Original Research: Focus on Platelets

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
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. [33P]2-Methylthio-adenosine diphosphate, and MRS2179 were from PerkinElmer. [33P]2-Methylthio-adenosine diphosphate, and MRS2179 were from PerkinElmer. 

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.8 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). Before blood withdrawal, flow cytometry tubes were also prepared. Tubes contained 2 μL of PAR4 TRAP (0.3, 1, 3, or 10 nmol/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 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 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 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 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 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 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 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 anti-CD62P, and 31 μL of PBS.
tracheostomy was performed to facilitate breathing, and the internal jugular vein was cannulated to allow intravenous administration of maintenance anesthesia and diocetyloxacarbocyanine 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 was spread and pinned across the raised circular area of the microscopic stage and superfused with thermocirculated 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 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 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).

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).

Bleeding Time
Ticagrelor extended bleeding time in P2Y12+/+ mice to the same level as seen in P2Y12−/− mice (Figure 2A). The extent of inhibition of P-selectin expression was 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).

Figure 2. A: Plasma levels of ticagrelor 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).
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/H11002/H11002 mice in mice treated with ticagrelor and P2Y12/H11002/H11002 mice (both P<0.001). No additional effect of ticagrelor was seen in P2Y12/H11002/H11002 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 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 therapy compared with clopidogrel.21 The laser injury studies

Figure 5. Effects of dosing 100 mg/kg ticagrelor (T), compared with vehicle (control, n=8), given as a single dose before FeCl3 injury (T(pre), n=6), double dose before injury and 4 hours after injury (T(pre&4h), n=6), and double dose 4 and 24 hours after injury (T(4&24h), n=5) on mean intima:media ratio (A) and maximum neointima area (B) at 21 days postinjury. Data are mean±SD; *P<0.01 (Mann-Whitney test).

Discussion

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.\textsuperscript{30} Glycoprotein IIb/IIIa antagonists do not appear to consistently reduce the incidence of restenosis,\textsuperscript{30} although therapeutic doses of these drugs have limited inhibitory effects on platelet microaggregation and less inhibitory effects compared with P2Y\textsubscript{12} antagonists on proinflammatory responses of platelets that may drive vascular inflammation.\textsuperscript{31,32}

In conclusion, our study demonstrates that highly effective and reversible inhibition of the platelet P2Y\textsubscript{12} receptor by ticagrelor gives rise to significantly less thrombus and neointima formation in mice. Effective P2Y\textsubscript{12} 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 P2Y\textsubscript{12} inhibitors on restenosis are warranted.

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