thiol group, D-AM was synthesized *in situ* by incubating its precursor, deuterated 2-oxo-clopidogrel, with RLM. To ensure complete quenching, an excess of D-AM was added, which was later removed during the separation of platelet membranes. The dissociation of AM and D-AM from their covalent bonds with $P2Y_{12}$ receptors was achieved by treatment with TCEP. Subsequently, the liberated AM and D-AM were immediately transformed into more stable 3′-methoxyacetophenone (MP) derivatives for further analysis. These derivatives served as surrogate markers for occupied and unoccupied $P2Y_{12}$ receptors, respectively, and were quantified using LC−MS/MS. The real-time occupancy of platelet $P2Y_{12}$ receptors was then determined by comparing the chromatographic peak areas (A) of these analytes as Eq. (1):

$$P2Y_{12}\ \text{occupancy}(\%) = \frac{A_{\text{AM-MP}}}{A_{\text{AM-MP}} + A_{\text{D-AM-MP}}} \times 100 \quad (1)$$

2.2.1. Optimized procedures for the assessment of platelet P2Y12 receptor occupancy

A. Platelet sample preparation:

(1) 4 mL of blood were collected in tubes pretreated with anticoagulant and centrifuged at $200 \times g$ for 10 min to prepare platelet-rich plasma (PRP);

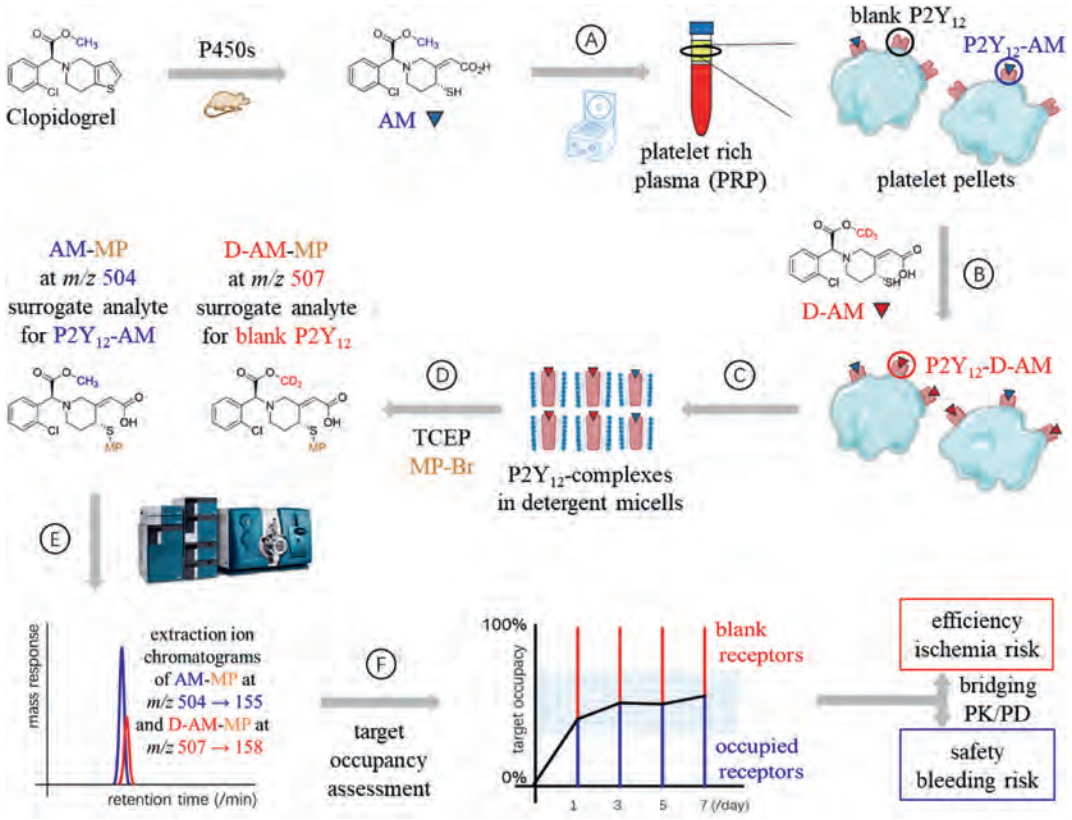

Figure 1 Strategy of platelet $P2Y_{12}$ receptor occupancy assessment, (A) Platelet sample preparation; (B) Quenching of unoccupied $P2Y_{12}$ receptor; (C) Isolation of $P2Y_{12}$ complexes; (D) Surrogate analytes disassociation and derivatization; (E) LC−MS/MS sample preparation; (F) Assessment of receptor occupancy.

(2) 400 μL PRP were suspended in ACD buffer (65 mmol/L citric acid, 80 mmol/L trisodium citrate, and 110 mmol/L glucose, pH 5.4, PRP:ACD = 10:1.25, *v*/*v*) and centrifuged at 600×*g* for 10 min, supernatant was removed;
(3) 600 μL of PBS buffer containing 5 mmol/L EDTA was added to the platelet pellet and centrifuged at 600×*g* for 10 min, supernatant was removed;
(4) the rinsed platelet pellet was resuspended in 200 μL HEPES buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, 5 mmol/L EDTA, 15 mmol/L HEPES, pH 7.4).

B. Quenching of blank $P2Y_{12}$ receptor:
(1) 0.1 mmol/L (final concentration) of deuterated 2-oxo-clopidogrel was incubated in an RLM system, 10 mmol/L NADPH was added for initiating the reaction;
(2) the reaction mixtures (final volume 200 μL) were incubated at 37 °C for 60 min under constant stirring (100 rpm) and light shielding, the mixtures were cooled to 4 °C and centrifuged at 13,000×*g* for 5 min;
(3) 185 μL supernatant of the incubation medium was added to the platelet pellet sample from A(4) and incubated at 20 °C for an additional 1 h.

C. Isolation of $P2Y_{12}$ complexes:
(1) 1 mmol/L PMSF was added to the quenched platelet sample from B(3) by ultrasonication in an iced water bath for 2 min;
(2) the lysate was centrifuged at 17,000×*g* for 10 min at 4 °C, the supernatant was ultracentrifuged at 200,000×*g* for 1 h at 4 °C;
(3) the precipitated membrane fraction was resuspended in 200 μL *β*-OG buffer (25 mmol/L Tris, 150 mmol/L NaCl, 2% *β*-OG, pH 7.4) to extract the $P2Y_{12}$ complexes.

D. Surrogate analytes disassociation and derivatization:
(1) the extracted $P2Y_{12}$ complexes from C(3) were treated with 50 mmol/L TCEP (final concentration) and heated at 90 °C for 3 min;
(2) After heating, 100 mmol/L MPBr (final concentration) solution in acetonitrile was added immediately and incubated at room temperature for 10 min.

E. LC−MS/MS sample preparation:
(1) *μ*-Elutionplate HLB® cartridges were preconditioned with 200 μL methanol and 200 μL water;
(2) 100 μL aliquots of samples from D(2) were doped with 20 μL working solution of internal standard and 300 μL of 0.1% formic acid-water and loaded onto preconditioned cartridges;
(3) rinsing the loaded cartridges with 300 μL of 0.1% formic acid-water and 300 μL of 30% methanol−water, followed by eluting with 60 μL acetonitrile;
(4) the eluates were diluted with 40 μL water and ready for LC−MS/MS analysis.

2.2.2. *Identification of P2Y12-AM complexes*

The extracted $P2Y_{12}$-AM complexes from C(3) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Supporting Information Fig. S1). SDS-PAGE was performed using 4%−20% flat gels (GenScript, Nanjing, China) under both non-reducing and reducing (10 mmol/L

DTT) conditions, followed by transfer to polyvinylidene difluoride membranes. The membranes were rinsed three times with TBST buffer, followed by the addition of 15 mL blocking buffer, and shaken at room temperature for 1 h. Mouse anti-$P2Y_{12}$ monoclonal antibody 1:2000 was added as the primary antibody, and incubated at 4 °C overnight. After rinsing, horseradish peroxidase-coupled rabbit anti-mouse IgG 1:5000 was added as the secondary antibody and shaken at room temperature for 2 h. Immunoreactive proteins were visualized using the Odyssey Imaging System (LI-COR, Lincoln, USA).

2.2.3. Chromatography and MS conditions

LC−MS/MS was conducted using a Waters I-Class UPLC system (massachusetts, USA) coupled with a Sciex Qtrap 6500 mass spectrometer (Ontario, Canada). Chromatographic separation was carried out on an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm; Waters, Milford, USA) maintained at 45 °C. The mobile phase consisted of acetonitrile/water/formic acid (45/55/0.0275, *v*/*v*/*v*) delivered at a flow rate of 0.45 mL/min. The autosampler temperature was set at 4 °C, and the injection volume was 30 μL. The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive ionization mode and multiple reaction monitoring (MRM) mode. The MS parameters were configured as follows: curtain gas 30 psi; nebulizer gas 40 psi; turbo gas 40 psi; ion spray voltage 5500 V; source temperature 550 °C; and dwell time 80 ms. The optimized MS parameters are listed in Supporting Information Table S1.

2.2.4. Assessment of platelet aggregation

ADP-induced platelet aggregation was measured by the light transmission aggregometry (LTA). Citrated blood was centrifuged at 200×*g* for 10 min to prepare PRP and further at 3500×*g* for 10 min to prepare platelet-poor plasma (PPP). Platelet aggregation was assessed by LBY-NJ4A automated platelet aggregation analyser (PRECIL, Beijing, China). Maximum platelet aggregation percentage (MPA) was determined at 37 °C within 5 min after the addition of agonist (5 μmol/L ADP). Inhibition of aggregation (IPA) was defined as follows, where MPA_0 is the MPA at baseline and MPA_t is the MPA at the given time point (t) as Eq. (2):

$$\mathrm{IPA}\ (\%) = \frac{\mathrm{MPA}_0 - \mathrm{MPA}_t}{\mathrm{MPA}_0} \times 100 \tag{2}$$

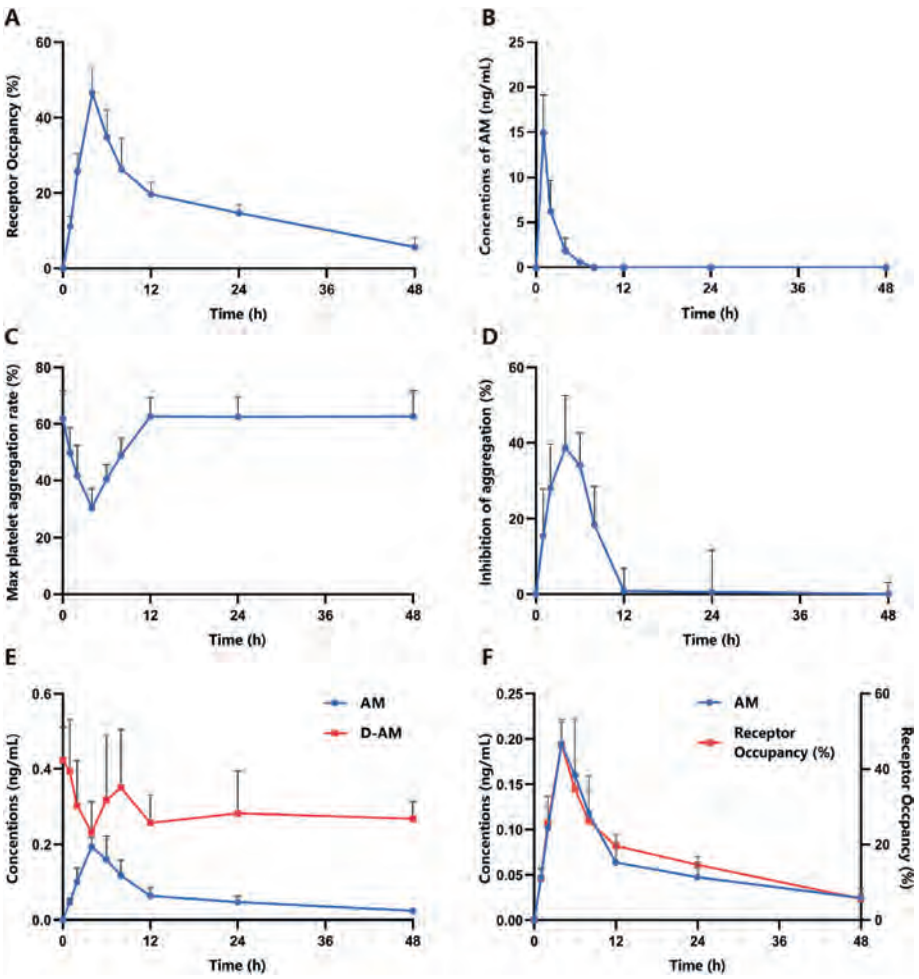

Figure 2 Single administration of 10 mg/kg clopidogrel to male rats (n = 6), (A) mean $P2Y_{12}$ occupancy−time curve; (B) mean concentration−time curve of AM in plasma; (C) mean MPA−time curve; (D) mean IPA−time curve; (E) quantitation of dissociated AM and D-AM; (F) comparison between $P2Y_{12}$ occupancy and target-driven PK.

2.2.5. Statistical analysis

Statistical analysis and graphical representations were conducted using Prism 9.5 software (Graphpad, La Jolla, USA). Data are presented as mean ± standard deviation (SD). The Mann−Whitney U test was employed for comparisons between two groups, while comparisons among three groups were made using the Kruskal−Wallis test. A significance level of $P < 0.05$ was considered statistically significant for all comparisons. Linear regression analysis was utilized to assess assay linearity.

3. Results and discussions

3.1. Optimization of the procedural conditions

The operational procedure is optimized for processing a 4 mL blood sample, with a focus on steps B(2) and D(1). To ensure sufficient and suitable quenching, we initially assessed the quantities of the deuterated quenching agent in step B(2). Platelet samples from step A(4) were treated with 100, 200, and 300 μL of 0.1 mmol/L deuterated 2-oxo-clopidogrel incubation medium[23], resulting in D-AM concentration in B(4) of approximately 60, 100, and 140 ng/mL, respectively. Subsequently, the quenched platelet samples underwent steps C−E. The resulting LC−MS/MS analyses of D-AM-MP were compared. There was no statistically significant difference between the results obtained from the groups with 200 and 300 μL of incubation medium. The experimental results, depicted in Supporting Information Fig. S2A, indicate that saturated quenching of $P2Y_{12}$ receptors in the 400 μL blank platelet sample was achieved by preparing 200 μL of incubation medium in step B(2).

Both $P2Y_{12}$-AM and $P2Y_{12}$-D-AM are disulfide-conjugate. The dissociation of the corresponding thiols, AM and D-AM, following the treatment with TCEP, was subsequently optimized[24]. The disassociation conditions in step D(1) were optimized for temperature (20, 37, and 90 °C), reaction time (1, 3, 10, and 30 min), and TCEP-concentration (10, 30, 50, and 100 mmol/L). The optimized reduction condition was found to be 50 mmol/L TCEP with heating at 90 °C for 3 min (Fig. S2B−S2D).

3.2. Validation of the surrogate analytes AM-MP and D-AM-MP

The dissociated AM and D-AM from the $P2Y_{12}$−AM and $P2Y_{12}$−D-AM complexes in step *D*(1) were immediately converted into stable MP-derivates in step *D*(2) following the established method[25−27], serving as surrogate analytes for the $P2Y_{12}$−AM complex and unoccupied $P2Y_{12}$ receptors, respectively. Therefore, the specificity of the strategy is critical for ensuring the accuracy of the results. It's important to evaluate any

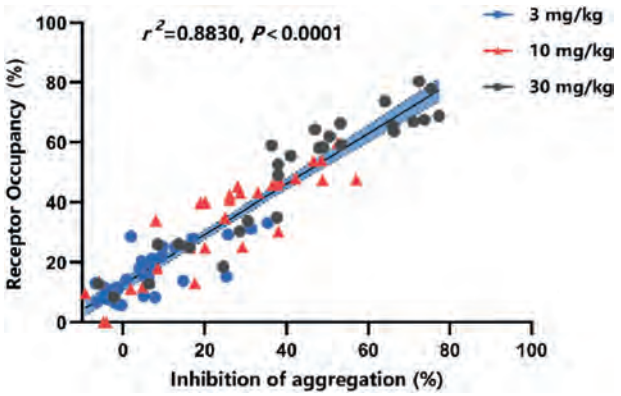

Figure 4 Linear fitting of $P2Y_{12}$ occupancy and IPA.

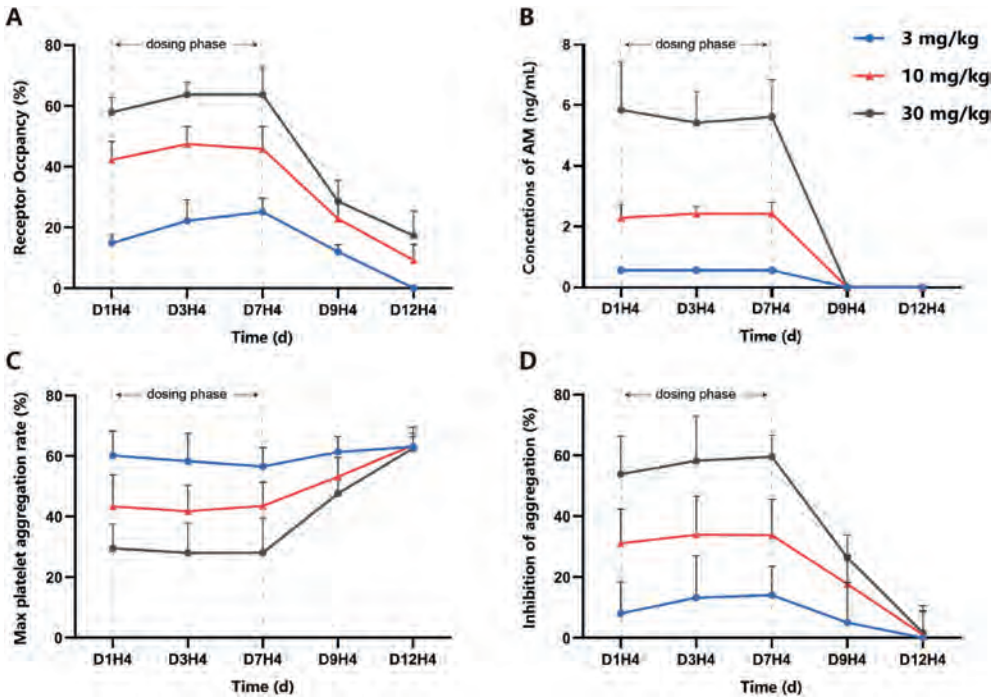

Figure 3 Repeated administration doses of 3, 10, and 30 mg/kg of clopidogrel to male rats ($n = 6$), (A) mean $P2Y_{12}$ occupancy−time curve; (B) mean concentration−time curve of AM in plasma; (C) mean MPA−time curve; (D) mean IPA−time curve.

residual AM or D-AM that remains in the samples. The extraction ion chromatograms of AM-MP and D-AM-MP from samples without (Supporting Information Fig. S3A and S3B) and with TCEP treatment in step D(1) (Fig. S3C and S3D) were compared. Only in the treated sample could AM-MP (retention time RT = 4.69 min) and D-AM-MP (RT = 4.78 min) be detected. Combining these results with those of immunoblotting, it was confirmed that AM and D-AM thiols were released from their respective $P2Y_{12}$-complexes.

The MS responses of the surrogate analytes AM-MP and D-AM-MP were assessed. Six portions of standard solutions of AM-MP and D-AM-MP were diluted to 5 nmol/L and analyzed. No significant difference was observed between the MS responses of AM-MP and D-AM-MP (Supporting Information Table S2). The analytical method's regression model was evaluated over the concentration range 0.02−20.0 ng/mL. The relationship between concentrations and MS responses of AM-MP and D-AM-MP in the surrogate matrix (solubilization buffer) was fitted using linear least squares regression (weighted $1/X^2$, Supporting Information Fig. S4). The linear regression equation and correlation coefficient for AM-MP were: $y = 1.05 \times 10^6x - 4.89 \times 10^3$ ($r = 0.9978$) and for D-AM-MP were: $y = 0.94 \times 10^6x - 1.25 \times 10^3$ ($r = 0.9972$). The precision and accuracy for the determination of AM-MP and D-AM-MP in the solubilization buffer were summarized in Supporting Information Tables S3−S4.

3.3. *Platelet P2Y12 occupancy under single dosing*

To explore the immediate response of receptor occupancy as a PD biomarker, single-dose studies were conducted. Following the protocol in Section 2.2.1, the PK profile of single-dosed Clop in rats was presented as platelet $P2Y_{12}$ occupancy (Fig. 2A). In comparison to the plasma exposure of AM (Fig. 2B), this profile exhibited a typical PK curve with a time course similar to the PD response measured by LTA. The peak of maximal inhibitory effects on platelet aggregation (MPA, Fig. 2C) and the inhibition of aggregation (IPA, Fig. 2D) occurred at 4 h post-administration, corresponded with the maximal $P2Y_{12}$ occupancy. The absolute quantitation of the dissociated AM and D-AM (in the form of their MP derivatives) was conducted at a subsequent stage and is depicted in Fig. 2E. This target-driven AM curve aligns well with the target occupancy curve (Fig. 2F), affirming the reliability of the analysis approach.

In the single-dose group, diverse onset and offset platelet responses in terms of platelet $P2Y_{12}$ occupancy were observed. During the occupancy-increasing phase (0−4 h), platelet response was detectable at relatively low $P2Y_{12}$ occupancy levels. Conversely, in the occupancy-decreasing phase (4−48 h), the antiaggregating effect diminished at 19.7 ± 3.1% $P2Y_{12}$ occupancy. Given clopidogrel's prodrug nature, its "absorption" phase in the conventional plasma concentration−time profile primarily involves the hepatic release of AM. Since platelets and AM are uniformly distributed in plasma, tissue penetration is not

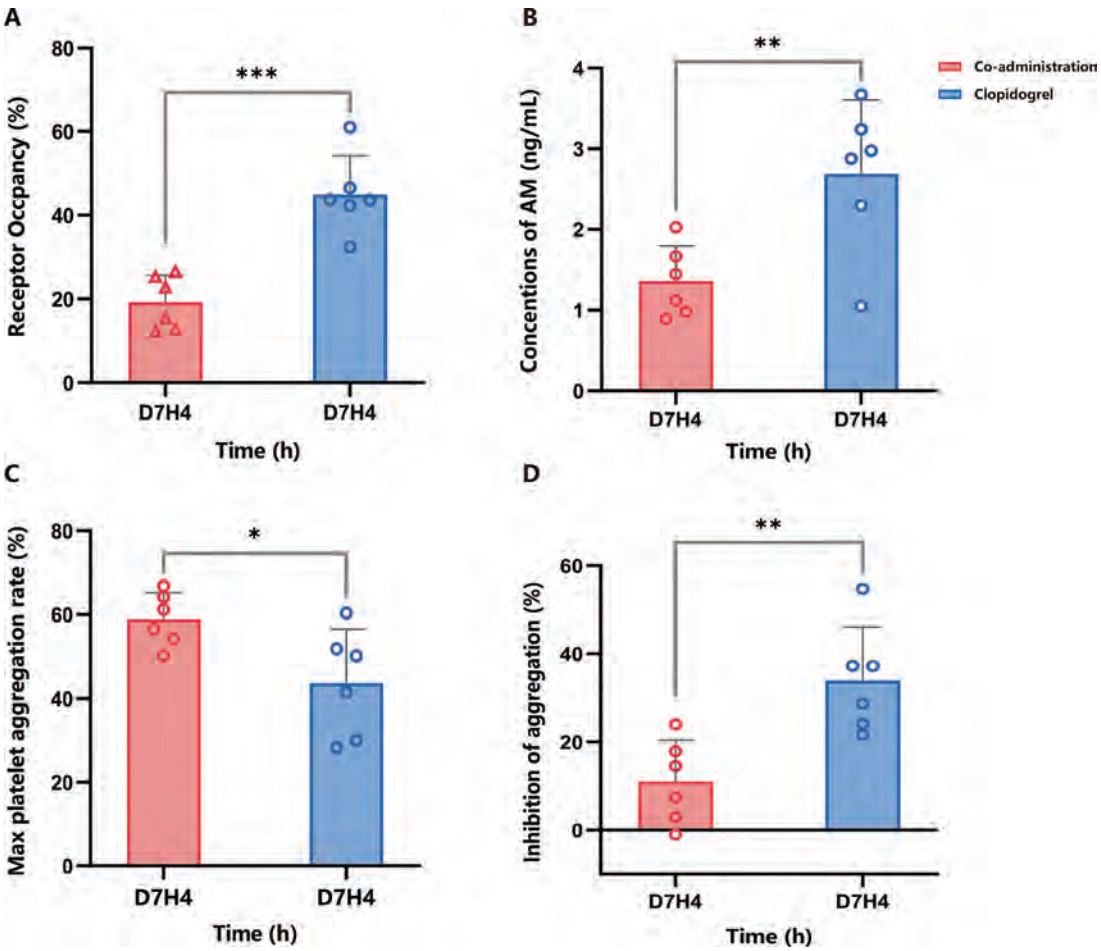

Figure 5 Co-administration of 10 mg/kg clopidogrel and 10 mg/kg omeprazole to male rats ($n = 6$), (A) mean $P2Y_{12}$ occupancies; (B) mean concentrations of AM in plasma; (C) mean MPA; (D) mean IPA measured on the seventh day.

an issue. The binding of AM to the platelet $P2Y_{12}$ receptor occurs swiftly and efficiently. Within the first 8 h post-administration, AM can be still detected in plasma (Fig. 2B). Throughout the MPA assessment, AM binding continues, resulting in a relatively robust platelet response during the occupancy-increasing phase.

3.4. *Platelet P2Y12 occupancy under repeated dosing*

To explore dose-response relationships and simulate various IIV responses to Clop, three repeated-dose groups were administered doses of 3, 10, and 30 mg/kg Clop for 7 consecutive days. The $P2Y_{12}$ occupancies at these doses exhibited significant distinctions (Fig. 3A). In the 3 mg/kg dose group, representing Clop low responders, a gradual and significant increase in $P2Y_{12}$ occupancy was observed between D1H4 and D7H4 ($P < 0.01$). This finding aligns with clinical observations where subjects identified as low responders showed a modest decrease in platelet reactivity index[28].

The plasma concentrations of AM 4 h post-administration remained relatively stable across different doses in the repeated-dose groups (Fig. 3B). However, no clear proportional correlation was observed between the dose of Clop and the plasma level of AM[29], nor between the plasma level of AM and $P2Y_{12}$ occupancy. This lack of correlation within the "Clop dose-AM exposure-antiaggregating effect" continuum directly contributes to the dissociation between PK and PD. After the cessation of administration, platelet activity takes five days to recover across all three dose groups (Fig. 3C and D), consistent with the recommended minimal interruption of Clop before surgery[30], and supporting the observation that platelet function recovery depends on platelet turnover[31]. Notably, the decrease in platelet response in the 30 mg/kg dose group occurs at 17.3 ± 8.3% $P2Y_{12}$ occupancy upon cessation, similar to the decrease observed in the single-dose group, suggesting a threshold for platelet response in rats.

The inhibitory effect of Clop on rat platelet aggregation, measured by MPA and IPA, increased with the dose (Fig. 3C and D). The mean IPA was 14.0 ± 12.5% in the 3 mg/kg group, 33.8 ± 11.8% in the 10 mg/kg group, and 59.6 ± 10.1% in the 30 mg/kg group. A linear relationship between $P2Y_{12}$ occupancy and IPA has been confirmed ($y = 0.839x + 12.55$, $r^2 = 0.883$), providing a basis for using target occupancy as a PD biomarker (Fig. 4). The correlation between platelet $P2Y_{12}$ occupancy and IPA observed with our real-time strategy exceeds that of non-real-time methods reported in the literature[14].

3.5. *DDIs with omeprazole*

Simultaneous administration of Clop and omeprazole results in a DDI, a recognized clinical factor that reduces the antiplatelet efficacy of Clop. Co-administration of 10 mg/kg Clop and 10 mg/kg omeprazole led to a 54.8% reduction ($P < 0.001$) in $P2Y_{12}$

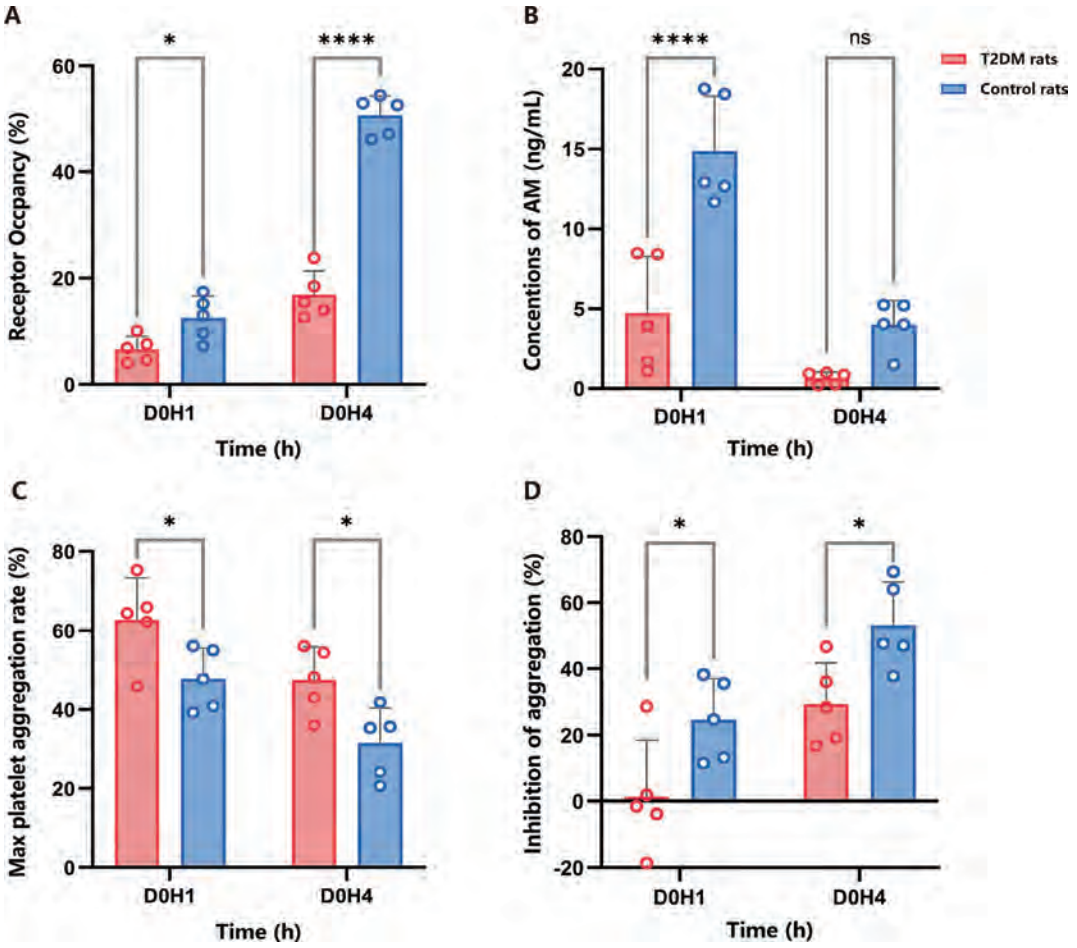

Figure 6 Single administration of 10 mg/kg clopidogrel to male T2DM rats ($n = 5$), (A) mean $P2Y_{12}$ occupancies; (B) mean concentrations of AM in plasma; (C) mean MPA; (D) mean IPA measured at 1 and 4 h post-administration.

occupancy compared to the group administered Clop alone (Fig. 5A). Correspondingly, in the co-administration group, the plasma concentration of AM was reduced by 42.7% ($P < 0.01$) (Fig. 5B). After 7 days co-administration, MPA increased by 21.1% ($P < 0.05$), and IPA decreased by 57.3% ($P < 0.01$) (Fig. 5C and D).

The DDI with omeprazole involves the inhibition of CYP2C19. The biotransformation of Clop to its AM involves CYP3A4 and 2C19, with lesser contributions from CYP1A2, 2B6, and 2C9. Clop also interacts with various P450 enzymes, potentially leading to DDIs. The inhibitory effects of Clop on several P450 enzymes in human liver microsomes were investigated using a previously established "cocktail" method[32]. These factors influencing Clop metabolism can ultimately affect platelet $P2Y_{12}$ occupancy.

3.6. T2DM

T2DM patients often show a high prevalence of poor response to Clop[33]. To evaluate the relevance of $P2Y_{12}$ occupancy as a PD biomarker in T2DM, a single-dose study was conducted in a rat model of T2DM. As shown in Fig. 6A, following a single oral dose of 10 mg/kg Clop, $P2Y_{12}$ occupancy in T2DM rats decreased by 50.7% ($P < 0.05$) at 1 h and by 64.4% ($P < 0.001$) at 4 h post-administration. The plasma concentration of AM was significantly reduced 1-h post-administration compared to the control group (Fig. 6B). Four hours after Clop administration, the T2DM group exhibited a 33.2% increase ($P < 0.05$) in MPA and a 48.3% decrease ($P < 0.05$) in IPA (Fig. 6C and D).

T2DM is associated with altered activities of P450 enzymes and upregulation of P-glycoprotein, which are crucial for the bioactivation of Clop. Additionally, platelets in T2DM patients often display increased expression of surface receptors, enlarged surface area, and altered size, which are factors associated with heightened aggregability and platelet activation[34]. The results from the T2DM group indicate that variations in receptors and platelet characteristics manifest in platelet $P2Y_{12}$ occupancy.

4. Conclusions

A strategy has been developed for real-time assessment of target engagement during clopidogrel-therapy employing LC−MS/MS technique. This method quantifies the proportion of irreversibly inhibited $P2Y_{12}$ receptors on platelets through personalized PK processes following clopidogrel administration. $P2Y_{12}$ occupancy demonstrates a robust correlation with the antiplatelet aggregation effect mediated by the $P2Y_{12}$ signaling pathway, highlighting its potential as a valuable PD biomarker. Establishing a therapeutic window based on target receptor occupancy for guiding individualized precision medicine could effectively address IIV during clopidogrel-therapy, given that the real-time and normalized $P2Y_{12}$ occupancy achieved through this strategy offers precise insights.

This study underscores the feasibility of using receptor occupancy as a standardized PD biomarker to establish the PK/PD relationship of clopidogrel. Validation through rat model studies has affirmed its capability to reflect diverse clinical scenarios, encompassing variations in dosage, T2DM, and DDI with omeprazole. This approach holds promise for clinical application in personalizing clopidogrel-therapy. Nevertheless, certain variables such as age, sex, weight, and dual antiplatelet therapy were not addressed in this study. Further validation through prospective, randomized studies is essential to consolidate its efficacy and broaden its clinical applicability.

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Author contributions

Haipeng Li: Writing − original draft, Validation, Methodology, Investigation. Yueming Gu: Data curation. Yumeng Zhao: Data curation. Aiyun Xu: Data curation. Dong Sun: Writing − original draft, Project administration, Funding acquisition, Conceptualization. Jingkai Gu: Writing − review & editing, Supervision, Resources, Conceptualization.

Conflict of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2024.08.008.

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