Ticagrelor Effectively and Reversibly Blocks Murine Platelet P2Y\textsubscript{12}-Mediated Thrombosis and Demonstrates a Requirement for Sustained P2Y\textsubscript{12} Inhibition to Prevent Subsequent Neointima*  

Shankar B. Patil, Laura E. Jackman, Sheila E. Francis, Heather M. Judge, Sven Nylander, Robert F. Storey

Objective—Our goal was to study the effects of ticagrelor on murine platelet function and thrombosis and characterize the time course of P2Y\textsubscript{12} 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 P2Y\textsubscript{12}\textsuperscript{+/+} and P2Y\textsubscript{12}\textsuperscript{-/-} mice. Neointima formation in FeCl\textsubscript{3}-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 P2Y\textsubscript{12}\textsuperscript{+/+} mice. Neointima formation was markedly reduced in mice treated with ticagrelor before and 4 hours after injury (neointima area: control, 39,921 ± 22,749 \textmu m\textsuperscript{2}, versus ticagrelor, 3705 ± 2600 \textmu m\textsuperscript{2}; P<0.01), whereas administration of ticagrelor either before injury only or from 4 hours postinjury was ineffective.

Conclusion—Ticagrelor effectively and reversibly inhibits P2Y\textsubscript{12}-mediated platelet function and thrombosis in mice. P2Y\textsubscript{12} 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 P2Y\textsubscript{12} 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, P2Y\textsubscript{1} and P2Y\textsubscript{12}.\textsuperscript{1-3} The P2Y\textsubscript{12} receptor strongly amplifies and sustains platelet activation and associated platelet responses, including aggregation, granule secretion, and procoagulant activity.\textsuperscript{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 P2Y\textsubscript{12} amplifies the platelet responses to PAR activation.\textsuperscript{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 P2Y\textsubscript{12} receptor has proven to be a successful strategy in preventing and treating arterial thrombosis.\textsuperscript{7} Recently, we have shown that platelet P2Y\textsubscript{12} receptors play an important role in late neointima formation in murine arterial injury models, and pharmacological blockade with clopidogrel or genetic deletion of P2Y\textsubscript{12} receptors significantly attenuates this response.\textsuperscript{8} The platelet P2Y\textsubscript{12} 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 P2Y\textsubscript{12} receptor, and these agents have proven efficacy in stable atherosclerotic disease and acute coronary syndromes.\textsuperscript{8-11} 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.\textsuperscript{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 P2Y\textsubscript{12} 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.\textsuperscript{15-19} The
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, prostaglandin E1, apyrase, 2-methylthio-adenosine diphosphate, and MR52179 were from Sigma. [33P]-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), platelet aggregation, as predicted by the platelet aggregation and P-selectin expression were assessed as previously described.3 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 mmol/L ADP + 3 mmol/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). Before blood withdrawal, flow cytometry tubes were also prepared. Plates contained 2 μL of PAR4 TRAP (0.3, 1, 3, or 10 mmol/L), 2 μL of saline or 1 μmol/L cangrelor, 10 μL of fluorescein isothiocyanate anti-CD62P, and 31 μL of PBS. Aliquots of blood (5 μL) were added to each tube and incubated in the dark for 20 minutes at room temperature. Following incubation, 2 mL of FACSFLOW (BD Biosciences, San Jose, Calif) was added, and the sample was then analyzed by flow cytometry. Nonspecific binding was determined using rat anti-mouse IgG–fluorescein isothiocyanate in place of the CD62P antibody. A gate was applied to the platelet region in a forward- versus side-scatter dot plot, and 2000 platelet events were collected. P-selectin expression was determined as the median fluorescence of the entire platelet population, and the results were expressed as an increase over baseline values.

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 used 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.

Table. Dosing Schedule in Ferric Chloride Injury Study

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Control</th>
<th>Ticagrelor Preinjury Only</th>
<th>Ticagrelor Preinjury and 4 Hours Postinjury</th>
<th>Ticagrelor Postinjury Only (4 and 24 Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour preinjury</td>
<td>Vehicle (lactose in water)</td>
<td>Ticagrelor (100 mg/kg)</td>
<td>Ticagrelor (100 mg/kg)</td>
<td>Vehicle (lactose in Water)</td>
</tr>
<tr>
<td>4 hours postinjury</td>
<td>Vehicle (lactose in water)</td>
<td>Vehicle (lactose in water)</td>
<td>Ticagrelor (100 mg/kg)</td>
<td>Vehicle (lactose in Water)</td>
</tr>
<tr>
<td>24 hours postinjury</td>
<td>Vehicle (lactose in water)</td>
<td>Vehicle (lactose in water)</td>
<td>Vehicle (lactose in water)</td>
<td>Vehicle (lactose in Water)</td>
</tr>
</tbody>
</table>

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 Institute® 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/mL; 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. 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 mmol/L ADP + 3 mmol/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). Before blood withdrawal, flow cytometry tubes were also prepared. Plates contained 2 μL of PAR4 TRAP (0.3, 1, 3, or 10 mmol/L), 2 μL of saline or 1 μmol/L cangrelor, 10 μL of fluorescein isothiocyanate anti-CD62P, and 31 μL of PBS. Aliquots of blood (5 μL) were added to each tube and incubated in the dark for 20 minutes at room temperature. Following incubation, 2 mL of FACSFLOW (BD Biosciences, San Jose, Calif) was added, and the sample was then analyzed by flow cytometry. Nonspecific binding was determined using rat anti-mouse IgG–fluorescein isothiocyanate in place of the CD62P antibody. A gate was applied to the platelet region in a forward- versus side-scatter dot plot, and 2000 platelet events were collected. P-selectin expression was determined as the median fluorescence of the entire platelet population, and the results were expressed as an increase over baseline values.

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, Gloucestor, United Kingdom). Body temperature was maintained at 37°C using a heat pad (Pdtronics, Sheffield, United Kingdom).

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tracheostomy was performed to facilitate breathing, and the internal jugular vein was cannulated to allow intravenous administration of maintenance anesthesia and diocetylcarboxycyanine 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 muscle was exteriorized through a small incision in the 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 induction. 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) and bright-field (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 excised, 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 less than 0.01 to allow for multiple group comparisons. Based on a control group neointima area of 0.06±0.015 mm² and a neointima area of 0.02 mm² with sustained P2Y₁₂ inhibition, a minimum group size of 5 mice was required to have 90% power of showing a significant difference with an α of 0.01.

**Results**

**Pharmacokinetic and Platelet Aggregation Studies**

Ticagrelor plasma levels peaked at the 1-hour time point after dosing and had fallen substantially by 2 hours, indicating that ticagrelor has a short half-life in mice (Figure 1A). Platelet aggregation was inhibited in a dose-dependent fashion, with 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 5.1, versus ticagrelor 20 mg/kg, 5.8). 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: mean (range) bleeding times were 3.2 (2.5 to 4.0) minutes in control animals, 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
groups). 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/H11002/H11002 mice.

Laser Injury Model

Thrombus formed rapidly after laser injury in P2Y12/H11001/H11001 mice but was markedly attenuated and more unstable in P2Y12/H11002/H11002 mice and mice treated with ticagrelor (Figure 3A to 3D). Area under the curve data (Figure 4) showed a significant difference between P2Y12/H11001/H11001 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.

**P<0.001 (Mann-Whitney test).
therapy compared with clopidogrel. The laser injury studies reflect 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, 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, consistent with a selective action of ticagrelor on the P2Y₁₂ receptor, although the extent of thrombus formation in P2Y₁₂⁻/⁻ mice was small, and so an additional effect of ticagrelor could not be completely excluded.

There is an injury-repair process from the time a stent is implanted in a coronary artery. In the first 24 hours, there is accumulation of platelets, fibrin, and neutrophils that subsequently drive fibrin deposition and vascular smooth cell proliferation, resulting in neointima formation within the stent.22 Despite the use of drug-eluting stents, in-stent restenosis remains a problem, with an incidence of approximately 10%, and contributes significantly to PCI-related morbidity and mortality.23,24 Furthermore, drug-eluting stents are associated with delayed endothelialization, which commits patients to a prolonged course of dual antiplatelet therapy (currently aspirin and thienopyridine), and ongoing studies seek to resolve the rare but serious problem of thrombosis occurring more than 6 to 12 months after implantation of drug-eluting stents following cessation of thienopyridine.25,26 Consequently, alternative solutions to prevent restenosis continue to merit investigation, the goal being to allow endothelialization of the stent and adequate coverage of the stent struts (which is sometimes prevented by drug-eluting stents) without excessive neointima formation leading to flow-limiting restenosis.

The gold standard for confirming the presence of restenosis is coronary angiography, but many clinical trials look at surrogate markers, such as the need for target vessel revascularization, although these will include episodes of coronary arterial thrombosis. Although many studies of clopidogrel and prasugrel have assessed their effects on target vessel revascularization, these studies have not used regimens that ensure a consistently high level of P2Y₁₂ inhibition at the time of PCI.10,27,28 Clopidogrel is known to achieve variable and inconsistent levels of P2Y₁₂ inhibition, with some individuals exhibiting a low level of inhibition, so even high loading doses of clopidogrel before PCI do not ensure consistent P2Y₁₂ inhibition at the time of PCI.29 A loading dose of prasugrel achieves a consistently high level of P2Y₁₂ inhibition, but the TRITON-TIMI 38 study, which showed the superiority of prasugrel over clopidogrel in reducing ischemic events, allowed for administration of study medication following the PCI procedure so that prasugrel-treated patients did not necessarily have effective P2Y₁₂ inhibition at the time of PCI, and only 25% of patients received clopidogrel or prasugrel before PCI.28 In our study, we demonstrated the need for effective P2Y₁₂ 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 P2Y₁₂ 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 P2Y₁₂ inhibition can reduce the incidence of angiographic restenosis.
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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