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# Potent and Orally Bioavailable Antiplatelet Agent, PLD-301, with the Potential of Overcoming Clopidogrel Resistance

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**Abstract:** PLD-301, a phosphate prodrug of clopidogrel thiolactone discovered by Prelude Pharmaceuticals with the aim to overcome clopidogrel resistance, was evaluated for its *in vivo* inhibitory effect on ADP-induced platelet aggregation in rats. The potency of PLD-301 was similar to that of prasugrel, but much higher than that of clopidogrel. The results of pharmacokinetic analysis showed that the oral bioavailability of clopidogrel thiolactone converted from PLD-301 was 4- to 5-fold higher than that of the one converted from clopidogrel, suggesting that in comparison with clopidogrel, lower doses of PLD-301 could be used clinically. In summary, PLD-301 presents a potent and orally bioavailable antiplatelet agent that might have some advantages over clopidogrel, such as overcoming clopidogrel resistance for CYP2C19-allele loss-of-function carriers, and lowering dose-related toxicity due to a much lower effective dose.

**Keywords:** Clopidogrel, active metabolite, drug resistance, platelet ADP receptor, platelet aggregation, thrombosis.

## 1. INTRODUCTION

Clopidogrel, an oral thienopyridine-class antiplatelet agent, has been widely used to inhibit blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It is the most prescribed antiplatelet drug for the prevention of vascular events in acute coronary syndrome (ACS) patients and stent thrombosis after percutaneous coronary intervention (PCI) [1]. Clopidogrel works by irreversibly inhibiting a receptor named P2Y12, an adenosine diphosphate (ADP) chemoreceptor on platelet cell membranes [2-4]. Clopidogrel is a prodrug that requires *in vivo* conversion by the hepatic cytochrome P450 (CYP) system to generate an active metabolite called clopidogrel thiolactone, which is further converted to the clopidogrel active metabolite (AM) [5-7] (Fig. **1**).

Up to 30% of treated Caucasian patients show non-responsiveness or poor responsiveness to clopidogrel therapy [8, 9]. In particular, among poor metabolizers (PMs) who carry CYP2C19 loss-of-function polymorphisms, plasma levels of the AM of clopidogrel are much lower than those of non-carriers, leading to lower platelet inhibition, and these patients have an increased risk of death from cardiovascular causes, myocardial infarction, or stroke compared with non-carriers [10-12]. The frequencies of the CYP2C19 PM genotypes observed in Chinese Han (18.7%), Chinese Hui (25.0%), and Chinese Mongolian (10.9%) subjects were significantly higher than that in Caucasians (1.7–3.0%, $P < 0.01$) [13-16]. Clinically, this phenomenon is referred to as clopidogrel resistance (CR) [17], and has led to the requirement that healthcare professionals and patients are warned that CYP2C19 PMs are at a high risk of therapeutic failure when they are treated with clopidogrel [18-20]. To overcome clopidogrel resistance, clinicians generally use a higher dose of clopidogrel or newer P2Y12 receptor antagonists (e.g. prasugrel, ticagrelor, cangrelor) [21, 22]; however, these approaches may present a significant risk of major bleeding (including fatal bleeding) and reduce medication adherence.

Novel antiplatelet agents with rapid onset of inhibition, low risk of bleeding, and low *in vivo* variability are needed to support effective treatment of ACS and its complications, and such agents will be especially useful in the clinical management of clopidogrel resistance [23, 24]. Clopidogrel presents a good platform for drug discovery because it is among the most commonly prescribed drugs in the world and its long-term safety profile has been well established by more than 15 years of clinical use. We anticipated that phosphate prodrugs might be readily transformed into clopidogrel thiolactone by intestinal alkaline phosphatase-mediated hydrolysis during absorption, and subsequently to the clopidogrel AM [25, 26]. Here we report the identification of PLD-301 as an antiplatelet agent with high potency and sufficient oral bioavailability which could be an ideal drug candidate for overcoming clopidogrel resistance without increasing bleeding risk and other adverse events associated with other antiplatelet agents.

## 2. METHODS

### 2.1. Metabolic Stability in Human and Rat Intestine Microsomes

Pooled human intestine microsomes and pooled male rat intestine microsomes were purchased from Xenotech

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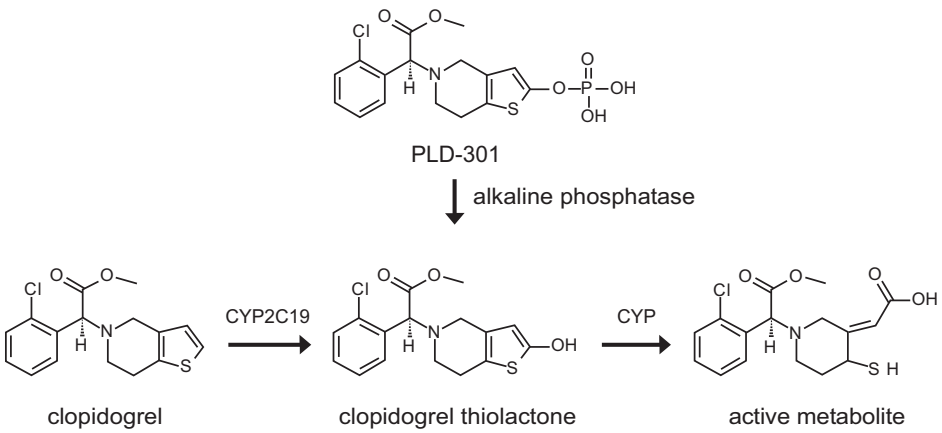


Fig. (1). Metabolic Pathways of Clopidogrel and PLD-301.

(Lenexa, Kansas, USA). Microsomes were stored at -80°C prior to use. The *in vitro* metabolic stability of PLD-301 and bioconversion of PLD-301 to clopidogrel thiolactone were assessed in pooled human and male rat intestine microsomes in the absence of NADPH cofactor. Samples were collected at 0, 15, 30, 45, and 60 min after the initiation of the reactions, and the concentrations of test compounds in the reaction systems were evaluated by LC/MS/MS to estimate the stability of PLD-301 in pooled human and male rat intestine microsomes. Bioconversion of fosphenytoin to phenytoin was evaluated as a positive control.

### 2.2. Animal Maintenance

All *in vivo* studies were performed under an animal protocol approved by Pharmaron, Inc. (Beijing, China), in line with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were quarantined for at least 7 days before dosing. The general health of the animals was evaluated by a veterinarian, and complete health checks were performed. Animals with abnormalities were excluded prior to the study. The animals were housed 3 per cage in polypropylene cages that were kept in an environmentally monitored, well-ventilated room maintained at a temperature of 20–25°C and a relative humidity of 40%–70%. Fluorescent lighting provided illumination for approximately 12 h per day. Each animal was assigned an identification number.

### 2.3. *In Vivo* Platelet Aggregation Evaluation in Rats

Sprague-Dawley male rats were fasted for 16 h prior to the test. The rats were randomly assigned to the experimental groups using a computer-generated randomization procedure that was based on body weight.

Compounds were formulated in 30% PEG400 at a concentration of 0.6 mg/mL, and orally administered at a volume of 5 mL/kg. One hour after dosing, a blood sample from each rat was collected into a tube containing 3.8% (w/v) sodium citrate solution as an anticoagulant (blood:sodium citrate = 9:1). Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 200 × *g* for 10 min. Platelet-poor plasma (PPP) was obtained by subsequent centrifugation of the PRP at 800 × *g* for 10 min. The aggregation of platelets in the PRP was measured using a PRECIL LBY-NJ4 platelet aggregation analyzer at 37°C with stirring. Aggregation curves were induced by the addition of ADP (50.0 μmol/L) and were recorded and evaluated by a computer program, which estimated 4 parameters of platelet aggregation: the aggregation rates after 1, 3, and 5 min, and the maximal aggregation rate (max rate).

One-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test were applied to the groups, and a result of $p < 0.05$ was accepted as significant.

### 2.4. Pharmacokinetic Studies

The pharmacokinetic (PK) parameters of PLD-301 and clopidogrel were studied in male Sprague-Dawley rats following oral (PO) and intravenous (IV) administration. Clopidogrel thiolactone was analyzed using LC/MS/MS method to determine the PK parameters for PLD-301 and clopidogrel. Dosing solutions of PLD-301 and clopidogrel were prepared in 30% PEG400/saline for oral gavage (24 μmol/kg). A solution of clopidogrel thiolactone in 30% PEG400/saline was prepared for intravenous bolus tail vein injection (8 μmol/kg) to determine the oral bioavailability of PLD-301 and clopidogrel. Blood samples (200 μL) were collected from the retro-orbital plexus from 3 animals in each treatment group at 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h for the IV group, and 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h for the PO group. Calibration standards for clopidogrel thiolactone ranged from 0.2 to 1000 ng/mL were obtained by serial dilution of pretreated rat plasma. The supernatants containing the organic components of each sample were used for analysis. The lower limit of quantification was 0.2 ng/mL.

## 3. RESULTS

### 3.1. Chemistry

PLD-301 is a proprietary, novel platelet inhibitor for clinical development, which was discovered by Prelude Pharmaceuticals (Beijing, China) founded by the corresponding author. The synthesis and characterization of PLD-301 have been published in the patent application [27].

**Table 1. Bioconversion of PLD-301 to Clopidogrel Thiolactone in Human and Rat Intestine Microsomes.**

| Compound | Monitored Species | Percentages of Monitored Species in Reaction Mixtures (without NADPH) | | | |
|---|---|---|---|---|---|
| | | Human Intestine Microsomes | | Rat Intestine Microsomes | |
| | | 0 min | 60 min | 0 min | 60 min |
| Fosphentoin | Fosphentoin | 100 | 53.5 | 100 | 61.4 |
| | Phentoin | 0.8 | 21.1 | 0.5 | 14.0 |
| Clopidogrel thiolactone | Clopidogrel thiolactone | 100 | 82.0 | 100 | 61.6 |
| Clopidogrel | Clopidogrel | 100 | 78.4 | 100 | 71.3 |
| | Clopidogrel thiolactone | 0.6 | 0.6 | 0.5 | 0.4 |
| PLD-301 | PLD-301 | 100 | 45.0 | 100 | 49.6 |
| | Clopidogrel thiolactone | 0.9 | 56.4 | 0.8 | 29.2 |

Optically pure PLD-301, clopidogrel, and Clopidogrel thiolactone were synthesized at a CRO facility, Sundia MediTech Company Ltd. (Shanghai, China). All final compounds showed ≥95% purity which was determined by analytical HPLC. The optical purity (% ee) of the compounds was measured using chiral HPLC.

### 3.2. Bioconversion of PLD-301 to Clopidogrel Thiolactone in Human and Rat Intestine Microsomes

PLD-301 was readily converted into clopidogrel thiolactone in both human and rat intestinal microsomes, whereas clopidogrel was not converted. These data suggest that bioconversion of PLD-301 to clopidogrel thiolactone in both human and rat intestinal microsomes was performed by intestinal alkaline phosphatase (Table **1**).

### 3.3. Inhibition of ADP-Induced Platelet Aggregation in Rats

The inhibitory effects of PLD-301 and clopidogrel on ADP-induced platelet aggregation in rats (3 mg/kg, 10 animals in each group) were evaluated, with prasugrel as a positive control. ADP-induced platelet aggregation was measured according to the method of Born [28, 29] (Table **2**). In previous study, clopidogrel exhibited high potency at a dose of 10 mg/kg (data not shown), but was nearly inactive at a dose of 3 mg/kg.

**Table 2. Inhibitory Effect of PLD-301 and Clopidogrel on ADP-Induced Platelet Aggregation in Rats at a Dose of 3 mg/kg.**

| Compound | Platelet Aggregation (%) |
|---|---|
| Vehicle | 67.10 ± 4.59 |
| Prasugrel | 17.30 ± 9.20 ** |
| Clopidogrel | 63.24 ± 6.99 |
| PLD-301 | 15.40 ± 12.37 ** |

Experimental details are given in the Methods section.
*Ex vivo* platelet aggregation was measured 1 h after oral administration. Data are presented as mean ± SD (n = 10). ** $P < 0.01$ versus vehicle. The free base forms of PLD-301, clopidogrel, and prasugrel were used in the study.

Among the tested compounds, PLD-301 showed the highest inhibitory activity against platelet aggregation. Prasugrel also showed a strong inhibition of platelet aggregation and was only slightly less potent than PLD-301.

### 3.4. Pharmacokinetic Parameters of Clopidogrel Thiolactone after Oral Administration of PLD-301 and Clopidogrel in Rats

Male Sprague-Dawley rats were orally administered PLD-301 or clopidogrel (both at 24 μmol/kg). Blood samples were collected at 0 h (before administration), and 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after administration. Clopidogrel thiolactone (8 μmol/kg) was intravenously administered to male Sprague-Dawley rats to determine the conversion rate of PLD-301 and clopidogrel into clopidogrel thiolactone and its subsequent bioavailability. Blood samples were collected at 0 h (before administration), and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after administration. The clopidogrel thiolactone concentrations in the plasma samples were determined by LC/MS/MS analysis after sample cleanup (Table **3**). After oral dosing of clopidogrel, the $C_{max}$, $T_{max}$, $T_{1/2}$, and $AUC_{0-\infty}$ of clopidogrel thiolactone in plasma were 1.07 ± 0.2 ng/mL, 1 h, 2.29 ± 0.36 h, and 4.08 ± 0.42 ng•h/mL, respectively. After oral dosing of PLD-301, the $C_{max}$, $T_{max}$, $T_{1/2}$, and $AUC_{0-\infty}$ of clopidogrel thiolactone in plasma were 6.44 ± 2.27 ng/mL, 1 h, 2.09 ± 0.39 h, and 22.8 ± 6.1 ng•h/mL, respectively.

These data suggest that orally administered PLD-301 could be easily converted into clopidogrel thiolactone, whose bioavailability was 4- to 5-fold higher than that converted from clopidogrel at the same dose.

## 4. DISCUSSION

A series of loss-of-function alleles in CYP2C19 have been shown to lead to clinically relevant clopidogrel resistance. The risk of cardiovascular events associated with

**Table 3. Pharmacokinetic Parameters of Clopidogrel Thiolactone upon PLD-301 and Clopidogrel Oral Dosing in Rats.**

| Compound | Dose (μmol/kg) | CL (mL/min/kg) | $T_{1/2}$ (h) | $T_{max}$ (h) | $C_{max}$ (ng/mL) | $AUC_{0\text{-last}}$ (ng*h/mL) | $AUC_{0\text{-}\infty}$ (ng*h/mL) | *F* (%) |
|---|---|---|---|---|---|---|---|---|
| Clopidogrel thiolactone (IV) | 8 | 132 ± 12 | 1.05 ± 0.16 | NA | 971 ± 187 | 343 ± 34 | 344 ± 33 | NA |
| Clopidogrel (PO) | 24 | NA | 2.29 ± 0.36 | 1 | 1.07 ± 0.2 | 3.22 ± 0.84 | 4.08 ± 0.42 | 0.39 |
| PLD-301 (PO) | 24 | NA | 2.09 ± 0.39 | 1 | 6.44 ± 2.27 | 15.8 ± 4.6 | 22.8 ± 6.1 | 2.2 |

Study details are described in the Methods section. Data are presented as mean ± SD (n = 3). The free base forms of PLD-301, clopidogrel, and clopidogrel thiolactone were used in the study.

clopidogrel resistance is critical for ACS patients undergoing PCI. In large-scale clinical trials of clopidogrel, the study population included mostly Caucasians, among whom only about 2% were CYP2C19 PMs [8, 9]. In contrast, among the Asian population, the percentage of CYP2C19 PMs is much higher, at about 15−23% [13-16]. Therefore, clinical studies on clopidogrel resistance are required to enhance our understanding of the risk of adverse cardiovascular events in CYP2C19 PMs receiving clopidogrel therapy.

Phosphate prodrugs of the clopidogrel thiolactone metabolite were synthesized with the primary aim of overcoming clopidogrel resistance. PLD-301 was evaluated for its inhibitory activity on ADP-induced platelet aggregation in rats. Animal studies indicated a potent inhibitory activity of PLD-301 on platelet aggregation, which was similar to prasugrel but much more potent than that of clopidogrel. *In vitro* metabolism studies showed that PLD-301 was readily converted into the clopidogrel AM by rat intestine alkaline phosphatase during absorption. The results of preliminary pharmacokinetic study revealed that the bioavailability of clopidogrel thiolactone converted from PLD-301 was 4- to 5-fold higher than that of the clopidogrel thiolactone converted from clopidogrel, implying a clinically effective dose significantly lower than that of clopidogrel and therefore a lower dose-dependent toxicity.

Taken together, our results show that PLD-301 is a potent and safe antiplatelet agent that possesses several advantages over clopidogrel. PLD-301 inhibits platelet aggregation as potently among CYP2C19 PMs as it does among normal individuals. PLD-301 is absorbed more efficiently than clopidogrel, suggesting that it has lower potential for dose-dependent toxicity owing to a much lower effective dose. PLD-301 is converted into clopidogrel thiolactone quickly because of its metabolic activation, which suggests a faster onset of action. Within a proper dose range, the efficacy of PLD-301 should be comparable to that of clopidogrel, suggesting that the bleeding risk associated with PLD-301 should also be comparable to that associated with clopidogrel. PLD-301 is currently under preclinical evaluation, and should soon advance to clinical development.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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