Ticagrelor Effectively and Reversibly Blocks Murine Platelet P2Y12-Mediated Thrombosis and Demonstrates a Requirement for Sustained P2Y12 Inhibition to Prevent Subsequent Neointima*

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Objective—Our goal was to study the effects of ticagrelor on murine platelet function and thrombosis and characterize the time course of P2Y12 inhibition required to inhibit neointima formation following vascular injury.

Methods and Results—Mice were treated with ticagrelor or vehicle. Platelet aggregation and P-selectin expression were assessed over time, and thrombus formation was assessed in laser-injured cremasteric arterioles of P2Y12+/− and P2Y12−/− mice. Neointima formation in FeCl3-injured carotid artery was assessed in C57BL/6 mice treated with different regimens of ticagrelor. Ticagrelor inhibited platelet aggregation and P-selectin expression in a dose-dependent, reversible manner. Ticagrelor inhibited thrombus formation to the same extent as seen in P2Y12−/− mice. Neointima formation was markedly reduced in mice treated with ticagrelor before and 4 hours after injury (neointima area: control, 39 921±22 749 μm², versus ticagrelor, 3705±2600 μm²; P<0.01), whereas administration of ticagrelor either before injury only or from 4 hours postinjury was ineffective.

Conclusion—Ticagrelor effectively and reversibly inhibits P2Y12-mediated platelet function and thrombosis in mice. P2Y12 inhibition is required both at the time of and after injury to effectively inhibit neointima formation. Additional studies are warranted to evaluate the role of P2Y12 inhibition in preventing restenosis. (Arterioscler Thromb Vasc Biol. 2010;30:2385-2391.)

Key Words: platelet receptor blockers ■ platelets ■ restenosis ■ thienopyridines ■ thrombosis

A DP is released from platelet-dense granules following activation of platelets and acts on 2 platelet surface G-protein-coupled receptors, P2Y1 and P2Y12.1–3 The P2Y12 receptor strongly amplifies and sustains platelet activation and associated platelet responses, including aggregation, granule secretion, and procoagulant activity.1,4 For example, thrombin activates platelets via protease-activated receptor (PAR)1 and PAR4 in humans or PAR3 and PAR4 in mice, and released ADP acting on P2Y12 amplifies the platelet responses to PAR activation.5,6 Platelets play a central role in arterial thrombosis and its clinical manifestations, such as myocardial infarction, stroke, and sudden cardiac death, and targeting the platelet P2Y12 receptor has proven to be a successful strategy in preventing and treating arterial thrombosis.7 Recently, we have shown that platelet P2Y12 receptors play an important role in late neointima formation in murine arterial injury models, and pharmacological blockade with clopidogrel or genetic deletion of P2Y12 receptors significantly attenuates this response.8 The platelet P2Y12 receptor contribution to neointima formation indicates that it is a potential target for preventing arterial restenosis in patients treated by percutaneous coronary intervention (PCI). Thienopyridines, such as clopidogrel, are converted to active metabolites in the liver, which then bind irreversibly to the P2Y12 receptor, and these agents have proven efficacy in stable atherosclerotic disease and acute coronary syndromes.9–11 There is wide variability of response to clopidogrel among individuals, and patients who have a poor pharmacodynamic response to clopidogrel therapy are at increased risk of arterial thrombotic events.12–14 Irreversibility of action may also pose a disadvantage in patients who require major surgery.

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Ticagrelor is an oral, reversibly binding P2Y12 receptor inhibitor that yields, in a dose-dependent fashion, greater and more consistent inhibition of platelet aggregation than standard regimens of clopidogrel in patients with stable atherosclerotic disease and acute coronary syndromes.15–19 The

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results from the Study of Platelet Inhibition and Patients Outcomes (PLATO) assessing the efficacy of ticagrelor compared with clopidogrel in patients with acute coronary syndromes showed a significant decrease in the rate of death from vascular causes, myocardial infarction, or stroke in patients treated with ticagrelor.20 The regimen of ticagrelor studied in the PLATO study was intended to deliver a high level of P2Y12 inhibition at the time of PCI, in those patients undergoing this procedure, which would then be sustained following PCI. We assessed the effects of ticagrelor on platelet function and thrombosis in mice with or without functional P2Y12 receptors and used the reversible properties of ticagrelor to study the timing of P2Y12 inhibition required to prevent neointima formation following arterial injury. We hypothesized that effective P2Y12 inhibition would be required both at the time of injury and sustained thereafter to prevent the subsequent formation of neointima.

Methods

Materials

ADP, 5-hydroxytryptamine, EDTA, prostanandin E1, apyrase, 2-methylthio-adenosine diphosphate, and MR52179 were from Sigma.18 32P-2-Methylthio-adenosine diphosphate was obtained from PerkinElmer. PAR4 thrombin receptor activating peptide (TRAP) with the sequence AYPGKF was custom synthesized by AnaSpec USA. The fluorescein isothiocyanate–labeled antibodies for P-selectin (rat anti-mouse, RB40.34) and rat IgG isotype control (A95–1) were from BD Pharmingen. Cangrelor (AR-C69931MX), another P2Y12 inhibitor suitable for in vitro studies,1 was provided by AstraZeneca R&D (Molndal, Sweden). Hirudin was recombinant desulfatohirudin (Revasc), a gift from Rhone Poulenc Rorer. Fixative solution consisted of saline with KH2PO4, and 0.16% (wt/vol) formaldehyde, pH 7.4.

Drug Administration

Male mice (C57BL/6, P2Y12−/−, and P2Y12+/+) were gavaged with either ticagrelor (up to 100 mg/kg in 200 μL of tap water) or tap water alone, using a gavage needle (20 gauge curved metal, Fine Science Tools, Heidelberg, Germany) and syringe. Ticagrelor (100 mg/kg in normal saline) or saline control was also administered via intraperitoneal injection at 4 hours postinjury in the FeCl3 injury model because gavage carries the risk of aspiration if performed during anesthesia. Sufficient doses and time points were studied to demonstrate dose-dependent effects and reversibility of action of ticagrelor while also aiming to limit the number of animals used to the minimum necessary; hence, some time points were not studied for the lowest doses of ticagrelor. All animal experiments were carried out under a UK Home Office project license. In the FeCl3 injury studies, ticagrelor or vehicle was dosed preinjury, 4 hours postinjury, and 24 hours postinjury (Table), the latter time point being selected as a next-day dose before complete recovery of platelet aggregation, as predicted by the platelet aggregation and P-selectin studies.

Animals

Male wild-type C57BL/6 (Harlan, Bicester, United Kingdom) and P2Y12−/− mice or +/− litter mates (derived from breeding pairs supplied by Schering Plough Research Institute6) weighing between 23 and 26 g were used in these experiments. Four mice were used in each group for the platelet aggregation and P-selectin studies and for the laser injury studies. Eight mice per group were used for the neointima studies with the intention of having at least 5 mice per group to allow for procedural fatality.

Blood and Plasma Preparation

Blood (0.5 to 1 mL) was withdrawn by cardiac puncture into 50 to 100 μL of hirudin (5 μg/μL; 900 anti-IIa units/mL) and incubated in polystyrene tubes at 37°C. Plasma was prepared by centrifugation of blood from ticagrelor-treated mice and frozen at −80°C before pharmacokinetic analysis by AstraZeneca.

Platelet Aggregation and P-Selectin Expression in Whole Blood

Platelet aggregation and P-selectin expression were assessed as previously described.9 Before blood withdrawal, the following polystyrene tubes were prepared to test for platelet aggregation: EDTA (4 μL of 20 mmol/L EDTA +16 μL of blood) and 30 μmol/L ADP +3 μmol/L 5-hydroxytryptamine (10 μL of each agonist +220 μL of blood). Aliquots of anticoagulated blood (220 μL) were placed into each of the tubes in a heated stir-block (37°C; stirring speed, 800 rpm) for 4 minutes, after which 500 μL of fixative solution was added. Fixed samples were counted using a KX21 Hematology Analyser (Sysmex Corporation), and the percentage of aggregation was calculated as the percentage of loss of single platelets compared with total single platelet count (EDTA sample).

Bleeding Time Measurement

Bleeding time assessments were performed by transecting the end 5 mm of the tails of anesthetized mice and placing them in a beaker of saline at room temperature. Bleeding was observed for up to 30 minutes, and the time to hemostasis was recorded.

Laser Injury Model of Vascular Thrombosis

Mice were anesthetized with an intraperitoneal injection (12.5 μL/g) of a mixture consisting of 10 mg/mL ketamine hydrochloride (Ketaset, Willows Francis Veterinary, Crawley, United Kingdom), 1 mg/mL xylazine hydrochloride (Bayer, Suffolk, United Kingdom), and 0.02 mg/mL atropine sulfate (Phoenix Pharmaceuticals, Gloucester, United Kingdom). Body temperature was maintained at 37°C using a heat pad (Pdtronics, Sheffield, United Kingdom).
tracheostomy was performed to facilitate breathing, and the internal jugular vein was cannulated to allow intravenous administration of maintenance anesthesia and diocetyloxycarbocyanine perchlorate (DiOC6), a dye that nonspecifically labels both platelets and leukocytes. The mice were then placed ventral side up on a viewing stage consisting of a base plate with a central area containing a raised circular area (diameter, 24 mm; height, 15 mm) topped with a permanently attached glass cover slip. The central area has a raised edge to contain the buffer (which drips onto the preparation to keep it warm and moist). Excess buffer was drained away by suction. The cremaster muscle was exteriorized through a small incision in the scrotum. The muscle pouch was opened by an anterior linear incision, carefully avoiding the major vessels. The opened cremaster scrotum. The muscle pouch was opened by an anterior linear incision, carefully avoiding the major vessels. The opened cremaster muscle was spread and pinned across the raised circular area of the microscope stage and superfused with thermocontrolled bicarbonate buffer solution (131.9 mmol of NaCl, 18 mmol/L NaHCO3, 4.7 mmol of KCl, 2.0 mmol of CaCl2·2H2O, and 1.2 mmol of MgCl2), through which a gas mixture of 5% CO2 in N2 was passed. DiOC6 (5 µL of a 100 µmol solution/g of body weight) was infused through the jugular cannula 10 minutes before induction of the first thrombus was begun. One to 3 arterioles were visible in each cremaster muscle preparation. Arterioles with undisrupted flow were chosen, and endothelial injury was induced using a pulsed nitrogen dye laser at 440 nm that was focused onto the blood vessel wall through the microscope optics. Wide-field-fluorescence (660 nm excitation wavelength, 60 milliseconds) and bright-field (40 milliseconds) images were collected alternately for up to 3 minutes after injury formation. Thrombi were visualized using a Nikon fluorescence microscope with a ×40 water immersion objective lens (numeric aperture, 0.9) and recorded using a 3CCD Nikon SensiCam digital camera. Images were taken in a rapid-repeating sequence to visualize platelets (660 nm excitation wavelength, 500 milliseconds) followed by a bright-field image (20 milliseconds). Data were collected and analyzed using Slide Book imaging software, version 4.0 (Intelligent Imaging Innovations, Denver, Colo), to determine fluorescence area, and graphical analysis was performed using Sigma Plot (SPSS, Chicago, Ill). Thrombus areas were measured over time by determining the area at 1-second intervals for 100 seconds.

FeCl3 Injury
Mice were anesthetized by intraperitoneal injection (0.01 mL/g) of Hypnorm solution (0.02 mg/mL fentanyl citrate, 1.25 mg/mL fluanisone) and midazolam (2.5 mg/mL). Under aseptic conditions with minimal incision, the right carotid artery was exposed, and a 1×2-mm strip of filter paper soaked in 10% (wt/vol) FeCl3 solution was applied to the common carotid artery for 3 minutes. The surface of the artery was washed with saline, and the dermis was subsequently approximated and sutured. The animals were allowed to recover in an incubator.

Statistical Analysis
Data are presented as mean and SD and were analyzed using GraphPad Prism (version 5.00). The correlation between P-selectin expression and plasma ticagrelor levels was assessed by the Spearman rank correlation coefficient. The Mann-Whitney test was used to compare the differences among different groups with significance attached to probability values as follows: ticagrelor, 20.0 ± 1.0 minutes in ticagrelor-treated P2Y12−/− mice; 26.6 (18.3 to 30) minutes in ticagrelor-treated animals, 30 minutes in P2Y12−/− mice, and 29.8 (29.3 to 30) minutes in ticagrelor-treated P2Y12−/− mice (n = 4 all peak levels of inhibition seen at around 2 hours after dosing, after which there was reversal of the inhibitory effect of ticagrelor (Figure 1B).

Platelet P-Selectin Expression
Ticagrelor inhibited TRAP-induced P-selectin expression in a dose-dependent and reversible fashion, as seen with the aggregation results (Figure 2A). The extent of inhibition of P-selectin expression was determined by the ticagrelor plasma level (r = −0.7; P < 0.0001) (Figure 2B). At the peak levels of inhibition following 30 to 100 mg/kg ticagrelor, there was no additional inhibition seen on adding 1 µmol/L cangrelor in vitro (for example, 4 hours after 30 mg/kg ticagrelor, median fluorescence with 3 mmol/L TRAP were as follows: ticagrelor, 20.0 ± 5.1, versus ticagrelor + cangrelor, 19.7 ± 11.7 median fluorescence units; P = 0.96). Similar levels of inhibition of P-selectin expression were seen in mice dosed with ticagrelor via the intraperitoneal route: control, 34 ± 7, versus ticagrelor, 20 ± 3 median fluorescence units, 3 mmol/L TRAP, P < 0.01).

Bleeding Time
Ticagrelor extended bleeding time in P2Y12+/+ mice to the same level as seen in P2Y12−/− mice (Figure 1A). Platelet aggregation was inhibited in a dose-dependent fashion, with

| Figure 1. Plasma levels of ticagrelor (A) and mean percentage platelet aggregation (B) determined by whole-blood single-platelet counting 4 minutes after addition of ADP and 5-hydroxytryptamine to hirudin-anticoagulated whole blood, before and after dosing by gavage of 10 to 100 mg/kg ticagrelor. Data are mean ± SD (n = 4 at each time point). | Patil et al Ticagrelor and Neointima | 2387 |
Because the bleeding times were at or near the maximal time studied (30 minutes), it was not possible to determine whether ticagrelor had any additional effect in P2Y12/−/−/− mice.

Laser Injury Model

Thrombus formed rapidly after laser injury in P2Y12+/+/+ mice but was markedly attenuated and more unstable in P2Y12−/−/− mice and mice treated with ticagrelor (Figure 3A to 3D). Area under the curve data (Figure 4) showed a significant difference between P2Y12+/+/+ mice and both P2Y12+/−/+ mice treated with ticagrelor and P2Y12−/−/− mice (both P<0.001). No additional effect of ticagrelor was seen in P2Y12−/−/− mice.

FeCl3 Injury

The intima:media ratio (mean) and neointima (maximum) area were significantly decreased in mice treated with ticagrelor both before and after injury (Figure 5A, 5B and 6). Intima:media ratios and neointima area in mice treated with ticagrelor either before injury only or postinjury only were not significantly reduced compared with controls.
We have shown that ticagrelor effectively inhibits platelet aggregation and P-selectin expression in mice in a dose-dependent fashion. Ticagrelor has a short plasma half-life in mice, which substantially limits its duration of action, and high doses of ticagrelor (30 to 100 mg/kg) were required in mice to achieve maximum inhibition of platelet function over the course of 4 hours postdosing. The rapid metabolism of ticagrelor is associated with marked reversibility of effect such that a dose of 10 mg/kg was associated with a peak effect on platelet aggregation at 1 to 2 hours after dosing and a substantial reversal of antiplatelet effect by 4 hours, reflecting the fall in plasma ticagrelor levels. These results are analogous to results of clinical studies showing more rapid recovery of platelet function following cessation of ticagrelor therapy compared with clopidogrel.21 The laser injury studies demonstrated the need for effective P2Y\textsubscript{12} inhibition both at the time of injury and sustained thereafter to effectively inhibit subsequent neointima formation, suggesting that transient accumulation of platelets at the site of arterial injury either immediately or hours after injury is sufficient to drive neointima formation. In the PLATO study, patients randomly selected to receive ticagrelor were pretreated with a loading dose of ticagrelor before PCI, and this is known to achieve a high level of P2Y\textsubscript{12} inhibition.28 Consequently, further analysis of target vessel revascularization rates in the PLATO study are warranted to assess any potential therapeutic effect. In addition, additional angiographic studies are warranted to determine whether sustained high-level P2Y\textsubscript{12} inhibition can reduce the incidence of angiographic restenosis.

![Graph](image-url)
The effects on restenosis and target vessel revascularization of antiplatelet drugs targeting other pathways of platelet activation and aggregation have been assessed in a number of studies, with the most data being available on glycoprotein IIb/IIIa antagonists. Glycoprotein IIb/IIIa antagonists do not appear to consistently reduce the incidence of restenosis, although therapeutic doses of these drugs have limited inhibitory effects on platelet microaggregation and less inhibitory effects compared with P2Y12 antagonists on proinflammatory responses of platelets that may drive vascular inflammation.

In conclusion, our study demonstrates that highly effective and reversible inhibition of the platelet P2Y12 receptor by ticagrelor gives rise to significantly less thrombus and neointima formation in mice. Effective P2Y12 inhibition both at the time of injury and sustained for more than 8 hours after injury is required to effectively inhibit subsequent neointima formation. Additional studies of the effects of P2Y12 inhibitors on restenosis are warranted.

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**Disclosures**

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**References**


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