Thienopyridines, but Not Elinogrel, Result in Off-Target Effects at the Vessel Wall That Contribute to Bleeding

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ABSTRACT
Clinical studies with clopidogrel or prasugrel show that although increased inhibition of P2Y₁₂ and platelet function improves efficacy, bleeding is also increased. Other preclinical and clinical studies have suggested a greater therapeutic index (TI) with reversible inhibitors and disproportionate effects of thienopyridines on bleeding at high doses. We used multiple in vivo (FeCl₃-induced arterial thrombosis in mesenteric arteries, blood loss after tail transection, and platelet deposition and wound closure time in a micropuncture model in mesenteric veins) and ex vivo (light transmittance aggregometry, prothrombin time; PT, prothrombin time; H11032, methyleneadenosine 5'-triphosphate) mouse models to 1) compare the TI of clopidogrel, prasugrel, and elinogrel, a reversible, competitive antagonist, with that in P2Y₁₂(-/-) mice and 2) determine whether the bleeding consequences of the thienopyridines are attributed only to the inhibition of P2Y₁₂. Data indicated greater (elinogrel) and decreased (thienopyridines) TI compared with that in P2Y₁₂(-/-) mice. The impaired TI associated with the thienopyridines was not attributed to non-P2Y₁₂ activities on platelet function or coagulation but was related to a direct effect at the vessel wall (inhibition of vascular tone). Further analysis showed that the prasugrel off-target effect was dose- and time-dependent and of a reversible nature. In conclusion, the TI of thienopyridines in the mouse may be decreased by P2Y₁₂-independent off-target effects at the vessel wall, whereas that of elinogrel may be enhanced by the reversible, competitive nature of the antiplatelet agent.

Introduction
The thienopyridine clopidogrel has emerged as a primary drug for the treatment and the prevention of arterial thrombosis in patients with cardiovascular disease (CAPRIE Steering Committee, 1996; Mehta et al., 2001; Yusuf et al., 2001). The pharmacologic activity of clopidogrel requires metabolism by cytochrome P450 to generate a highly unstable active metabolite to inhibit platelet function (Savi et al., 1992, 2000). Although the pharmacologic target of this drug was not known upon approval, work in our laboratory using an expression cloning strategy identified the target as P2Y₁₂, a Gₛ-coupled receptor on platelets (Foster et al., 2001; Hollenbach et al., 2001). In a subsequent study, we generated P2Y₁₂(-/-) mice and showed that P2Y₁₂ functions in arterial thrombosis by maintaining thrombus stability (André et al., 2003a). Although increased doses of clopidogrel provide increased inhibition of P2Y₁₂ and increased efficacy, these are invariably associated with increased bleeding (Mehta and Van de Werf, 2009). That this is a cause-and-effect relationship is supported by recent data (Rehmel et al., 2006) showing that prasugrel, another thienopyridine that also generates an active metabolite to irreversibly inhibit P2Y₁₂ and platelet function, provides increased efficacy and increased bleeding (Wiviott et al., 2007).

Although it is commonly anticipated that bleeding propensity will parallel the anti-aggregatory effects of a P2Y₁₂ antagonist, there have been multiple reports of excessive bleeding associated with thienopyridines. For example, studies performed in rabbits suggest that unexplained bleeding occurs at levels of clopidogrel greater than those required to provide complete inhibition of platelet aggregation (Wong et al., 2009). In particular, high doses of clopidogrel providing limited incremental benefits in preventing arterial thrombosis have been associated with disproportionate levels of
bleeding in both rabbits and rats (Schumacher et al., 2007; Wong et al., 2007). Similar discrepancies have been reported in humans, with CYP2C19*2 carriers having a higher rate of thrombotic events but a rate of bleeding similar to those of noncarriers (Mega et al., 2009). In addition, marked bleeding has been reported in the TRITON-TIMI 38 trial with prasugrel (Wiviott et al., 2007). In contrast, reversible antagonism of P2Y$_{12}$ has been proposed to offer a potent and safer alternative to thienopyridine agents (Storey et al., 2002; Husted et al., 2006; Wang et al., 2007; van Giezen et al., 2009), although a potential off-P2Y$_{12}$ effect on platelet function has also been reported for canegrelor (Srinivasan et al., 2009). Thus, although the antithrombotic activities of the thienopyridines can be directly linked to the inhibition of P2Y$_{12}$, platelet function, and thrombosis, it is not known whether the bleeding consequences of the thienopyridines are entirely P2Y$_{12}$-dependent.

The present study was designed to establish whether bleeding induced by the thienopyridines is entirely P2Y$_{12}$-dependent. We found that although dosing of mice with the three agents had no effects on coagulation parameters and achieved the same inhibition of arterial thrombosis and the same levels of antiaggregatory activities in vitro as in P2Y$_{12}$(-/-) mice, both thienopyridines caused more bleeding in two primary hemostasis models than those that occurred in P2Y$_{12}$(-/-) mice or in WT animals treated with a reversible P2Y$_{12}$ antagonist (elinogrel). Furthermore, dosing of P2Y$_{12}$(-/-) mice with clopidogrel or prasugrel further increased bleeding. Additional data using a mouse model of vasoconstriction indicated that one component of bleeding induced by thienopyridines is caused by inhibition of a target other than P2Y$_{12}$ at the vessel wall and that, unlike the effect on platelet P2Y$_{12}$ receptors, it is of a reversible nature.

**Materials and Methods**

**Drugs.** Clopidogrel was from Sequoia Research Products Ltd. (St. James Close, Pangbourne, UK). Prasugrel was from Albany Molecular Research (Albany, NY). Elinogrel (Ueno et al., 2010) was from Portola Pharmaceuticals Inc. (South San Francisco, CA).

**Animals.** C57BL/6J mice (Charles River Laboratories, Inc., Wilmington, MA) were used for determination of antithrombotic activities of clopidogrel and prasugrel and determination of their effects on primary hemostasis and ex vivo platelet aggregation. P2Y$_{12}$ knockout mice (on a pure C57BL/6J background (>10 times backcrossed) were used for thrombosis and hemostasis studies. All experiments were performed by investigators blinded to the different treatments. All procedures conformed to institutional guidelines and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

**Thrombosis Study.** Thrombosis on mouse mesenteric arteries (1000–1300 s$^{-1}$) was achieved and recorded as described previously with minor modifications (André et al., 2003b). Platelets were labeled in situ using rhodamine 6G (0.2 mg/ml) administered through the tail vein 10 min before visualization of the arteries. Vessel wall injury was induced by a 1 × 1-mm filter paper saturated with a 10% FeCl$_3$ solution. After 5 min, the filter paper was removed, and mesenteric arteries were rinsed with warmed saline (37°C). Platelet-vessel wall interactions were recorded for 40 additional min or until full occlusion occurred and lasted for more than 40 s. C57BL/6J mice were orally gavaged 48, 24, and 2 h before injury with the vehicle control (0.5% methylcellulose), clopidogrel (0.25, 1.5, 15, and 50 mg/kg), or prasugrel (0.1, 0.3, 1, 3, and 10 mg/kg) or 2 h (on the basis of the elinogrel murine pharmacokinetic profile [Supplemental Fig. 1A]) before injury with elinogrel (0.83, 2.5, 7.5, 20, and 60 mg/kg).

**Video Analysis.** Thrombosis was analyzed in real time using SimplePCI software (André et al., 2003a). The fluorescence intensity was recorded at a rate of 2 Hz for 40 min and plotted over time. Time to occlusion and time for appearance of the first thrombus were analyzed.

**Elinogrel Plasma Concentration Determination.** Plasma samples were analyzed for elinogrel concentration using liquid chromatography-tandem mass spectrometry. In brief, whole blood was collected on trisodium citrate (1:9 volumes), and platelet-poor plasma was prepared. Plasma samples were processed in a 96-wellCaptiva plate (0.2 μm; Varian, Inc., Palo Alto, CA). Aliquots of plasma samples were precipitated with acetonitrile containing 500 ng/ml internal standard [5-chloro-N-(4-(7-methylamino)-2,4-dioxo-1,2-dihydridouquinazolin-3-(4H)-yl)]phenylcarbonyl][thiophene-2-sulfonamide (CT55951)] or 5-chloro-N-(4-(6-fluoro-7-(d3-methylamino)-2,4-dioxo-1,2-dihydridouquinazolin-3-(4H)-yl)]phenylcarbamoyl][thiophene-2-sulfonamide (PRT061200)], and protein was precipitated. The mixture was filtered into a 96-well collection plate. The filtrate was injected onto a Sciex API4000 liquid chromatography-tandem mass spectrometry system equipped with a TurbolonSpray source. Elinogrel and internal standard were separated on a Hypersil-Keystone Betasil C$_{18}$ column (4.6 × 100 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA). A mobile phase gradient mixture of 80% mobile phase A (0.5% formic acid in water) and 20% mobile phase B (0.5% formic acid in 90% acetonitrile) to 85% mobile phase B was programmed over 2.2 min. The peak areas of the m/z 524 → 301 product ion (elinogrel) were measured against those of the m/z 506 → 283 (CT55951) or m/z 527 → 304.2 (PRT061200) product ion (internal standard) in positive ion mode. The analytical range was 2.00 to 5000 ng/ml.

**Ex Vivo Platelet Aggregation Study and Coagulation Assays.** In a separate set of experiments, C57BL/6J mice were orally gavaged for 3 consecutive days with 0.5% methylcellulose (b.i.d., in 100 μl), clopidogrel (50 mg/kg q.d. in 100 μl), prasugrel (10 mg/kg q.d. in 100 μl), or elinogrel (60 mg/kg b.i.d. in 100 μl). The last dose was given 3 h before blood collection. Measurements were performed using a four-channel lumiaggregometer using Aggrolink software (Chrono-log Corporation, Havertown, PA). Blood samples (0.6 ml; anesthetized mouse) were obtained via intracardiac puncture (on 3.2% trisodium citrate, 1 volume of trisodium citrate and 9 volumes of blood) and centrifuged at 200g for 10 min, and platelet-rich plasma (PRP) was collected. For each dose, PRP of three animals was pooled for aggregation measurements. Experiments for each dose were repeated between two and three times, six animals each. Aliquots (250 μl) of PRP were placed in cuvettes containing magnetic stirrer bars, warmed at 37°C, and stirred for 1 min to obtain a stable baseline. Aggregation in PRP was induced using ADP (Chrono-log) at 1 and 10 μM final concentrations, collagen (Chrono-log) at 10 and 5 μg/ml, and murine TRAP at 2.5 and 1 mM, and the change in light transmittance was recorded for an additional 8 min.

Frozen platelet-poor plasma samples were thawed to room temperature and coagulation parameters, activated partial thromboplastin times (aPTT) and prothrombin times (PT), were measured using an MLA Electra 800 automatic coagulation timer. Assay reagents for aPTTs were Dade actin FS activated PTT reagent (purified soy phosphatides in 100 μl), and 0.025 M calcium chloride solution. The reagent for the PT assay was Dade Thromboplastin C Plus (rabbit brain thromboplastin). All reagents were purchased from Siemens Healthcare Diagnostics Products (Deerfield, IL).

**Tail Bleeding Time Measurement.** Male mice (6–8 weeks old) were anesthetized (by subcutaneous injection) with ketamine cocktail (40 mg/kg ketamine, 2.5 mg/kg xylazine, and 0.75 mg/kg acepromazine; Henry Schein, Melville, NY) 6 min before tail transsection. Mice were then positioned in lateral recumbence on a heating pad (used to maintain body temperature) placed on a firm dissecting board (Richard-Allan Scientific, Kalamazoo, MI) with the tail straight out. Tails were transected 2 mm from the tip with a number
A 10 scalpel blade (Bard-Parker; BD, Franklin Lakes, NJ) and immediately immersed into a 20-ml scintillation vial (Wheaton Science Products, Millville, NJ) filled with 10 ml of normal saline held at 37°C by an unstirred digitally controlled water bath (VWR International, Buffalo Grove, IL). A stopwatch was started immediately upon transsection to determine time to cessation of bleeding, frequency of bleeding, and duration of rebleeding for a 15-min period. C57BL/6J mice were orally gavaged with the vehicle control, clopidogrel (1.5, 15, and 50 mg/kg), or prasugrel (1, 3, and 10 mg/kg) 48, 24, and 2 h before tail transsection or 2 h before transsection with P2Y12(-/-) or WT mice were observed using brightfield intravital microscopy. In a first set of experiments, mice were orally gavaged (using the same regimen) with maximal doses of clopidogrel, prasugrel, elinogrel, and vehicle control.

Tail Blood Loss Measurement. Blood loss was assessed when collection of blood for bleeding time determination had concluded. To measure blood loss volume, any blood collected as described above after tail transsection was frozen at −80°C overnight. After thawing the following day, 10 ml of deionized water was added to further induce hemolysis. Aliquots of each sample were analyzed via spectrophotometry (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA) and diluted further (1:5, 1:10, or 1:20) if necessary. The resulting OD490 nm values (% T) were compared against a previously determined standard curve to estimate the blood volume lost.

**Micropuncture-Induced Primary Hemostasis In Vivo in Mesenteric Veins.** The in vivo primary hemostasis model was performed via micropuncture (using a 27-gauge needle) of mesenteric veins according to the model developed by van Gestel et al. (2007). In brief, 3- to 4-week-old anesthetized P2Y12(-/-) and WT mice, previously orally gavaged (48, 24, and 2 h before injury) with either 0.5% methylcellulose, clopidogrel (50 mg/kg), prasugrel (10 mg/kg), or elinogrel (60 mg/kg 2 h before micropuncture), were given injections of rhodamine 6G (0.2 mg/ml) to fluorescently label platelets in situ. By using videomicroscopy, a 27-gauge needle (mounted on a 1-ml syringe) was pressed on the mesenteric vein to induce rupture of the vascular wall. Bleeding time and platelet recruitment at the site of micropuncture were then recorded for 20 min and quantified in real time using intravital microscopy.

**In Vivo Vasoconstriction Studies in Mesenteric Veins.** Mesenteric veins (100–190-μm diameter) from 3- to 4-week-old anesthetized P2Y12(-/-) or WT mice were observed using brightfield inverted microscopy. In a first set of experiments, mice were orally gavaged with 0.5% methylcellulose (daily), clopidogrel (50 mg/kg, prasugrel (10 mg/kg), or elinogrel (60 mg/kg 2 h before micropuncture), were given injections of rhodamine 6G (0.2 mg/ml) to fluorescently label platelets in situ. By using videomicroscopy, a 27-gauge needle (mounted on a 1-ml syringe) was pressed on the mesenteric vein to induce rupture of the vascular wall. Bleeding time and platelet recruitment at the site of micropuncture were then recorded for 20 min and quantified in real time using intravital microscopy.

**Fig. 1.** A, clopidogrel (1.5, 15, and 50 mg/kg), prasugrel (1, 3, and 10 mg/kg), and elinogrel (7.5, 20, and 60 mg/kg) were identified as providing similar levels of inhibition on thrombus stability. n = 5 to 18/group. **p < 0.01; ***p < 0.0001 versus vehicle control (V.Ctl). All animals treated with the maximal doses had patent arteries at the end of the observation period as did P2Y12(-/-) mice (2400 s). B, clopidogrel, prasugrel, and elinogrel effects on thrombus growth: prasugrel and elinogrel (not clopidogrel) dose-dependently delayed time for appearance of first thrombus. *, p < 0.05, prasugrel (H) dose versus P2Y12(-/-); ***, p < 0.0001, maximal clopidogrel dose versus P2Y12(-/-). C, representative profiles of platelet deposition in response to FeCl3-induced injury in WT mice treated with the maximal doses of elinogrel, clopidogrel, and prasugrel that prevented vascular occlusion throughout the 40-min observation period. I, intermediate; H, high; M, maximal.
q.d.), prasugrel (10 mg/kg q.d.), or elinogrel (60 mg/kg b.i.d.) for 3 consecutive days. Veins were stimulated by a superfusion of α,β-metATP (10 μl of a 1 μM solution; Sigma-Aldrich) 2 h after the last oral gavage. Challenge and evaluation of the vascular tone were performed by an investigator blinded to the treatment regimen. A similar experimental design was applied to WT mice treated with prasugrel (1, 3, and 10 mg/kg) or P2Y12(−/−) mice treated with prasugrel (10 mg/kg q.d.) for 3 consecutive days, which were rendered thrombocytopenic (anti-glycoprotein Ibα antibodies, 2 mg/kg; Emlert Analytics GmbH & Co. KG, Eibelstadt, Germany) 16 h before superfusion of α,β-metATP. Finally, another group of WT mice was orally gavaged with prasugrel (10 mg/kg q.d.) for 3 consecutive days, and their mesenteric veins were challenged 1, 2, 5, 10, 24, and 48 h after the last oral gavage. The diameter of the mesenteric veins was monitored for 5 min postchallenge.

**Statistical Analysis.** Analysis was performed using the standard Student’s *t* test. Statistical analysis of the blood loss study comparing doses of clopidogrel, prasugrel, and elinogrel to naive or 0.5% methylcellulose P2Y12 knockout mice was performed using one-way analysis of variance (Bonferroni multiple comparison test). All values are expressed as means ± S.E.M. unless otherwise specified.

**Results**

**Determination of Clopidogrel, Prasugrel, and Elinogrel Doses Reproducing the Thrombotic Profile of P2Y12(−/−) Mice.** By using the FeCl3 thrombosis model, maximal doses of clopidogrel (50 mg/kg), prasugrel (10 mg/kg), and elinogrel (60 mg/kg) that shared the phenotypic characteristics associated with the genetic targeting of P2Y12 were identified (destabilization of growing arterial thrombi preventing vascular occlusion) (Fig. 1A). Although these maximal doses totally inhibited occlusion, subtle differences were observed compared with those in P2Y12(−/−) mice because the maximal clopidogrel dose did not reach levels of inhibition similar to those achieved by P2Y12 gene targeting on the initial rate of thrombus growth \( [p < 0.0001 \text{ versus } P2Y12(−/−) \text{ for time for appearance of first thrombus} ] \) (Fig. 1B). In addition, although the maximal dose of clopidogrel prevented vascular occlusion, there were more fluorescent platelets deposited throughout the 40-min observation period (Fig. 1C). In contrast, the maximal doses of prasugrel and elinogrel had a thrombosis phenotype identical to that of P2Y12(−/−) mice. Doses of clopidogrel (1.5 and 15 mg/kg), prasugrel (1 and 3 mg/kg), and elinogrel (7.5 and 20 mg/kg) that provided similar but lower levels of inhibition on thrombus stability were also identified and were classified as intermediate and high, respectively. The elinogrel plasma concentration determined 2 min after vascular occlusion or 42 min after injury (for patent arteries) indicated that maximum levels of inhibition with elinogrel were achieved beyond 1 μg/ml (Fig. 2A). Of interest, in a separate group of animals, we found that the ex vivo pharmacodynamic monitoring (by measurement of ADP-induced platelet aggregation) of elinogrel was biased by the concentration of ADP used to induce platelet aggregation. This finding was highlighted by the fact that the 60 mg/kg dose provided an effect equivalent to that of prasugrel and clopidogrel on thrombosis in vivo but failed to block platelet aggregation induced by 10 μM ADP, although it abolished that induced by 1 μM ADP (Fig. 2B). Further analysis of the pharmacologic effects of the maximal dose of the three agents on platelet aggregation induced by collagen and murine TRAP demonstrated that similar levels of inhibition were achieved that closely reproduce the phenotype associated with P2Y12 deficiency (Fig. 3A) (André et al., 2003a). Furthermore, analysis of prothrombin time and aPTT indicated that neither the pharmacologic modulation nor P2Y12 gene targeting affected coagulation parameters (Fig. 3, B and C).

**P2Y12 Antagonists and P2Y12 Gene Targeting Differentially Affect Bleeding Time and Blood Loss After Tail Transsection.** We studied the effects of clopidogrel, prasugrel, and elinogrel on bleeding frequency and blood loss
and compared their effects to the phenotype of P2Y12(-/-) mice. Animals treated with placebo (n = 40) stopped bleeding before 2 min after tail transection but subsequently displayed multiple periods of rebleeding and cessation of blood loss (Fig. 4). At the intermediate doses, 7 of 10 (clopidogrel), 10 of 10 (prasugrel), and 2 of 10 animals (elinogrel) had continuous bleeding that lasted for the duration of the 15-min observation period (Fig. 4). At the high doses, 10 of 10 (clopidogrel), 9 of 10 (prasugrel), and 3 of 10 (elinogrel) animals displayed continuous bleeding. For the maximal doses, all animals treated with clopidogrel and prasugrel and P2Y12(-/-) mice but only 4 of 10 animals treated with elinogrel had continuous bleeding (Fig. 4).

Blood loss measurements also revealed differences between the three P2Y12 antagonists and P2Y12(-/-) mice. Maximal clopidogrel and prasugrel doses significantly increased the volume of blood loss compared with that in P2Y12(-/-) mice (clopidogrel, 551 ± 43 μl; prasugrel, 561 ± 73 μl; P2Y12(-/-) mice, 293 ± 38 μl, p < 0.0005). In addition, animals treated with the intermediate doses of clopidogrel and prasugrel had equal (clopidogrel) or greater (prasugrel) volume of blood loss compared with the P2Y12(-/-) mice (Fig. 5A), despite the fact that they were not able to prevent vascular occlusion (Fig. 1A), indicating that some levels of bleeding could not be accounted for by the sole antiplatelet effect. Data obtained with thienopyridines were in sharp contrast with the volume of blood loss associated with elinogrel, which in all cases, was less than the blood loss observed in P2Y12(-/-) mice.

**Clopidogrel and Prasugrel Increase Blood Loss When Dosed in P2Y12(-/-) Mice.** Because the maximal doses of clopidogrel and prasugrel extended blood loss beyond the level of the P2Y12(-/-) mouse, we next asked whether they would affect blood loss when administered to P2Y12-deficient animals. Compared with their respective vehicle controls, in P2Y12(-/-) mice, oral administration of clopidogrel and prasugrel but not elinogrel significantly increased the volume of blood loss (Fig. 5C).
Effects of P2Y₁₂ Antagonists and P2Y₁₂ Gene Targeting on Platelet Deposition and Bleeding and Closure Time After Mesenteric Vein Micropuncture. The micropuncture model is characterized by a rapid accumulation of platelets at the vascular wound in the ~20 s after vascular injury independently of the treatment or genotype (Fig. 6A) followed by a reopening of the wound. Animals treated with the maximal doses of the P2Y₁₂ antagonists had slightly less (clopidogrel and prasugrel) or longer (elinogrel) periods of cessation of bleeding than those that occurred in P2Y₁₂(−/−) mice (Fig. 6B). Vessels from animals treated with the thienopyridines had delayed closure and reduced platelet deposition (Fig. 6, C and D) compared with those of P2Y₁₂(−/−) mice. In contrast, elinogrel presented a faster closure of the vascular wound compared with P2Y₁₂(−/−) or P2Y₁₂(−/−)MC. B, lower therapeu tic index (ratio of the fold increase for time to occlusion (Thr.) versus vehicle control (V. Ctl)) and the fold increase in blood loss (B.L.) versus vehicle control associated with the use of thienopyridines. C, effects of maximal doses of clopidogrel (clop, n = 10), prasugrel (pras, n = 8), elinogrel (elin, n = 10), and methylcellulose (MC, n = 9) on blood loss measurement in P2Y₁₁(−/−) mice (n = 19), *p < 0.05 versus P2Y₁₁(−/−)MC; **p < 0.01 versus P2Y₁₁(−/−) or P2Y₁₁(−/−)MC. ns, not significant versus either P2Y₁₁(−/−) or P2Y₁₁(−/−)MC. BLQ, below the limit of quantitation; I, intermediate; H, high; M, maximal.

Fig. 5. A, doses of clopidogrel, prasugrel, and elinogrel providing equivalent anti-thrombotic activity differentially affect volume of blood loss. *, p < 0.005 versus clopidogrel; †, p < 0.005 versus prasugrel; ††, p < 0.0005 versus P2Y₁₁(−/−); ●, p < 0.05 versus P2Y₁₁(−/−). B, lower therapeutic index (ratio of the fold increase for time to occlusion (Thr.) versus vehicle control (V. Ctl)) and the fold increase in blood loss (B.L.) versus vehicle control associated with the use of thienopyridines. C, effects of maximal doses of clopidogrel (clop, n = 10), prasugrel (pras, n = 8), elinogrel (elin, n = 10), and methylcellulose (MC, n = 9) on blood loss measurement in P2Y₁₁(−/−) mice (n = 19), *p < 0.05 versus P2Y₁₁(−/−)MC; **p < 0.01 versus P2Y₁₁(−/−) or P2Y₁₁(−/−)MC. ns, not significant versus either P2Y₁₁(−/−) or P2Y₁₁(−/−)MC. BLQ, below the limit of quantitation; I, intermediate; H, high; M, maximal.

Fig. 6. Differential effects of maximal doses of elinogrel (E., n = 8), clopidogrel (C., n = 8), and prasugrel (P., n = 9) versus vehicle control (V. Ctl., n = 11) and genetic targeting (n = 7) on bleeding time frequency in the micropuncture model applied to mesenteric veins. A, schematic diagram of platelet deposition at site of micropuncture in mesenteric veins. B, bleeding frequency. C, reduction in micropuncture lumen (µm²) of WT-treated animals or P2Y₁₁(−/−) mice. Thienopyridine-treated mice have delayed occlusion of the vascular wound compared with P2Y₁₁(−/−) mice. Elinogrel-treated animals displayed a faster rate of closure than either thienopyridine-treated or P2Y₁₁(−/−) mice that paralleled an increased amount of platelet deposition (D). M, maximal dose.
tion in response to mechanical injury. To further investigate a potential effect of these pharmacologic agents on vascular tone, we next studied the response of mesenteric veins to in situ stimulation by α,β-metATP (1 μM), a P2X<sub>1</sub> agonist that is not sensitive to CD39-mediated degradation in vivo.

Mesenteric veins from P2Y<sub>12</sub>(−/−), elinogrel-treated, or vehicle control-treated mice rapidly constricted after stimulation by α,β-metATP. The constriction of mesenteric veins from P2Y<sub>12</sub>(−/−) mice was not sustained compared with that in veins from WT mice treated with 0.5% methylcellulose or the maximal dose of elinogrel, indicating a possible role of vascular P2Y<sub>12</sub> in the sustained constriction of the vessel wall (as suggested by Wihlborg et al., 2004).

In contrast, mesenteric veins from animals treated with the maximal doses of clopidogrel and prasugrel only partially (clopidogrel) or minimally (prasugrel) responded to α,β-metATP (Fig. 7A). Further analysis revealed that the inhibition of vasoconstriction by prasugrel was dose- and time-dependent (Fig. 7, B and C), reaching a maximum 2 to 5 h after the third oral gavage and then gradually returning to normal within 48 h. This time-dependent inhibitory activity was not observed after a single oral administration (data not shown). Because platelets can influence endothelium-dependent vasodilatation via secretion of superoxide anions (Krötz et al., 2004), we next studied the effects of prasugrel on vasoconstriction induced by α,β-metATP after platelet depletion in P2Y<sub>12</sub>(−/−) mice (mean platelet count of 29.16 ± 2.1 ×10<sup>7</sup>/μL). Under these conditions, prasugrel treatment inhibited vasoconstriction, indicating both a vascular P2Y<sub>12</sub>- and platelet-independent effect (Fig. 7D).

**Discussion**

The aim of the present study was to determine whether in the mouse, all of the hemostatic effects of clopidogrel and prasugrel are attributed to P2Y<sub>12</sub> inhibition. Doses of clopidogrel, prasugrel, and a direct-acting reversible P2Y<sub>12</sub> inhibitor (elinogrel) were normalized to provide equivalent inhibition of thrombosis. Data indicated that 1) in WT mice, maximal doses of clopidogrel and prasugrel recapitulating the phenotype of P2Y<sub>12</sub>(−/−) mice on arterial thrombosis and platelet aggregation and lacking activities on PT and aPTT were accompanied by more bleeding than that associated with P2Y<sub>12</sub> gene targeting, 2) the same doses increased bleeding in P2Y<sub>12</sub>(−/−) mice, and 3) at all doses, the thienopyridines provided more bleeding than their elinogrel counterpart doses. In addition, studies of primary hemostasis and vascular tone in mesenteric veins indicated that the thienopyridines had effects that could not be accounted for by P2Y<sub>12</sub> inhibition. Taken together, these data indicate that the thienopyridines have off-target effects in the vasculature of the mouse that directly contribute to bleeding.

Although previous studies performed in rats and rabbits showed that clopidogrel produced an unexpectedly large amount of bleeding compared with the inhibition of platelet aggregation or thrombosis (Schumacher et al., 2007; Wong et al., 2007, 2009), the strategy reported herein allows evaluation for the first time of whether the thienopyridine effects on thrombosis and hemostasis are restricted to P2Y<sub>12</sub>. The doses of thienopyridines normalized to achieve inhibition of thrombosis similar to that occurring as a consequence of P2Y<sub>12</sub> gene targeting clearly resulted in more bleeding. The data demonstrated that a dose of clopidogrel (1.5 mg/kg) that provided a volume of blood loss similar to that occurring in the P2Y<sub>12</sub>(−/−) mouse provided less inhibition of thrombosis, whereas the low dose of prasugrel (1 mg/kg) that was also associated with a suboptimal inhibition of thrombosis led to a greater volume of blood loss than that in the genetically engineered mice. These results are particularly interesting if one extrapolates these murine doses to their human counterparts, because the doses would correspond to 105- and 70-mg
doses in healthy volunteers, which are within a range of chronic and loading doses similar to those used in humans for clopidogrel and prasugrel, respectively. Finally, the increased blood loss induced by dosing of P2Y12-deficient animals with clopidogrel and prasugrel clearly established the fact that a portion of the bleeding induced by these drugs was due to an off-target effect.

Off-target effects resulting from administration of thienopyridines are not necessarily unexpected because this class of P2Y12 inhibitors was not screened for its preferential selectivity for P2Y12 over other possible targets, and the selectivity of the active metabolites and possible roles of other “inactive” metabolites have not yet been fully characterized. For example, at the time of regulatory approval of clopidogrel, the molecular identity of the platelet target was unknown, although it was attributed to blockade of the platelet P2Y12 receptor, later identified as P2Y12 (Hollopeter et al., 2001). Likewise, it was later demonstrated that thienopyridines inhibit P2Y12 through irreversible binding of a highly reactive thiol species generated as the active metabolite of the parent compound (Savi et al., 2000). In light of the highly reactive, transient nature of the intermediate, it is possible that it could react with other thiol species present on the surface of all cell types and potentially modify plasma proteins in circulation. Indeed, although sparse, off-platelet effects of clopidogrel have been reported, with clopidogrel showing possible proinflammatory (Waehre et al., 2006) and anti-inflammatory (Molero et al., 2005; Heitzer et al., 2006) as well as vasomodulatory activities (Yang and Fareed, 1997; Warnholtz et al., 2008). Whereas clopidogrel effects on the vascular tone have been tentatively attributed to inhibition of circulating platelets (Körtz et al., 2004; Giachini et al., 2009), inhibition of endothelium P2Y12 (Simon et al., 2002; Wihlborg et al., 2004; Shanker et al., 2006), and a direct modulation of nitric oxide bioavailability (Ziemianin et al., 1999; Heitzer et al., 2006; Warnholtz et al., 2008), it has also been suggested that the parent compound and active and inactive metabolites of clopidogrel could act on endothelial cells (Ziemianin et al., 1999; Jakubowski et al., 2005; Warnholtz et al., 2008).

The present data indicate that both clopidogrel and prasugrel blocked vasoconstriction induced by α,β-metATP in vivo in mice with prasugrel showing the strongest inhibitory activity. The prasugrel effect was time- and dose-dependent but also reversible (unlike its effect on platelet function, which is irreversible). In addition, this effect did not appear to involve the platelets nor the vascular P2Y12 because the same results were obtained in P2Y12-deficient mice rendered thrombocytopenic via an anti-glycoprotein Ibα antibody. The inhibition of vasoconstriction required multiple dosing cycles, suggesting possible up- or down-regulation of mediators of the vascular tone. However, a direct and sole effect on nitric oxide seems unlikely because thienopyridine treatment was associated with an immediate and localized inhibition of the vasoconstrictive effect mediated by α,β-metATP. Hence, further experiments will be required to precisely determine the mechanisms(s) by which thienopyridines inhibit vasoconstriction.

As opposed to the thienopyridines, elinogrel use was associated with less bleeding, a phenomenon probably explained by the competitive, reversible nature of the compound. Our ex vivo aggregation data showed that elinogrel activity is affected by ADP concentrations because doses that fully blocked arterial occlusion in vivo abolished 1 μM ADP-induced platelet aggregation but were ineffective in inhibiting the aggregatory activity of 10 μM ADP in vitro. Various observations come together to substantiate localized areas of greatly varying ADP concentrations within the blood vessel and growing thrombus as a whole. Born and Kratzer (1984) reported that damaged cells in the severed vessel wall are the primary source of ADP and ATP, thus creating an ADP concentration gradient across the blood vessel lumen in such a way that the highest concentrations are found closest to the vessel wall, i.e., the site of hemostasis or initiation of arterial thrombosis (mural thrombus). Because ADP concentrations can exceed 5 μM under a low shear environment such as that encountered in veins, a greater competition between ADP and elinogrel is expected to occur (as shown in our aggregation study) and to reduce the effect of the reversible inhibitor on the activation level and procoagulant activity of the platelets (Leon et al., 2004; van der Meijden et al., 2005). In agreement with this theory, we found an increased amount of platelets deposited at the wound of mesenteric veins of mice treated with elinogrel versus that in P2Y12(−/−) mice (Fig. 6D). In contrast, lower amounts of ADP are expected to be released by platelet-platelet interactions under arterial shear rates. Moake et al. (1988) have shown that only 4 to 5% (300 – 400 nM) of the ADP platelet content was released upon high shear stress-induced platelet aggregation in the presence of large von Willebrand factor multimers, and the mathematical model proposed by Polie and McIntire (1989) estimated the ADP concentration present in the vicinity of a thrombus forming at 1500 s−1 to be approximately 1 μM. Consistent with these findings, we found that limited concentrations of elinogrel induced dethrombosis in vivo in mice and in vitro in human blood (Supplemental Fig. 2, A and B) and that ADP concentrations as low as 300 nM were sufficient to confer stability to aspirinized human arterial thrombi in a perfusion chamber assay (Supplemental Fig. 2C). Finally, these results also indicate that the value of pharmacodynamic assays using exogenous and nonphysiologic concentrations of ADP in predicting antithrombotic activity of antiplatelet drugs may not apply to competitive, reversible antagonists.

Until recently, it has been accepted dogma that higher levels of platelet inhibition lead to greater efficacy, but at the cost of increased bleeding. This theory was substantiated by data from the recent TRITON-TIMI 38 trial (Wiviott et al., 2007), in which prasugrel, an irreversible thienopyridine that was dosed to provide greater levels of platelet inhibition than clopidogrel, demonstrated substantially greater clinical benefit, accompanied by significantly more bleeding. The observations described in the present study suggest that some of the additional bleeding observed with prasugrel may be due to off-target activity.

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Authorship Contributions

Participated in research design: André, Hollenbach, Phillips, and Conley.

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References


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