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THIENOPYRIDINES, BUT NOT ELINOGREL, RESULT IN OFF-TARGET EFFECTS AT THE VESSEL WALL THAT CONTRIBUTE TO BLEEDING

Patrick André, Francis DeGuzman, Helena Haberstock-Debic, Scott Mills, Yvonne Pak, Mayuko Inagaki, Anjali Pandey, Stanley Hollenbach, David R. Phillips, and Pamela B. Conley

Portola Pharmaceuticals Inc. (*P.A., F.D.G., H.H.D., S.M., Y.P., M.I., A.P., S.H., D.R.P., P.B.C.)* South San Francisco, CA

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Running title: Thienopyridines effects on bleeding

Corresponding author:

Pamela B. Conley, PhD
Portola Pharmaceuticals, Inc.
270 East Grand Avenue, South San Francisco, CA 94080.
Phone (650.246.7525),
Fax (650.246.7776).
Email: pconley@portola.com

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Abbreviations used in this paper: ADP, adenosine diphosphate; WT, wild-type mice

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Abstract

Clinical studies with clopidogrel or prasugrel show that while increased inhibition of $P2Y_{12}$ and platelet function improves efficacy, bleeding is also increased. Other preclinical and clinical studies have suggested greater therapeutic index (TI) with reversible inhibitors and disproportionate effects of thienopyridines on bleeding at high doses. We utilized multiple *in vivo* ($FeCl_3$-induced arterial thrombosis in mesenteric arteries; blood loss following tail trans-section; platelet deposition and wound closure time in a micropuncture model in mesenteric veins) and *ex vivo* (light transmittance aggregometry, PT, aPTT) mouse models to: i) compare the TI of clopidogrel, prasugrel and elinogrel, a reversible, competitive antagonist, with that of $P2Y_{12}^{-/-}$ mice. ii) determine whether the bleeding consequences of the thienopyridines are attributed only to the inhibition of $P2Y_{12}$. Data indicated greater (elinogrel) and decreased (thienopyridines) TI when compared to $P2Y_{12}^{-/-}$ mice. The impaired TI associated with the thienopyridines was not attributed to non-$P2Y_{12}$ activities on platelet function or coagulation but related to a direct effect at the vessel wall (inhibition of vascular tone). Further analysis showed that 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 $P2Y_{12}$-independent off-target effects at the vessel wall while that of elinogrel may be enhanced by the reversible, competitive nature of the antiplatelet agent.

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Introduction

The thienopyridine clopidogrel has emerged as a primary drug for the treatment and the prevention of arterial thrombosis in patients with cardiovascular disease (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; Savi et al., 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 $P2Y_{12}$, a Gi-coupled receptor on platelets (Foster et al., 2001; Hollopeter et al., 2001). In a subsequent study, we generated $P2Y_{12}^{-/-}$ mice and showed that $P2Y_{12}$ functions in arterial thrombosis by maintaining thrombus stability (Andre et al., 2003a). While increased doses of clopidogrel provide increased inhibition of $P2Y_{12}$ and increased efficacy, this is 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 $P2Y_{12}$ 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 $P2Y_{12}$ antagonist, there have been multiple reports of excessive bleeding associated with thienopyridines. For example, studies performed in rabbits suggest that unexplained bleeding occurs above levels of

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clopidogrel required to provide complete inhibition of platelet aggregation (Wong et al., 2009). Particularly, 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 higher rate of thrombotic events but similar rates of bleeding as non-carriers (Mega et al., 2009). Also, 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 cangrelor (Srinivasan et al., 2009). Thus, while the antithrombotic activities of the thienopyridines can be directly linked to the inhibition of $P2Y_{12}$, platelet function and thrombosis, it is unknown 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 while dosing of mice with the three agents had no effects on coagulation parameters, achieved the same inhibition of arterial thrombosis and the same levels of anti-aggregatory activities *in vitro* as in $P2Y_{12}^{-/-}$ mice, both thienopyridines caused more bleeding in two primary hemostasis models than occurred in $P2Y_{12}^{-/-}$ mice or in WT

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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, is of a reversible nature.

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Methods

Drugs

Clopidogrel was from Sequoia Research Products Ltd. (United Kingdom). Prasugrel was from Albany Molecular Research (USA). Elinogrel (Ueno et al. 2010) was from Portola Pharmaceuticals (USA).

Animals

C57/BL6J mice (Charles River) were used for determination of antithrombotic activities of clopidogrel and prasugrel, determination of their effects on primary hemostasis and *ex vivo* platelet aggregation. $P2Y_{12}$ knockout mice (on a pure C57/BL6J 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* (National Institutes of Health, Bethesda, Md).

Thrombosis study

Thrombosis on mouse mesenteric arteries (1000-1300 s^{-1}) was performed and recorded as previously described with minor modifications (Andre et al., 2003b). Platelets were labeled *in situ* using rhodamine 6G (0.2 mg/mL) administered through the tail vein 10 minutes before visualization of the arteries. Vessel-wall injury was induced by a 1x1-mm filter paper saturated with a 10% $FeCl_3$ solution. After 5 minutes, the filter paper was removed and mesenteric arteries rinsed with

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warmed saline (37°C). Platelet-vessel wall interactions were recorded for 40 additional minutes or until full occlusion occurred and lasted for more than 40 seconds. C57Bl6J mice were orally gavaged 48, 24 and 2 hours prior to injury with vehicle control (0.5% methylcellulose), clopidogrel (0.25, 1.5, 15 and 50 mg/kg) or prasugrel (0.1, 0.3, 1, 3, 10 mg/kg), or 2 hours (on the basis of elinogrel murine pharmacokinetic profile, see Supplemental Figure 1A) prior to injury with elinogrel (0.83, 2.5, 7.5, 20 and 60 mg/kg,).

Video analysis

Thrombosis was analyzed in real time using Simple PCI software (Andre et al., 2003a). The fluorescence intensity was recorded at a rate of 2 Hz for 40 minutes and plotted over time. Time to occlusion and time for appearance of first thrombus were analyzed.

Elinogrel plasma concentration determination

Plasma samples were analyzed for elinogrel concentration using a liquid chromatography tandem mass spectrometry (LC/MS/MS). In brief, whole blood was collected on trisodium citrate (1:9 vol) and platelet poor plasma prepared. Plasma samples were processed in a 96-well Captiva™ filter plate (0.2 µm, Varian, Inc., Palo Alto, CA). Aliquots of plasma samples were precipitated with acetonitrile containing 500 ng/mL of an internal standard (CT55951 or PRT061200) and protein precipitated. The mixture was filtered into a 96-well collection plate. The filtrate was injected onto a Sciex API4000 LC/MS/MS

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equipped with a turbo-ion spray source. Elinogrel and internal standard were separated on a Thermo Hypersil-Keystone Betasil C_{18} column (4.6 x 100 mm, 5µm; Fisher Scientific, Houston, TX). 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 minutes. 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, C57Bl6J mice were orally gavaged for 3 consecutive days with 0.5% methylcellulose (BID, in 100 µL), clopidogrel (QD, 50 mg/kg in 100 µL), prasugrel (QD, 10 mg/kg in 100 µL) or elinogrel (BID, 60 mg/kg in 100 µL). The last dose was given 3 hours prior to blood collection. Measurements were performed using a 4-channel Chronolog lumiaggregometer using Aggrolink software. Blood samples (0.6 ml per anesthetized mouse) were obtained via intracardiac puncture (on 3.2% trisodium citrate, 1vol TSC : 9 vol of blood), centrifuged at 200xg for 10 minutes and platelet rich plasma (PRP) collected. For each dose, PRP of three animals was pooled for aggregation measurements. Experiments for each dose were repeated between 2 and 3 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 minute to

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obtain a stable baseline. Aggregation in PRP was induced using ADP (Chronolog) at 1 and 10 μM final concentration, collagen (Chronolog) at 10 and 5 μg/mL, murine TRAP at 2.5 and 1 mM and change in light transmittance was recorded for an additional 8 minutes.

Frozen platelet poor plasma samples were thawed to room temperature and coagulation parameters, activated partial thromboplastin times (aPTT) and prothrombin times (PT), measured using an MLA Electra 800 automatic coagulation timer. Assay reagents for aPTTs were Dade® Actin® FS Activated PTT Reagent (purified soy phosphatides in 1 x 10^{-4} ellagic acid) and Calcium Chloride Solution, 0.025 Mol/L. Reagent for the PT assay was Dade® Thromboplastin C Plus (rabbit brain thromboplastin). All reagents were purchased from Siemens Healthcare Diagnostics Products, Germany.

Tail bleeding time measurement

Male mice (6-8 weeks old) were anesthetized (by subcutaneous injection) with ketamine cocktail (ketamine [40 mg/kg], xylazine [2.5 mg/kg], and acepromazine [0.75 mg/kg], Henry Schein, Melville, NY, USA) 6 minutes prior to tail trans-section. Mice were then placed in lateral recumbence on a heating pad (used to maintain body temperature) placed on a firm dissecting board (Richard-Allan Scientific, Kalamazoo, MI, USA) with the tail straight out. Tails were transected 2 mm from the tip with a number 10 scalpel blade (Bard-Parker; Becton Dickinson, Franklin Lakes, New Jersey, USA) and immediately immersed into a 20-ml scintillation vial (Wheaton Science Products, Millville, NJ, USA) filled with 10 mL

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normal saline held at 37°C by an unstirred digitally-controlled water bath (VWR International, Buffalo Grove, IL, USA). A stopwatch was started immediately upon trans-section to determine time to cessation of bleeding, frequency, and duration of re-bleed for a 15 minute period. C57Bl6J mice were orally gavaged with vehicle control, clopidogrel (1.5, 15 and 50 mg/kg) or prasugrel (1, 3, 10 mg/kg) 48, 24 and 2 hours prior to tail trans-section or 2 hours prior to trans-section with elinogrel (7.5, 20 and 60 mg/kg). In one set of experiments, $P2Y_{12}^{-/-}$ mice were also 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 bleed time determination had concluded. To measure blood loss volume, any blood collected as described above following tail transection 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, USA) and diluted further (1/5, 1/10, or 1/20) if necessary. Resulting OD_{490nm} 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

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The *in vivo* primary hemostasis model was performed via micropuncture (using a 27 G needle) of mesenteric veins according to the model developed by van Gestel and colleagues (van Gestel et al., 2007). Briefly, 3-4 week-old anesthetized $P2Y_{12}^{-/-}$ and WT mice previously orally gavaged (48, 24 and 2 hours prior to injury) with either 0.5% methycellulose, clopidogrel (50 mg/kg), prasugrel (10 mg/kg) or elinogrel (60 mg/kg 2 hours prior to micropuncture) were injected with R6G (0.2 mg/ml) to fluorescently label platelets *in situ*. Using videomicroscopy, a 27G 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 site of micropuncture were then recorded for 20 minutes 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 $P2Y_{12}^{-/-}$ or WT mice were observed using brightfield inverted microscopy. In a first set of experiments, mice were orally gavaged with 0.5% methylcellulose (QD), clopidogrel (50 mg/kg, QD), prasugrel (10 mg/kg, QD) or elinogrel (60 mg/kg, BID) for 3 consecutive days. Veins were stimulated by a superfusion of α,β metATP (Sigma-Aldrich; 10 μl of a 1 μM solution) 2 hours after 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 $P2Y_{12}^{-/-}$ mice treated with prasugrel (10 mg/kg, QD) for three consecutive days and which were

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rendered thrombocytopenic (antiGPIbα antibodies, 2 mg/kg; Emfret laboratories) 16 hours prior to superfusion of α,β metATP. Finally, another group of WT mice was orally gavaged with prasugrel (10 mg/kg, QD) for 3 consecutive days and their mesenteric veins challenged 1, 2, 5, 10, 24 and 48 hrs after the last oral gavage. The diameter of the mesenteric veins was monitored for 5 minutes post challenge.

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 naïve or 0.5% methylcellulose $P2Y_{12}$ knockout mice was performed using one-way ANOVA (Bonferroni's multiple comparison test). All values are expressed as mean ± SEM unless otherwise specified.

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Results

Determination of clopidogrel, prasugrel and elinogrel doses reproducing the thrombotic profile of $P2Y_{12}^{-/-}$ mice.

Using the $FeCl_3$ thrombosis model, maximal doses **(M)** of clopidogrel (50 mg/kg), prasugrel (10 mg/kg) and elinogrel (60 mg/kg) were identified that shared the phenotypic characteristics associated with the genetic targeting of $P2Y_{12}$ (destabilization of growing arterial thrombi preventing vascular occlusion; Figure 1A). Although these maximal doses totally inhibited occlusion, subtle differences were observed compared with $P2Y_{12}^{-/-}$ mice as maximal clopidogrel dose did not reach similar levels of inhibition achieved by $P2Y_{12}$ gene targeting on the initial rate of thrombus growth ($p<0.0001$ *vs* $P2Y_{12}^{-/-}$ for time for appearance of first thrombus; Figure 1B). In addition, while preventing vascular occlusion, there were more fluorescent platelets deposited throughout the 40 min observation period with the maximal dose of clopidogrel (Figure 1C). In contrast, the maximal doses of prasugrel and elinogrel had a thrombosis phenotype identical to that of $P2Y_{12}^{-/-}$ mice. Doses of clopidogrel (1.5 and 15 mg/kg), prasugrel (1 and 3 mg/kg) and elinogrel (7.5 and 20 mg/kg) were also identified that provided similar but lower levels of inhibition on thrombus stability and classified intermediate **(I)** and high **(H),** respectively. Determination of elinogrel plasma concentration performed 2 min after vascular occlusion or 42 minutes after injury (for patent arteries) indicated that maximum levels of inhibition with elinogrel were achieved beyond 1 μg/mL (Figure 2A). Interestingly, in a separate group of animals, we found that the *ex vivo* pharmacodynamic monitoring (by measurement of ADP-

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induced platelet aggregation) of elinogrel was biased by the concentration of ADP utilized to induce platelet aggregation. This was highlighted by the fact that the 60 mg/kg dose provided equivalent effect to prasugrel and clopidogrel on thrombosis *in vivo*, but failed at blocking platelet aggregation induced by 10 μM ADP although it abolished that induced by 1 μM ADP (Figure 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 $P2Y_{12}$-deficiency (Figure 3A; see also (Andre et al., 2003a)). Furthermore, analysis of prothrombin time and aPTT indicated that neither the pharmacological modulation nor $P2Y_{12}$ gene targeting affected coagulation parameters (Figures 3B, C).

$P2Y_{12}$ antagonists and $P2Y_{12}$ gene targeting differentially affect bleeding time and blood loss following tail trans-section

We studied the effects of clopidogrel, prasugrel and elinogrel on bleeding frequency, and blood loss and compared their effects to the phenotype of $P2Y_{12}^{-/-}$ mice. Animals treated with placebo (n = 40) stopped bleeding before 2 min following tail trans-section but subsequently displayed multiple periods of rebleed and cessation of blood loss (Figure 4). At the intermediate doses, 7 out of 10 (clopidogrel), 10 out of 10 (prasugrel) and 2 out of 10 animals (elinogrel) had continuous bleeding that lasted for the duration of the 15 min observation period (Figure 4). At the high doses, 10 out of 10 animals administered clopidogrel, 9

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out of 10 (prasugrel) and 3 out of 10 (elinogrel) animals displayed continuous bleeding. For the maximal doses, all animals treated with clopidogrel and prasugrel as well as $P2Y_{12}^{-/-}$ mice but only 4 out of 10 animals treated with elinogrel had continuous bleeding (Figure 4).

Blood loss measurements also revealed differences between the three $P2Y_{12}$ antagonists and $P2Y_{12}^{-/-}$ mice. Maximal clopidogrel and prasugrel doses significantly increased volume of blood loss when compared to $P2Y_{12}^{-/-}$ mice (clopidogrel, 551 $\pm$ 43 µl; prasugrel, 561 $\pm$ 73µl; $P2Y_{12}^{-/-}$ mice, 293 $\pm$ 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 when compared to the $P2Y_{12}^{-/-}$ mice (Figure 5A), despite the fact that they were not able to prevent vascular occlusion (Figure 1A), indicating that some levels of bleeding could not be accounted for by the sole anti-platelet effect. Data obtained with thienopyridines were in sharp contrast with the volume of blood loss associated with elinogrel which in all cases, were less than the blood loss observed in $P2Y_{12}^{-/-}$ mice.

Clopidogrel and prasugrel increase blood loss when dosed in $P2Y_{12}^{-/-}$ mice

Since the maximal doses of clopidogrel and prasugrel extended blood loss beyond the level of the $P2Y_{12}^{-/-}$ mouse, we next asked whether they would affect blood loss when administered to $P2Y_{12}$-deficient animals. When compared with their respective vehicle control, oral administration of clopidogrel and prasugrel

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but not elinogrel significantly increased the volume of blood loss in $P2Y_{12}^{-/-}$ mice (Figure 5C).

Effects of $P2Y_{12}$ antagonists and $P2Y_{12}$ gene targeting on platelet deposition, bleeding and closure time following mesenteric vein micropuncture

The micropuncture model is characterized by a rapid accumulation of platelets at the vascular wound in the ~ 20 seconds post-vascular injury independently of the treatment or genotype (represented in Figure 6A) followed by a reopening of the wound. Animals treated with the maximal doses of the $P2Y_{12}$ antagonists had slightly less (clopidogrel and prasugrel) or longer (elinogrel) periods of cessation of bleeding than occurred in $P2Y_{12}^{-/-}$ mice (Figure 6B). Vessels from animals treated with the thienopyridines had delayed closure and reduced platelet deposition (Figure 6C, D) when compared with $P2Y_{12}^{-/-}$ mice. In contrast, elinogrel presented a faster closure of the vascular wound that was in part related to an increase in platelet deposition (Figure 6C, D).

Clopidogrel and prasugrel block vasoconstriction in mesenteric veins

While studying platelet deposition in the micropuncture model, we noticed that the mesenteric veins of thienopyridine-treated mice had a reduced constriction in response to mechanical injury. To investigate further a potential effect of these pharmacological agents on the vascular tone, we next studied the response of

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mesenteric veins to an *in situ* stimulation by α,β metATP (1 μM), a $P2X_1$ agonist that is not sensitive to CD39-mediated degradation *in vivo*.

Mesenteric veins from $P2Y_{12}^{-/-}$, elinogrel- or vehicle control-treated mice rapidly constricted following stimulation by α,β metATP. The constriction of mesenteric veins from $P2Y_{12}^{-/-}$ mice was not sustained when compared with veins from WT mice treated with 0.5% methylcellulose or elinogrel (M), indicating a possible role of vascular $P2Y_{12}$ in the sustained constriction of the vessel wall (as suggested by Wihlborg and collaborators (Wihlborg et al., 2004)).

In contrast, mesenteric veins from animals treated with the maximal doses of clopidogrel and prasugel, only partially (clopidogrel) or minimally (prasugrel) responded to α,βmet ATP (Figure 7A). Further analysis revealed that the inhibition of vasoconstriction by prasugrel was dose- and time-dependent (Figure 7B, C), reaching a maximum 2 to 5 hours after the 3rd oral gavage, then gradually returning to normal within 48 hours. This time-dependent inhibitory activity was not observed after a single oral administration (data not shown). Since platelets can influence endothelium-dependent vasodilatation via secretion of superoxide anions (Krotz et al., 2004), we next studied the effects of prasugrel on vasoconstriction induced by α,βmet ATP following platelet depletion in $P2Y_{12}^{-/-}$ mice (mean platelet count 29.16 ± 2.1 K/μl). Under these conditions, prasugrel treatment inhibited vasoconstriction indicating both a vascular $P2Y_{12}$- and platelet-independent effect (Figure 7D).

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Discussion

The present study aimed at determining whether in the mouse, all of the hemostatic effects of clopidogrel and prasugrel are attributed to $P2Y_{12}$ inhibition. Doses of clopidogrel, prasugrel and a direct acting reversible $P2Y_{12}$ inhibitor (elinogrel) were normalized to provide equivalent inhibition of thrombosis. Data indicated that: i) in WT mice, maximal doses of clopidogrel and prasugrel recapitulating the phenotype of $P2Y_{12}^{-/-}$ mice on arterial thrombosis and platelet aggregation (LTA) and which lack activities on PT and aPTT were accompanied with more bleeding than that associated with $P2Y_{12}$ gene targeting. ii) the same doses increased bleeding in $P2Y_{12}^{-/-}$ mice. iii) 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 than could be accounted for by $P2Y_{12}$ inhibition. Taken together, these data indicate that the thienopyridines have off-target effects in the vasculature of the mouse that directly contribute to bleeding.

While previous studies performed in rats and rabbits showed that clopidogrel produced an unexpectedly large amount of bleeding compared to the inhibition of platelet aggregation or thrombosis (Schumacher et al., 2007; Wong et al., 2007; Wong et al., 2009), the strategy reported herein allows evaluation for the first time of whether the thienopyridine effects on thrombosis and hemostasis are restricted to $P2Y_{12}$. The doses of thienopyridines normalized to achieve a similar inhibition of thrombosis as occurred as a consequence of $P2Y_{12}$ gene targeting

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clearly resulted in more bleeding. The data demonstrated that a dose of clopidogrel (1.5 mg/kg) that provided similar volume of blood loss as occurred in the $P2Y_{12}^{-/-}$ mouse provided less inhibition of thrombosis, while the low dose of prasugrel (1 mg/kg) which was also associated with a sub-optimal inhibition of thrombosis lead to a greater volume of blood loss than the genetically engineered mice. This is particularly interesting if one extrapolates these murine doses to their human counterparts, as this would correspond to 105 mg and 70 mg doses in healthy volunteers, which are within a similar range of the chronic and loading dose utilized in human for clopidogrel and prasugrel, respectively. Finally, the increased blood loss induced by dosing $P2Y_{12}$-deficient animals with clopidogrel and prasugrel, clearly established 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 as this class of $P2Y_{12}$ inhibitors was not screened for its preferential selectivity for $P2Y_{12}$ 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 attributed to blockade of the platelet $P2_T$ receptor, later identified as $P2Y_{12}$(Hollopeter et al., 2001). Similarly, it was later demonstrated that thienopyridines inhibit $P2Y_{12}$ through irreversible binding of a highly reactive thiol species generated as the active metabolite of the parent compound (Savi et al.,

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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, as well as potentially modify plasma proteins in circulation. Indeed, although sparse, off-platelet effects of clopidogrel have been reported, with clopidogrel showing possible pro- (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., 2007). While clopidogrel effects on the vascular tone have been tentatively attributed to the inhibition of circulating platelets (Krotz et al., 2004; Giachini et al., 2009), endothelium $P2Y_{12}$ (Simon et al., 2002; Wihlborg et al., 2004; Shanker et al., 2006) and a direct modulation of NO bioavailability (Ziemianin et al., 1999; Heitzer et al., 2006; Warnholtz et al., 2008), it has also been suggested that parent compound, 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,βmet ATP *in vivo* in mice with prasugrel showing the strongest inhibitory activity. Prasugrel effect was time- and dose-dependent, but also reversible (unlike its effects on platelet function, which is irreversible). In addition, this effect did not appear to involve the platelets nor the vascular $P2Y_{12}$ as the same results were obtained in $P2Y_{12}$-deficient mice rendered thrombocytopenic via an anti-GPIbalpha antibody. Since the inhibition of vasoconstriction required multiple dosing cycles, this suggested possible up- or down-regulation of mediators of the vascular tone. A direct and sole effect on

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NO seems unlikely however, as thienopyridine treatment was associated with an immediate and localized inhibition of the vaso-constrictive effect mediated by α,βmet ATP. Hence, future experiments will be required to precisely determine the mechanism(s) by which thienopyridines inhibit vasoconstriction.

As opposed to the thienopyridines, elinogrel use was associated with less bleeding, a phenomenon likely explained by the competitive, reversible nature of the compound. Our *ex vivo* aggregation data showed that elinogrel activity is affected by ADP concentrations as doses that fully blocked arterial occlusion in vivo abolished 1 μM ADP-induced platelet aggregation but were ineffective at 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 reported that damaged cells in the severed vessel wall are the primary source of ADP and ATP (Born and Kratzer, 1984), thus creating an ADP concentration gradient across the blood vessel lumen in such a way that highest concentrations are found closest to the vessel wall, i.e. the site of hemostasis or initiation of arterial thrombosis (mural thrombus). Since ADP concentrations can exceed 5 μM under 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 pro-coagulant activity of the platelets (Leon et al., 2004; van der Meijden et al., 2005). In agreement with this theory, we found increased

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amount of platelets deposited at the wound of mesenteric veins of mice treated with elinogrel *vs* $P2Y_{12}^{-/-}$ mice (Figure 6D). Conversely, lower amounts of ADP are expected to be released by platelet-platelet interactions under arterial shear rates. Moake and collaborators have shown that only 4-5 % (300-400 nM) of the ADP platelet content was released upon high shear stress-induced platelet aggregation in presence of large vWF multimers (Moake et al., 1988) and the mathematical model proposed by Folie and McIntire estimated the ADP concentration present in the vicinity of a thrombus forming at $1500s^{-1}$ to be around 1 μM (Folie and McIntire, 1989). Consistent with these findings, we found that limited concentrations of elinogrel induced dethrombosis *in vivo* in mice and *in vitro* in human blood (Supplemental Figure 2A, B) and that ADP concentrations as low as 300 nM were sufficient to confer stability to aspirinated human arterial thrombi in a perfusion chamber assay (Supplemental Figure 2C). Finally, these results also indicate that the value of pharmacodynamic assays utilizing exogenous and non-physiological concentrations of ADP at 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 was substantiated by data from the recent TRITON trial (Wiviott et al., 2007), where prasugrel, an irreversible thienopyridine which was dosed to provide greater levels of platelet inhibition than clopidogrel, demonstrated substantially greater

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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.

Conducted experiments: Patrick André, Francis DeGuzman, Helena Haberstock-Debic and Scott Mills

Performed data analysis: Patrick André and Francis DeGuzman

Contributed new reagents or analytic tools: Yvonne Pak, Mayuko Inagaki and Anjali Pandey

Participated in research design: Patrick Andre, Stanley Hollenbach, David R. Phillips and Pamela B. Conley

Wrote or contributed to the writing of the manuscript: Patrick Andre, Pamela B. Conley

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Footnotes

Disclosure:
All co-authors are employees and shareholders of Portola Pharmaceuticals

To request reprints:

Pamela B. Conley, PhD

Portola Pharmaceuticals, Inc.

270 East Grand Avenue, South San Francisco, CA 94080.

Email: pconley@portola.com

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Legends for Figures

Figure 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 (intermediate (**I**), high (**H**) and maximal (**M**), respectively). N = 5 to 18 per group.**P<0.01, ***P<0.0001 *vs* V.Ctl (vehicle control). All animals treated with the maximal (**M**) doses had patent arteries at the end of the observation period as did $P2Y_{12}^{-/-}$ mice (2400 sec). **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 *vs* $P2Y_{12}^{-/-}$; ***, p<0.0001, maximal clopidogrel dose *vs* $P2Y_{12}^{-/-}$. **C)** Representative profiles of platelet deposition in response to $FeCl_3$-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.

Figure 2:

A) Time to occlusion/PK relationship. Elinogrel significantly inhibited thrombus stability (vascular occlusion) at concentrations > 1 μg/ml *in vivo***. B)** Percent inhibition of platelet aggregation by doses of antiplatelet agents providing maximum levels of inhibition of thrombosis. While clopidogrel and prasugrel activity on platelet aggregation were minimally affected by ADP concentration,

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elinogrel failed at inhibiting platelet aggregation induced by 10 μM ADP while it maximally inhibited that induced by 1 μM ADP.

Figure 3:

A) Aggregation measurements in PRP obtained from WT mice treated with 0.5% methylcellulose or the maximal doses of the three agents or $P2Y_{12}^{-/-}$ mice in response to 10 and 5 μg/ml collagen, 2.5 and 1 mM murine TRAP. Traces are representative of data obtained from two independent experiments (blood from six mice pooled for each experiment). V.CTl, vehicle control (0.5% methylcellulose). Elinogrel (M), 60 mg/kg. Clopidogrel (M), 50 mg/kg. Prasugrel (M), 10 mg/kg. Prothrombin time (PT) **(B)** and aPTT **(C)** measurements in PPP from WT mice treated with 0.5% methylcellulose, intermediate (I), high (H) or maximal (M) doses of the three agents or from $P2Y_{12}^{-/-}$ mice.

Figure 4:

Doses of clopidogrel, prasugrel and elinogrel providing equivalent antithrombotic activity (I, H and M, n = 10 animals per dose) differentially affect bleeding frequency. Solid lines indicate bleeding period, gap (white bars) period of cessation of blood loss. (WT + Vehicle control (V. Ctl), n = 30; $P2Y_{12}^{-/-}$, n =19))

Figure 5:

A) Doses of clopidogrel, prasugrel and elinogrel providing equivalent antithrombotic activity differentially affect volume of blood loss. *, $p<0.005$ *vs*

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clopidogrel; ‡, p<0.005 *vs* prasugrel; † P<0.0005 *vs* $P2Y_{12}^{-/-}$; • P<0.05 *vs* $P2Y_{12}^{-/-}$. **B)** Lower therapeutic index (ratio of the fold increase for time to occlusion *vs* V.Ctl. (vehicle control) and the fold increase in blood loss *vs* V.Ctl) associated with the use of thienopyridines. **C)** Effects of maximal doses of clopidogrel (C., n = 10), prasugrel (P., n = 8), elinogrel (E., n = 10) and methylcellulose (MC., n = 9) on blood loss measurement in $P2Y_{12}^{-/-}$ mice (n = 19).

Figure 6:

Differential effects of maximal doses of elinogrel (E., n = 8), clopidogrel (C., n = 8) and prasugrel (P., n = 9) *vs* vehicle control (n = 11) and genetic targeting (n = 7) on bleeding time frequency in the micropuncture model applied to mesenteric veins. **A)** Schematic of platelet deposition at site of micropuncture in mesenteric veins. **B)** Bleeding frequency. **C)** Reduction in micropuncture lumen of WT-treated animals or $P2Y_{12}^{-/-}$ mice. Thienopyridine-treated mice have a delayed occlusion of the vascular wound compared with $P2Y_{12}^{-/-}$ mice. Elinogrel-treated animals displayed a faster rate of closure than either thienopyridine-treated or $P2Y_{12}^{-/-}$ mice that paralleled an increased amount of platelet deposition (**D)**.

Figure 7:

Thienopyridines block vasoconstriction of mesenteric veins. **A)** Vasoconstriction of mesenteric veins induced by 1 μM α,β metATP is inhibited by 3 days (D3) oral gavage with the maximal doses of clopidogrel (n = 5) and prasugrel (n = 7) but not elinogrel (n = 9), Vehicle control (n = 11) of $P2Y_{12}$-deficiency (n = 10). **B)**

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Prasugrel dose response on α,β metATP-induced vasoconstriction (I, n = 9; H, n = 9; M, n = 4). **C)** Time-dependent effects of prasugrel (M, 10 mg/kg) on vasoconstriction 1 (n = 4), 2 (n = 4), 5 (n = 4), 10 (n = 7), 24 (n = 10) and 48 hours (n = 10) following administration of the third dose. **D)** Prasugrel blocks α,β metATP-induced vasoconstriction of mesenteric veins of $P2Y_{12}$ knockout mice which have been rendered thrombocytopenic.

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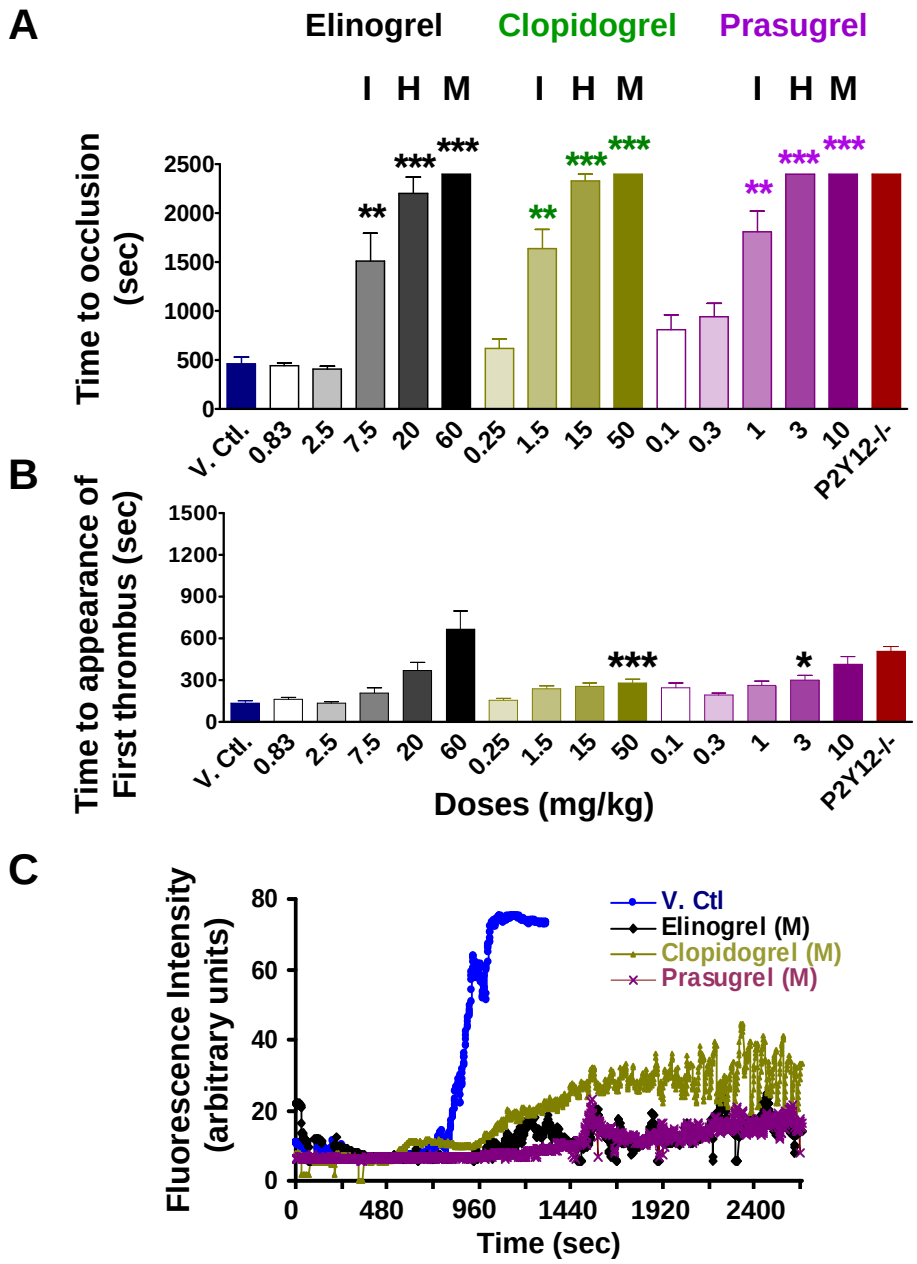

Figure 1

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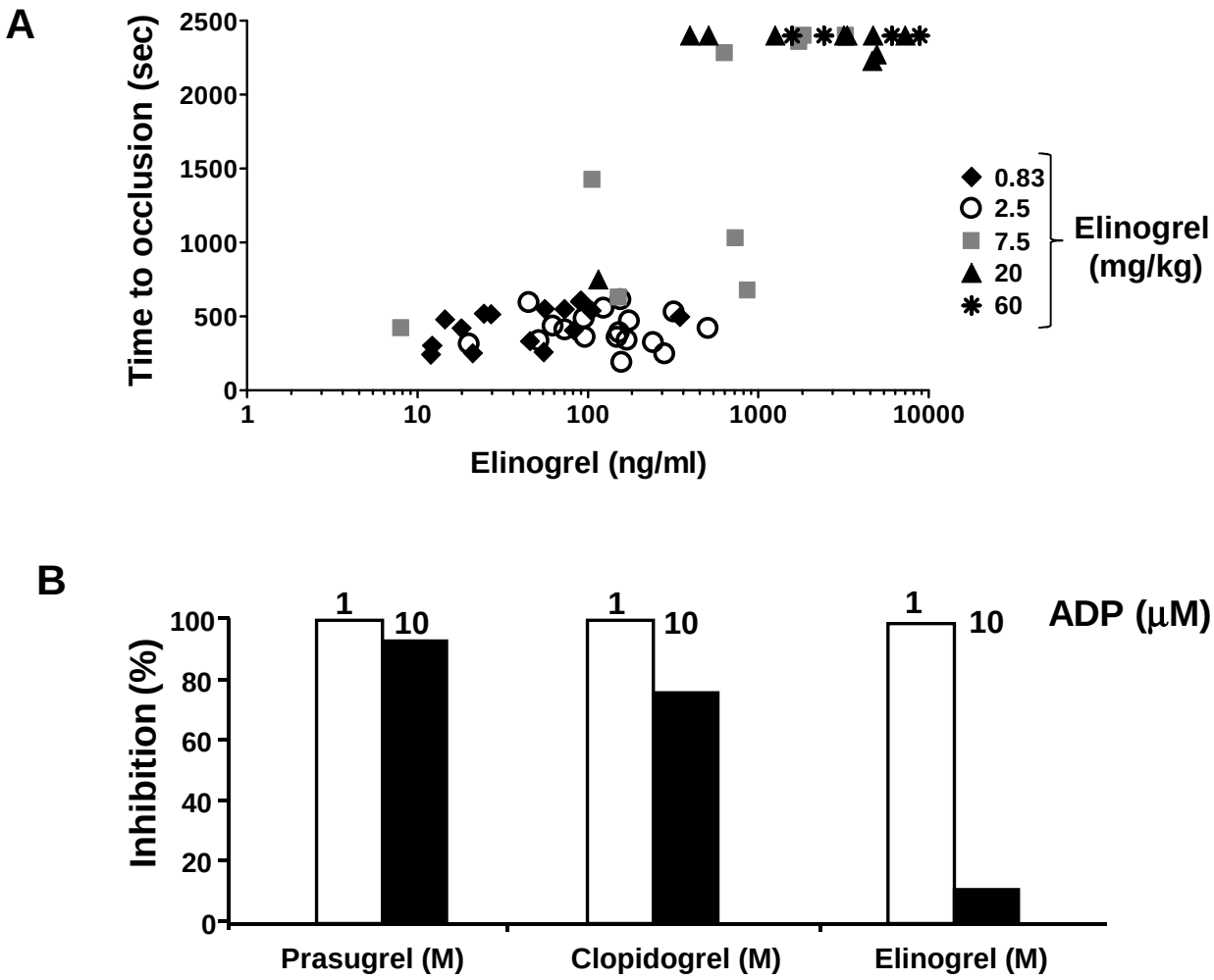

Figure 2

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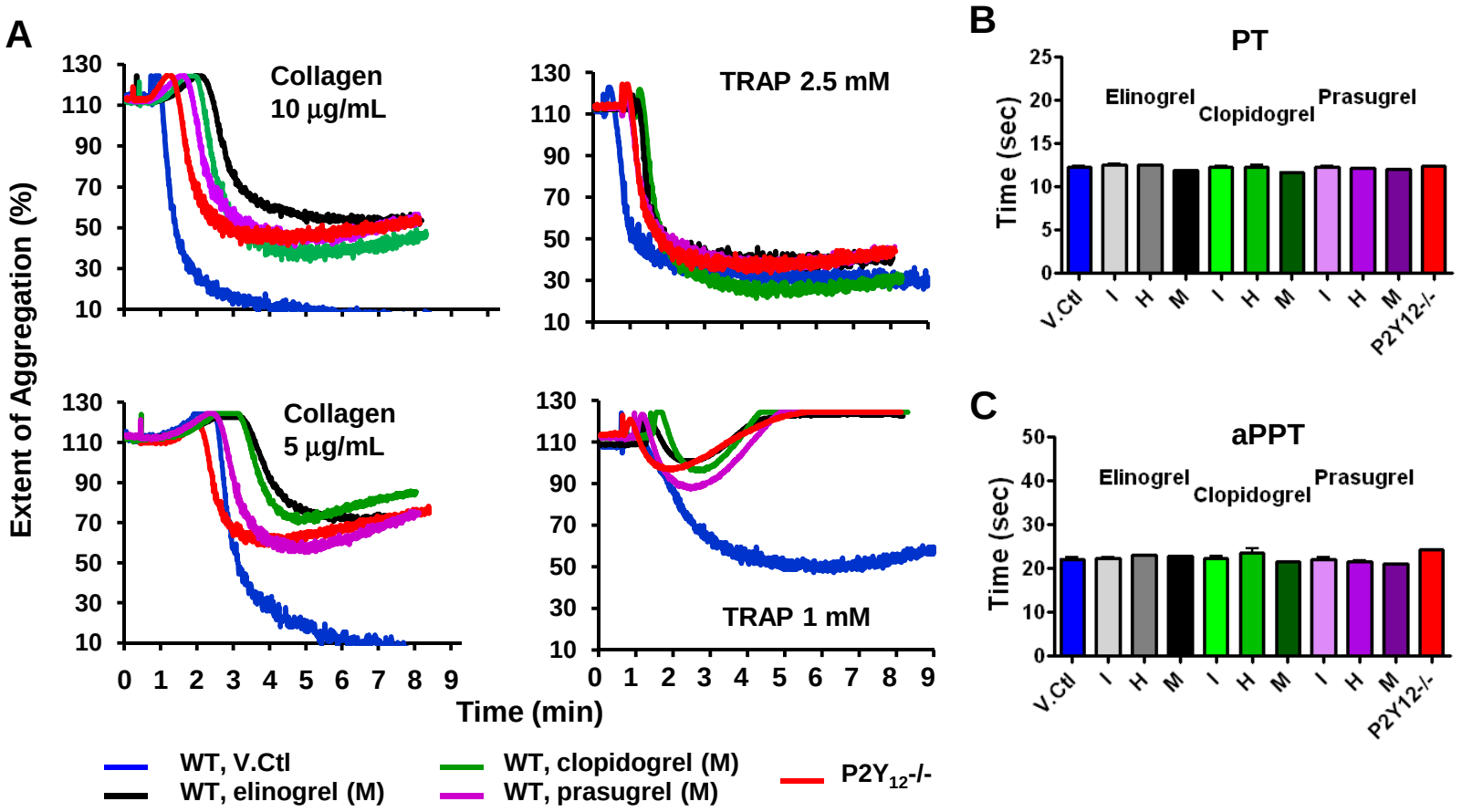

Figure 3

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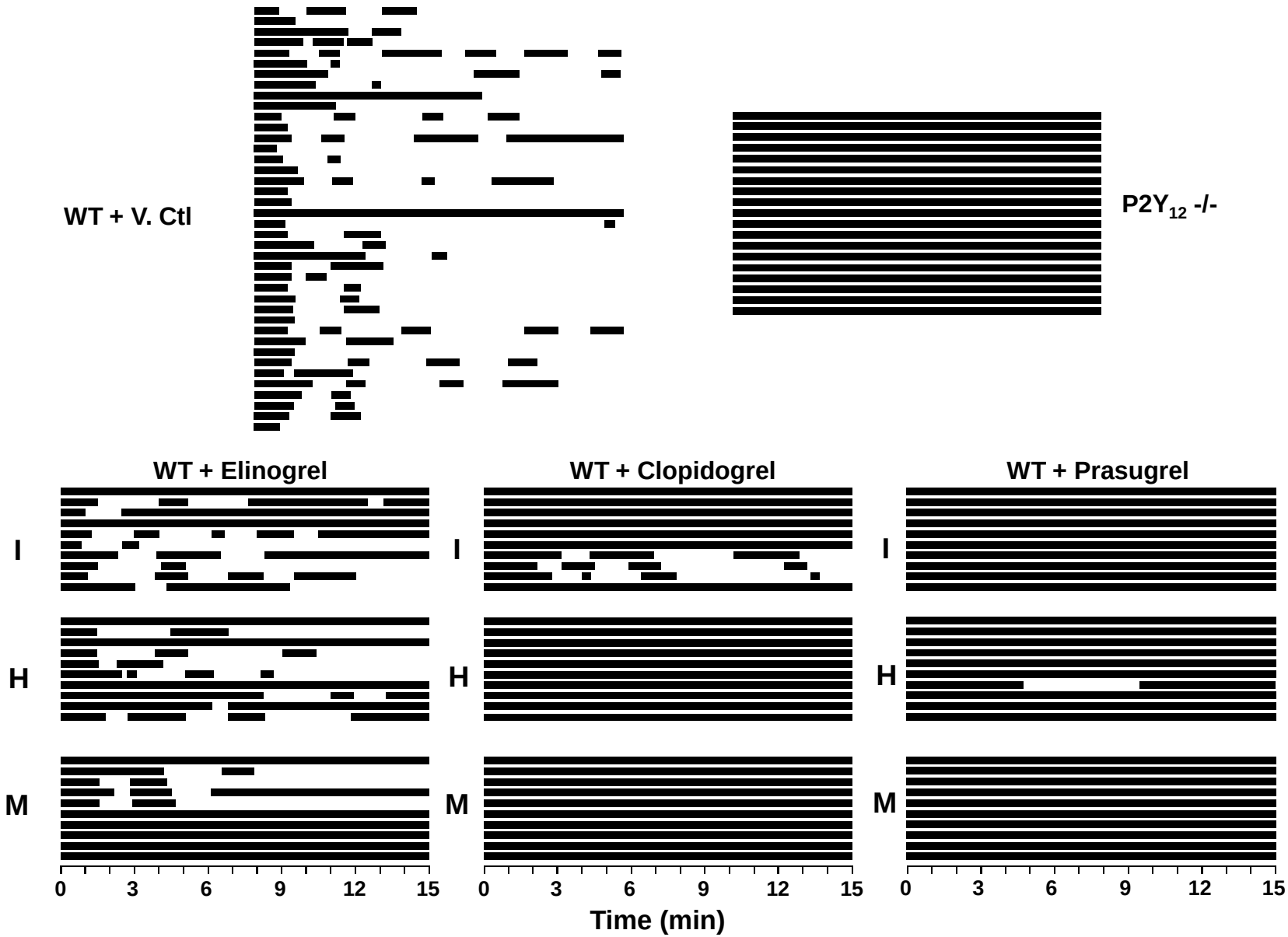

Figure 4

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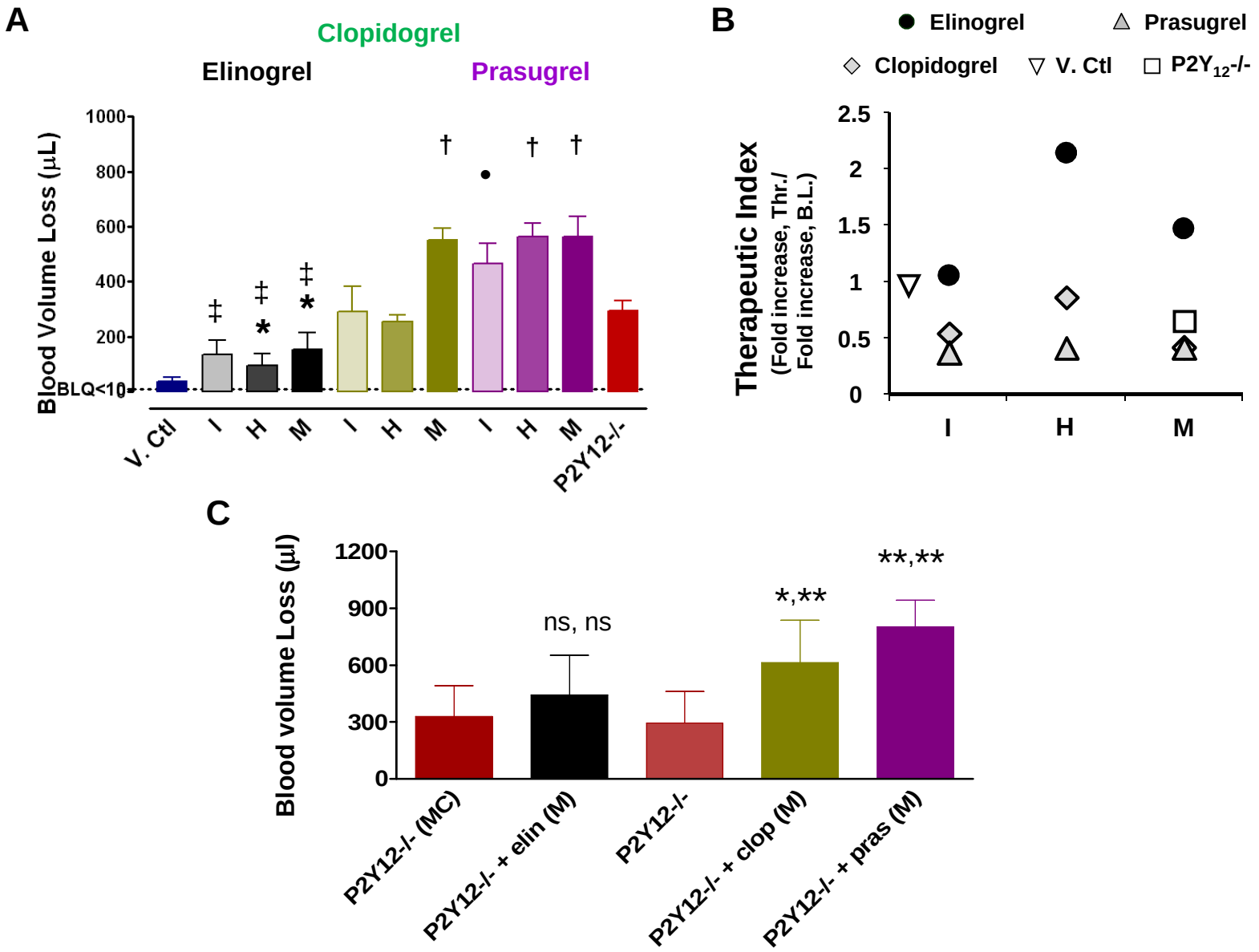

Figure 5

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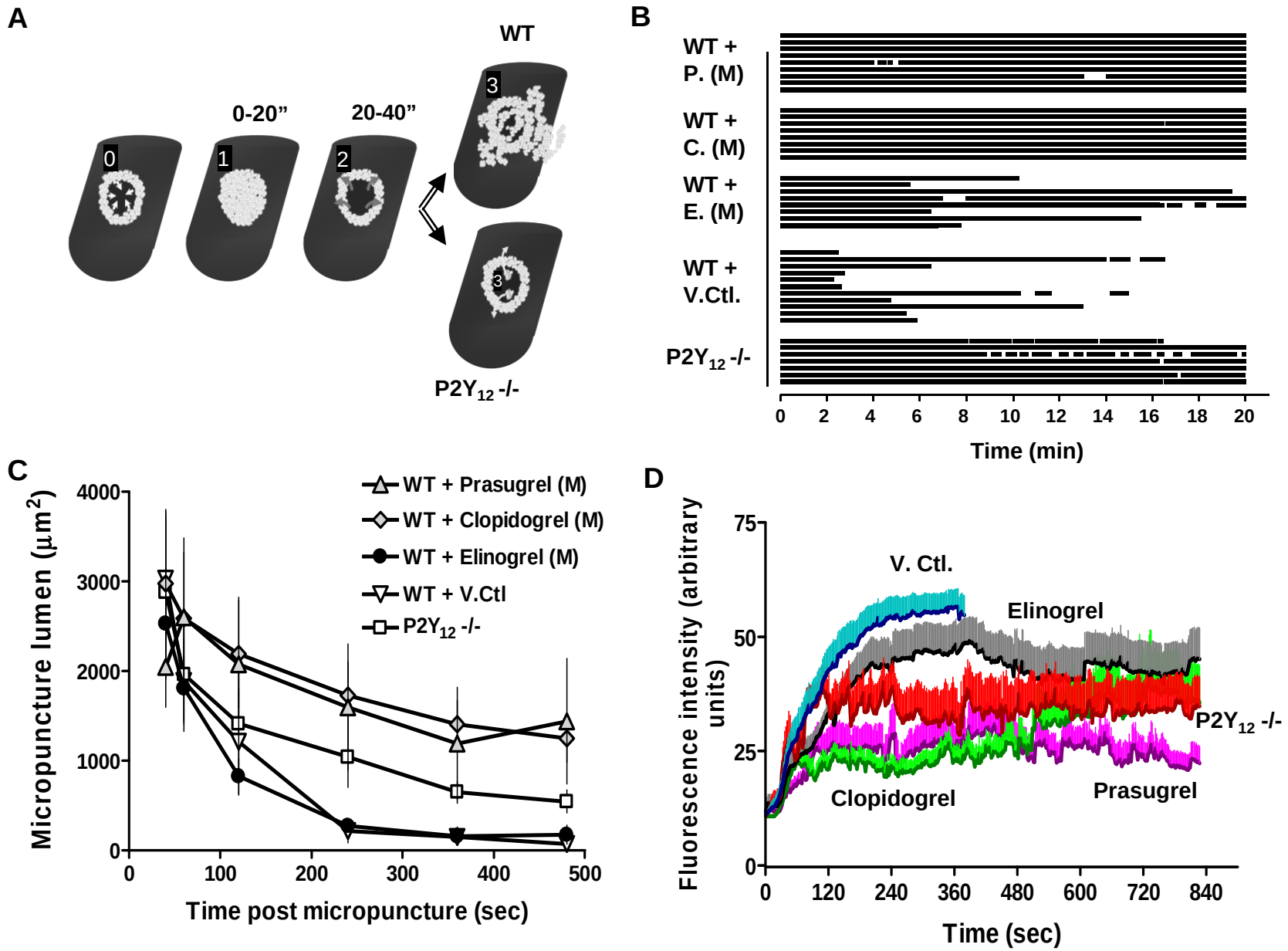

Figure 6

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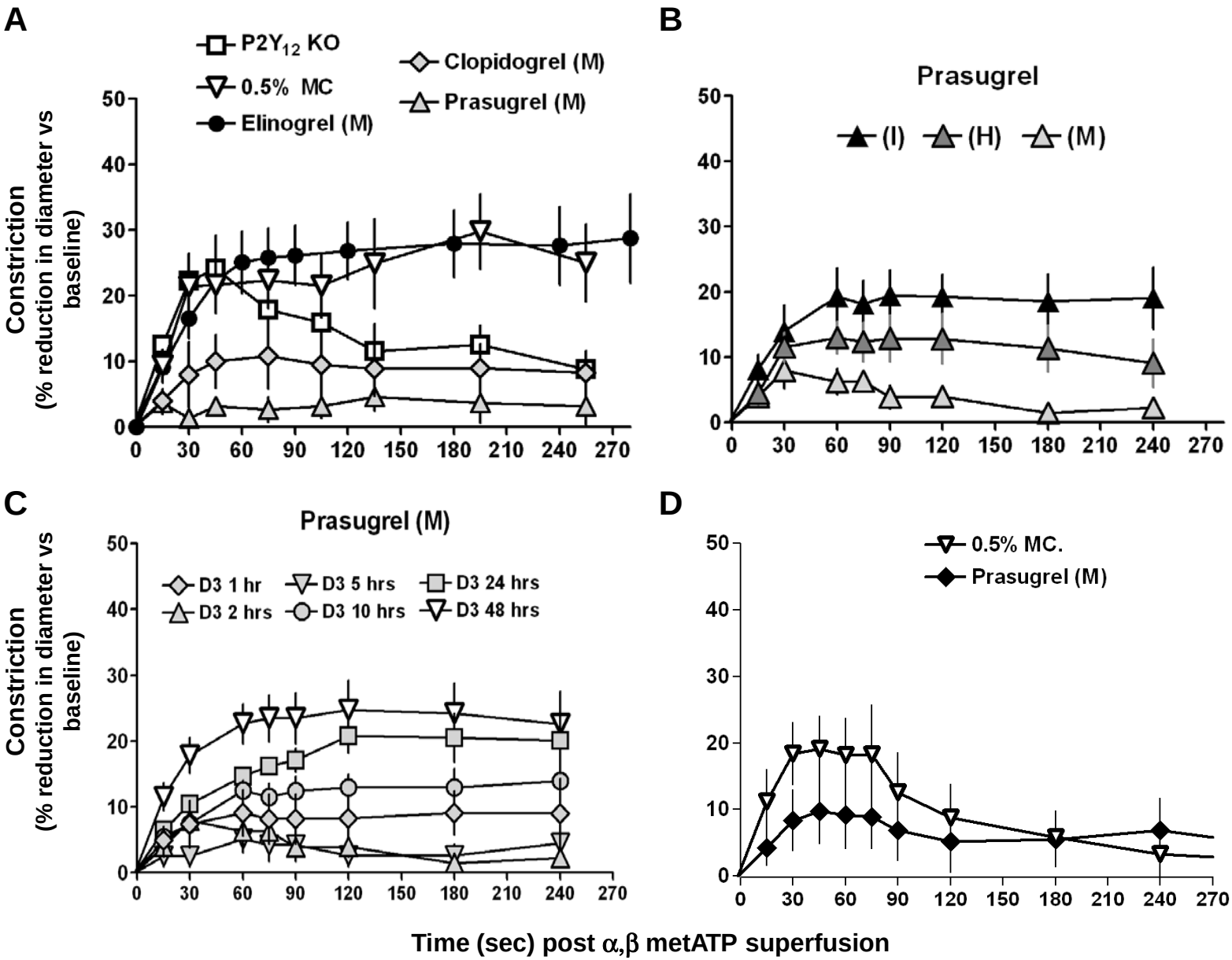

Figure 7

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