CYP-independent inhibition of platelet aggregation in rabbits by a mixed disulfide conjugate of clopidogrel

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Introduction

Dual antiplatelet therapy with co-administration of clopidogrel and aspirin has been the standard of care in the United States for patients with acute coronary syndromes (ACS) and/or undergoing percutaneous coronary interventions (PCI). However, the effectiveness of clopidogrel varies significantly among different sub-populations due to inter-individual variability. In this study we examined the antiplatelet potential of a novel mixed disulfide conjugate of clopidogrel with the aim to overcome the inter-individual variability. In the metabolic studies using human liver microsomes and cDNA-expressed P450s, we confirmed that multiple P450s are involved in the bioactivation of 2-oxoclopidogrel to H4, one of the diastereomers of the pharmacologically active metabolite (AM) possessing antiplatelet activity. Results from kinetic studies demonstrated that 2C19 is the most active in converting 2-oxoclopidogrel to H4 with a catalytic efficiency of 0.027 µM⁻¹min⁻¹ in the reconstituted system. On the basis of this finding, we were able to biosynthesise the conjugate of clopidogrel with 3-nitropyridine-2-thiol, referred to as clopNPT, and examined its antiplatelet activity in male New Zealand white rabbits. After administration as intravenous bolus at 2 mg/kg, the clopNPT conjugate was rapidly converted to the AM leading to the inhibition of platelet aggregation (IPA). Analyses of the blood samples drawn at various time points showed that intravenous administration of clopNPT led to ~70% IPA within 1 hour and the IPA persisted for more than 3 hours. Since the antiplatelet activity of clopNPT does not require bioactivation by P450s, the mixed disulfide conjugate of clopidogrel has the potential to overcome the inter-individual variability in clopidogrel therapy.

Keywords
Clopidogrel, antiplatelet, mixed disulfide conjugate, inter-individual variability, active metabolite

Summary
Dual antiplatelet therapy with clopidogrel and aspirin has been the standard of care in the United States for patients with acute coronary syndromes (ACS) and/or undergoing percutaneous coronary interventions (PCI). However, the effectiveness of clopidogrel varies significantly among different sub-populations due to inter-individual variability. In this study we examined the antiplatelet potential of a novel mixed disulfide conjugate of clopidogrel with the aim to overcome the inter-individual variability. In the metabolic studies using human liver microsomes and cDNA-expressed P450s, we confirmed that multiple P450s are involved in the bioactivation of 2-oxoclopidogrel to H4, one of the diastereomers of the pharmacologically active metabolite (AM) possessing antiplatelet activity. Results from kinetic studies demonstrated that 2C19 is the most active in converting 2-oxoclopidogrel to H4 with a catalytic efficiency of 0.027 µM⁻¹min⁻¹ in the reconstituted system. On the basis of this finding, we were able to biosynthesise the conjugate of clopidogrel with 3-nitropyridine-2-thiol, referred to as clopNPT, and examined its antiplatelet activity in male New Zealand white rabbits. After administration as intravenous bolus at 2 mg/kg, the clopNPT conjugate was rapidly converted to the AM leading to the inhibition of platelet aggregation (IPA). Analyses of the blood samples drawn at various time points showed that intravenous administration of clopNPT led to ~70% IPA within 1 hour and the IPA persisted for more than 3 hours. Since the antiplatelet activity of clopNPT does not require bioactivation by P450s, the mixed disulfide conjugate of clopidogrel has the potential to overcome the inter-individual variability in clopidogrel therapy.

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Introduction

Dual antiplatelet therapy with co-administration of clopidogrel and aspirin has been the standard of care in the United States for patients with acute coronary syndromes (ACS) and/or undergoing percutaneous coronary interventions (PCI) (1), as recommended by the American Heart Association and the American College of Cardiology. The thrombotic events associated with ACS and PCI are primarily driven by the activation of platelets. Therefore, inhibition of platelet aggregation (IPA) in the early stage of thrombus formation by antiplatelet agents such as aspirin and clopidogrel is vitally important in improving thrombotic events and patient prognosis (2). Despite its wide use in clinical practice, clopidogrel has the potential to overcome the inter-individual variability. A large body of evidence have demonstrated that mutations in the CYP2C19 gene are primarily responsible for the variable response to clopidogrel therapy, in addition to other factors such as age, body mass index (BMI), diabetes mellitus, etc (6–8). It is now understood that clopidogrel is a prodrug requiring metabolic bioactivation to its pharmacologically active metabolite (AM) by hepatic cytochromes P450 (P450s or CYPs) including CYP2C19 (9–11). The pharmacological effect of clopidogrel arises from irreversible modification of the P2Y12 receptor by the AM preventing ADP-induced platelet aggregations. Dansette et al. showed that clopidogrel is bioactivated to the AM in two consecutive steps of oxidation by P450s (12–15); clopidogrel is first oxidised to 2-oxoclopidogrel which is then oxidised to the AM via a glutathionyl conjugate as illustrated in Figure 1. According to Kazui et al., the main contributors to the first and second steps of bioactivation include CYP1A2, 2B6, 2C19 and CYP2B6, 2C9, 2C19, 3A4, respectively (11). These isoforms of P450s involved in the bioactivation processes are polymorphic and their catalytic activities in human...
livers are subjected to inhibition and induction by other xenobiotics. These factors are confounded to affect the effectiveness of clopidogrel in an unpredictable manner.

The antplatelet activity of clopidogrel is also stereo-selective. Savi et al. reported that metabolism of (7S)-clopidogrel in human liver microsomes (HLMs) produced four diastereomers, referred as to H1, H2, H3, H4, and only H4 was found to possess antiplatelet activity (16). According to Bluett et al. (17), the stereoisomer of H4 has a cis exocyclic double bond and (7S, 4R) configuration. Historically the mixture of H1, H2, H3 and H4 is referred to as the AM. In the human plasma from individuals receiving clopidogrel, only H3 and H4 were observed (18). Clearly bioactivation of clopidogrel to H4 is the most important factor determining the antplatelet activity of clopidogrel. However, the kinetics for the formation of H4 has not been carefully examined.

We previously reported that stable mixed disulfide conjugates of clopidogrel are capable of releasing the AM in the presence of glutathione (GSH) alone via a thiol exchange reaction (19). Furthermore, release of the AM does not involve P450s. Therefore, it is possible to utilise the stable mixed disulfide conjugates of clopidogrel as antiplatelet agents to overcome the inter-individual variability. In this work we have investigated the antiplatelet potential of a stable mixed disulfide conjugate of clopidogrel with 3-nitropyridine-2-thiol (clopNPT) in male New Zealand white rabbits. The results suggest that clopNPT was rapidly converted to the AM in vivo leading to potent IPA without the need for P450s.

Materials and methods

Materials

(7S)-2-oxoclopidogrel was purchased from Shanghai Chem-Partner Corporation (Shanghai, China). cis-clopidogrel-MP and isotope-labeled trans-clopidogrel-MP (13C, D) were purchased from Toronto Research Company (Toronto, ON, Canada). L-α-dilauroyl phosphatidylcholine (DLPC) was purchased from Doosan Serdary Research Laboratory (Toronto, ON, Canada). Pool HLMs were purchased from XenoTech LLC (Lenexa, KS, USA). All other chemical reagents such as 3’-methoxyphenyl bromide (MPB) and 3-nitropyridine-2-thiol (NPT) were purchased from Sigma Aldrich Corporation (St Louis, MO, USA). Recombinant truncated forms of P450s, full-length membrane forms of cytochrome P450 reductase (CPR) and cytochrome b5 (cyt b5) were heterologously over-expressed in bacterial C41(DE3) cells and purified to homogeneity as previously described (20).

Biosynthesis of the mixed disulfide conjugate of clopidogrel with 3-nitropyridine-2-thiol (clopNPT)

The clopNPT conjugate was synthesised enzymatically using recombinant 2C19 in the reconstituted systems. In a typical reaction, 2C19 (150 nmoles), CPR (450 nmoles), and cyt b5 (750 nmoles), and DLPC (9 mg) were reconstituted in 300 ml of 50 mM KPi (pH 7.4). 2-oxoclopidogrel and NPT were added to final concentrations of 0.1 and 0.3 mM, respectively. The reaction was initiated by the addition of an NADPH regenerating system containing 1 mM NADP, 3 mM glucose-6-phosphate and 1 unit/ml glucose-6-phosphate dehydrogenase. After an incubation of 37°C with orbital shaking at 100 rpm for 90 min the reaction was terminated by the addition of perchloric acid to 0.7%. The quenched reaction mixture was centrifuged at 16,000 g for 30 min to precipitate protein aggregates. The supernatant containing clopNPT was sub-
Inhibition of platelet aggregation by clopNPT in male New Zealand white rabbits

The procedures used to determine ex vivo platelet reactivity were in accordance with the guidelines of the University of Michigan University Committee on the Use and Care of Animals (National Institutes of Health publication No. 86–23). The University of Michigan Unit for Laboratory Animal Medicine provided all veterinary care.

ClopNPT was administered to untreated or liposomal GSH-treated rabbits (2.2–3.2 kg) at a dose of 2 mg/kg as intravenous bolus via the jugular vein within 1 min. In the case of liposomal treatment, the rabbits were fed with 5 ml of liposomal GSH solution at a dose of 157 mg/kg for three days. The intravenous solution was prepared by dissolving clopNPT powder in a mixture of N, N-dimethylacetamide (DMA), polyethylene glycol (PEG) 400, and saline at a volumetric ratio of 5:15:80 as previously reported (21). The concentration of clopNPT in the intravenous solution was 0.7–1 mg/ml. Blood samples were drawn from carotid artery into a plastic syringe containing 3.7% sodium citrate as the anticoagulant (1:10 volume ratio of citrate to blood) prior to the intravenous injection (IV) (t=0) and at t=1, 2, and 3 hours (h) after the IV. The drawn blood samples were divided into two parts, 4.8 ml of which were used to determine the platelet reactivity. The remaining blood samples were used to determine the amounts of the AM and clopNPT in the plasma using LC-MS/MS.

The platelet reactivity was determined in platelet-rich plasma (PRP) prepared from the whole blood samples using light transmission aggregometry as we previously reported (19). To quantify the amounts of the AM and clopNPT in the plasma, the drawn blood samples were divided into two parts, 4.8 ml of which were used to determine the platelet reactivity. The remaining blood samples were used to determine the amounts of the AM and clopNPT in the plasma using LC-MS/MS.

Formation of H4 by human liver microsomes

Metabolic oxidation of (7S)-2-oxoclopidogrel by the HLMs led to the formation of four diastereomers (H1 to H4). Under the chromatographic conditions used in this study, H4 was separated from the H1-H3 isomers, as we previously reported (19). This allowed us to quantify the amounts of H4 without the interference from other isomers. The dependence of H4 on the concentration of 2-oxoclopidogrel is shown in Figure 2A. At first glance, it appears that formation of H4 follows a typical hyperbolic curve. Fitting the curve gave an apparent V\text{max} and K\text{m} of 0.14 nmol/min/mg HLM and 56 μM, respectively. However, re-plotting the velocity vs S curve in the Eddie-Hofstee plot revealed a biphasic pattern, as shown in Figure 2B. This biphasic pattern indicated that multiple P450s were involved in the formation of H4 in the HLMs. Linear regression of the Eddie-Hofstee plot yielded two sets of kinetic parameters for the high-affinity and low-affinity sites. The V\text{max} and K\text{m} values for the high-affinity site were determined to be 0.082 nmol/min/mg HLM and 22 μM, respectively. For the low-affinity site, the V\text{max} and K\text{m} values were determined to be 0.16 nmol/min/mg HLM and 78 μM, respectively. The catalytic efficiency of the high-affinity site is approximately two-fold higher than that of the low-affinity site.
ing 1A2, 2B6, 2C8, 2C9, 2C19, and 2D6. The $K_m$ value of 2C19 is 16 µM, similar to the $K_m$ value of 22 µM observed for the high-affinity site in the HLMs. Of the six P450s, 2C19 has the highest catalytic efficiency ($0.027 \mu M^{-1} min^{-1}$) for the formation of H4. The catalytic efficiency for the formation of H4 follows the order of 2C19 ≈ 2B6 > 2C8 > 2D6 > 2C9 > 3A4. Unexpectedly, negligible amounts of H4 were produced by recombinant 3A4, in contrast to the results obtained with cDNA-expressed P450 3A4 supersomes (11).

**Biosynthesis of clopNPT using recombinant 2C19**

As shown in Figure 3, 2C19 has the highest catalytical efficiency for converting 2-oxoclopidogrel to H4. Therefore, we elected to biosynthesise clopNPT using recombinant 2C19. As shown in Figure 4, simultaneous formation of the diastereomers of clopNPT observed at 9.0 and 9.9 min in the presence of NADPH was correlated with the consumption of 2-oxoclopidogrel observed at 7.4 and 7.8 min. The clopNPT conjugate was formed as the only major metabolite. It was estimated that approximately 40% of 2-oxoclopidogrel was converted to clopNPT within 90 min of incubation. The clopNPT formed in the reconstituted system was purified using preparative reverse phase chromatography to give a purity of > 95% based on the results from LC-MS analyses. As shown in Figure 5, the total ion chromatogram (TIC) showed the diastereomers of clopNPT at 9.0 and 9.9 min without the presence of any major impurities. Furthermore, the MS at 9.9 min showed only a pair of isotopic peaks characteristic of the presence of one chlorine atom in clopNPT, and no major impurities.

**Antiplatelet activity of clopNPT in male New Zealand white rabbits**

To investigate the capability of clopNPT in inhibiting platelet aggregation in vivo, clopNPT was administered IV to male New Zealand white rabbits. Both the platelet reactivity and the amount of the AM formed and clopNPT remaining in the plasma were determined in the same blood samples. As shown in Figure 6, IV injection of clopNPT at a dose of 2 mg/kg led to rapid IPA. Within 1 hour (h) of IV, platelet aggregation was inhibited by ~70% in both untreated and liposomal GSH-treated rabbits (Figure 6, white and grey bars). Approximately the same levels of inhibition persisted for 3 h. Even at 24 h a significant level of IPA remained (see Suppl. Figure 1, available online at www.thrombosis-online.com). This observation is consistent with the irreversible inhibition of the P2Y$_{12}$ receptor by the AM. Suppl. Figure 1 (available online at www.thrombosis-online.com) also shows that the level of IPA depends on the dosage of clopNPT. In contrast, IV injection of clopidogrel at a dose of 2 mg/kg did not have any effect on platelet aggregation, consistent with the results reported by Shan et al. (21).

Figure 7 showed the quantitative results for the amounts of the AM and clopNPT in the plasma of untreated rabbits. To correlate the IPA with the formation of the AM, these results were obtained from the same blood samples as those used for the analyses.
Zhang et al. Rapid IPA by clopidogrel conjugate

As shown in Figure 7A, the baseline plasma at t=0 h contained neither the AM nor clopNPT. However, a significant increase in the amount of the AM was observed at 1 h after administration of clopNPT. The plasma concentration of the AM at 1 h was determined to be 8.5 ng/ml (see Figure 7B). The plasma level of the AM was rapidly decreased over time; less than 10% of the AM remained at 3 h. The amount of the AM in the plasma decreases to the baseline level at 24 h as shown in Suppl. Figure 2 (available online at www.thrombosis-online.com). The parent clopNPT compound was not observed in any of the blood samples (data not shown), indicative of rapid conversion of clopNPT to the AM in rabbits.

Discussion

In this study we have demonstrated the potential of a stable mixed disulfide conjugate of clopidogrel, clopNPT, as an effective antiplatelet agent. Our results showed that administration of clopNPT as intravenous bolus to rabbits at a dose of 2 mg/kg resulted in rapid IPA by ~70% within 1 h. The inhibitory effect of clopNPT persisted for more than 3 h. It was found that the IPA observed in untreated rabbits was similar to that observed in liposomal GSH-treated rabbits. As reported previously, cellular levels of GSH in liposomal GSH-fed rabbits were significantly raised in heart, liver, and brain tissues (22), which would accelerate the release of the AM from clopNPT. The comparable IPA values observed in both untreated and liposomal-GSH treated rabbits indicate that cellular levels of GSH in untreated rabbits are high enough to allow rapid release of the AM from clopNPT in vivo. Of note, IV injection of clopidogrel at the same dosage had no effects on platelet reactivity.

The thiol exchange reaction between the mixed disulfide conjugates of clopidogrel and GSH followed second-order kinetics with rate constants in the range of 1–30 M$^{-1}$s$^{-1}$ (19). Therefore, greater GSH concentrations promote faster release of the AM. Although the cellular levels of GSH vary considerably, the highest concentrations of GSH were found in the livers. It was reported that the concentrations of GSH in the liver were in the range of 1–10 mM (23). At such high concentrations of GSH, clopNPT would readily exchange thiols with GSH to release the AM. In addition, the thiol exchange reaction can be further facilitated by glutaredoxin. It was reported that glutaredoxin was involved in the formation of the pharmacologically active metabolites of clopidogrel and prasugrel from their GSH conjugates (24, 25). The rate for the formation of the AM from the GSH conjugate of clopidogrel was stimulated up to ~20-fold in the HLMs by human liver cytosols containing glutaredoxin (24). Hagihara et al. identified that glutaredoxin 1 (Grx1) in human livers is responsible for the formation of the AM (25). It is conceivable that clopNPT can be reduced by GSH/Grx1 in the liver to facilitate the formation of the AM. This seems consistent with the rapid IPA and the observation that no clopNPT remained in the plasma. This is likely due to rapid conversion of clopNPT to the AM either by GSH alone or by GSH/Grx1. Simultaneous formation of the AM with the rapid IPA indicates that there is no significant metabolic delay in responses to clopNPT.
addition, the long-lasting effect of IPA by clopNPT is also consistent with the irreversible modification of P2Y$_{12}$ receptor by the AM. These results unequivocally suggest that clopNPT is converted to the AM, which in turn irreversibly modifies the P2Y$_{12}$ receptor leading to potent IPA.

It is clear that conversion of clopNPT to the AM by GSH or GSH/Grx1 does not require metabolic activation by polymorphic P450s. In fact, stable mixed disulfide conjugates of clopidogrel retain the identical pharmacological "warhead" as that of clopidogrel while eliminating the "side effects" associated with the bioactivation of clopidogrel by P450s. As such, they present a solution to overcome the inter-individual variability and the slow onset time of clopidogrel since both of the shortcomings are closely related to the metabolic activation by P450s.

The biphasic pattern observed in the Eddie-Hofstee plot (see Figure 2B) supports the notion that multiple P450s are involved in the bioactivation of clopidogrel. This is not unexpected since results from the metabolic studies in the reconstituted systems indicated that conversion of 2-oxoclopidogrel to the AM was not exclusive to a single isoform of P450s (see Figure 3). Whether these isoforms of P450s contribute to the formation of H4 in vivo is likely dependent on their catalytic capabilities, levels of expressions, and the formation of 2-oxoclopidogrel in the first step of the bioactivation. The Eddie-Hofstee plot revealed a high-affinity site for the formation of H4 in the HLMs with a $K_m$ of 22 µM, which is similar to those of 2C19 (16.2 µM) and 2C9 (27.2 µM) obtained in the reconstituted systems. Since 2C19 is involved in both steps of bioactivation with high efficiency, it is expected that 2C19 plays a major role on the bioactivation of clopidogrel in humans. However, it is unexpected that H4 was not detected in the reconstituted system of 3A4, in contrast to previous studies in cDNA-expressed 3A4 supersomes (11). According to these authors, the catalytic efficiency of 3A4 was six-fold less than 2C19 for the conversion of 2-oxoclopidogrel to the AM in cDNA-expressed 3A4 supersomes. It is likely that the catalytic efficiency of 3A4 was further compromised in our reconstituted system due to truncation of the N-terminus of 3A4 and changes in lipid compositions. Our studies of conversion of 2-oxoclopidogrel in the reconstituted system was useful in guiding the biosynthesis, but did not rule out the role of 3A4 in the bioactivation of the AM in HLMs due to the fact that 3A4 constitutes as much as 60% of the P450s expressed in human livers (26).

A number of studies showed that clopidogrel is extensively metabolised by P450s and carboxylesterase to generate a number of inactive metabolites (16, 27, 28). These attrition pathways greatly attenuate the metabolic activation efficiency. It was estimated that less than 1% of clopidogrel ingested was converted to the AM (27). Early pharmacokinetic studies showed that clopidogrel was rapidly absorbed in the small intestine after oral administration since the major metabolite of clopidogrel, an inactive carboxylic acid, reached $C_{max}$ within 1 h (29).
What is known about this topic?

- Clopidogrel exhibits interindividual variability in pharmacodynamic response.
- Clopidogrel is a prodrug requiring bioactivation by hepatic P450s.
- Inefficient bioactivation of clopidogrel by P450s contributes to the inter-individual variability and slow onset time.

What does this paper add?

- A novel mixed disulphide conjugate of clopidogrel exhibits rapid and potent IPA in vivo.
- The conjugate does not require bioactivation by P450s.
- A biosynthetic method has been developed to synthesise the conjugate.

seemed contradictory to the slow onset time for the IPA. In fact, significant IPA was usually achieved between 3-7 days of dosing at 75 mg/day, and the onset time was significantly shortened to 6 h after a loading dose of 300 mg (30). These findings indicated that the slow onset time of clopidogrel is due to inefficient bioactivation of clopidogrel by P450s. The significant delay in pharmacological response limits the use of clopidogrel in emergent ACS situations. Compared with clopidogrel, prasugrel induces faster and more potent IPA. It was reported that the AM of prasugrel reached C\text{max} at 30 min after an oral dose in human or animal plasma and achieved 70% IPA approximately 6 h after a 60-mg dose in healthy volunteers (31).

As pointed out by Silvain and Montalescot (32), IV formulation of rapid P2Y\text{12} antagonists is still an unmet medical need. This is because the IV formulation of P2Y\text{12} antagonists is especially useful in emergent ACS situations where administration of antiplatelet agents in the early stage of thrombus formation improves patient survival and prognosis. In the emergent ACS situations, it is desirable to administer antiplatelet agents after angiography when the coronary anatomy can be defined. Administration of antiplatelet agents without angiography increases the risk of bleeding if patients need coronary artery bypass graft surgery. To work in this narrow window of time, availability of an IV formulation of fast-acting antiplatelet agents with proven record of efficacy for ACS/PCI patients is highly valuable. The two new P2Y\text{12} antagonists, prasugrel and ticagrelor, are unlikely to meet this need for several reasons: 1) both are oral drugs only, 2) both have shown delayed onset of action in patients with ST-elevated myocardial infarction (STEMI). Recent studies showed that prasugrel and ticagrelor do not provide adequate IPA in STEMI patients within 2 h of administration (33, 34). 3) Concerns with bleeding in older or underweight patients for prasugrel and dyspnea for ticagrelor may limit their use. The rapid onset time observed with clopNPT has the potential to fill this unmet need.

In conclusion, quantitative analysis of the formation of H4 in both HLMs and reconstituted systems confirmed that 2C19 plays a major role in the bioactivation of clopidogrel. More importantly, we have demonstrated that intravenous administration of clopNPT to rabbits led to rapid formation of the AM in plasma with concomitant IPA. Within 1 h of IV administration, clopNPT at a dose of 2 mg/kg resulted in an IPA of ~70% whereas clopidogrel administered at the same dosage had no effects. This rapid delivery of the AM without the need for P450s has the potential to overcome the inter-individual variability associated with clopidogrel therapy. In addition, an intravenous formulation of fast-acting clopNPT as antiplatelet agent may fill the unmet medical need in emergent ACS situations.

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Conflicts of interest

None declared.

References


Abbreviations

ACS, acute coronary syndrome; AM, active metabolite of clopidogrel; CPR, cytochrome P450 reductase; CYP or P450, cytochrome P450; Cyt b5, cytochrome b5; HLM, human liver microsome; HPLC, high performance liquid chromatography; IPA, inhibition of platelet aggregation; MPB, 3’-methylxophenacyl bromide; LC-MS/MS, liquid chromatography-tandem mass spectrometer; NPT, 3-nitropyridine-2-thiol; PCI, percutaneous coronary intervention; PRP, platelet-rich plasma.