

**A CLOPIDOGREL-INSENSITIVE INDUCIBLE POOL OF P2Y₁₂ RECEPTORS
CONTRIBUTES TO THROMBUS FORMATION: INHIBITION BY ELINOGREL,
A DIRECT-ACTING, REVERSIBLE P2Y₁₂ ANTAGONIST**

Helena Haberstock-Debic, Patrick Andre, Scott Mills, David R. Phillips, Pamela B.
Conley

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

JPET#184143

Running title: Clopidogrel-insensitive pool of P2Y₁₂ receptors

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

Number of text pages: 35

Number of tables: 1

Number of figures: 7

Number of supplemental figures: 1

Number of references: 30

Number of words in the Abstract: 245

Number of words in the Introduction: 702

Number of words in the Discussion: 1007

Abbreviations used in this paper: ADP, adenosine diphosphate; IV, intravenous; TP α , thromboxane receptor α ; $\alpha_{IIb}\beta_3$, integrin alpha IIb beta 3.

Recommended section: Cardiovascular

JPET#184143

Abstract

It is known that hepatic metabolism limits the anti-aggregatory activity of clopidogrel and by consequence, its clinical benefits. In this study, we investigated whether other factors exist, that could account for clopidogrel's sub-optimal antithrombotic activity. Using an *in vivo* murine FeCl₃ thrombosis model coupled with intravital microscopy, we found that at equivalent, maximal levels of inhibition of ADP-induced platelet aggregation, clopidogrel (50mg/kg oral) failed at reproducing the phenotype associated with P2Y₁₂-deficiency. On the other hand, elinogrel (60mg/kg oral), a direct-acting reversible P2Y₁₂ antagonist, achieved maximal levels of inhibition *in vivo* and its administration (1 mg/kg IV) abolished the residual thrombosis associated with clopidogrel dosing. Since elinogrel is constantly present in plasma, while the active metabolite of clopidogrel exists for ~ 2 hours, we evaluated whether an intracellular pool of P2Y₁₂ exists, that would be inaccessible to clopidogrel and contribute to its limited antithrombotic activity. Using saturation [³H]-2MeSADP binding studies, we first demonstrated that platelet stimulation with thrombin and convulxin (mouse) and TRAP (human) significantly increased surface expression of P2Y₁₂ relative to resting platelets. We next found that clopidogrel dose-dependently inhibited ADP-induced aggregation, signaling (cAMP) and surface P2Y₁₂ on resting mouse platelets, achieving complete inhibition at the highest dose (50mg/kg), but failed at blocking this inducible pool. Thus, an inducible pool of P2Y₁₂ exists on platelets that can be exposed upon platelet activation by strong

JPET#184143

agonists. This inducible pool is not completely blocked by clopidogrel, contributes to thrombosis *in vivo*, and can be blocked by elinogrel.

JPET#184143

Introduction

ADP, one of the most important mediators of hemostasis and thrombosis, binds to two G-protein coupled receptors on platelets. Interaction of ADP with P2Y₁ initiates the aggregation reaction and that with P2Y₁₂ drives sustained aggregation and secretion processes (Gachet, 2001; Andre et al., 2003a). Clinical studies using the thienopyridine clopidogrel, a prodrug undergoing biotransformation to an active metabolite that irreversibly antagonizes P2Y₁₂ receptors (Savi et al., 2001), have demonstrated a significant clinical benefit in patients with acute coronary syndrome (Mehta et al., 2001; Yusuf et al., 2001). Nevertheless, it is also now known that its efficacy is sub-optimal as illustrated by its slow onset of action, inter-individual variability and genetic variants that limit its metabolism in selected patients (Savi et al., 2000; Gurbel et al., 2003; Serebruany et al., 2005; Gurbel and Tantry, 2006). Interestingly, the results of the GRAVITAS study (Price et al., 2011) comparing single (75 mg) and double (150 mg) chronic doses of clopidogrel showed similar benefits, likely due to the fact that hepatic metabolism is still a limitation in patients who are poor responders, even at higher doses. However, since genetic polymorphisms in cytochrome P450 (*CYP*) and transporters (*ABCB1*) which are involved in clopidogrel metabolism and disposition only partially explain the correlation with suboptimal clopidogrel response (Campo et al., 2011), one could speculate that other factors exist that could account for a lack of dose response on clinical outcomes as seen in GRAVITAS.

JPET#184143

Inhibition of platelet aggregation by direct acting and reversible antagonists might be preferable to irreversible prodrug inhibitors because of faster onset and offset, less interpatient variability and higher levels of platelet inhibition with less impact on hemostasis. Ticagrelor, a direct acting and reversible allosteric modulator of P2Y₁₂, demonstrated greater clinical benefit over clopidogrel without a proportional increase in overall bleeding risk but increased non-CABG bleeding in a recent phase III study (Wallentin et al., 2009) . Elinogrel (Oestreich, 2010; Ueno et al., 2010), a potent, selective and direct acting reversible antagonist of the P2Y₁₂ receptor which completed Phase II clinical development (Leonardi et al., 2010; Lieu et al., 2007) has been demonstrated to overcome high platelet reactivity in patients with suboptimal response to clopidogrel (Gurbel et al., 2010).

It has been well established that several platelet receptors such as P2Y₁, the thromboxane receptor (TP α) (Nurden et al., 2003) and $\alpha_{IIb}\beta_3$ (Nurden et al., 1997) are present on the platelet surface but also on the membranes of α granules, constituting an inducible intracellular pool. Upon stimulation with thrombin or collagen, intracellular receptors redistribute to the surface and increase the total receptor number that can mediate biological responses. One example of the importance of blocking both populations of receptors has been shown in a study where internal $\alpha_{IIb}\beta_3$ receptors that were expressed on the surface following potent agonist stimulation were not occupied by the $\alpha_{IIb}\beta_3$ antagonists abciximab, possibly explaining the incomplete inhibition of platelet aggregation observed in response to TRAP (Quinn et al., 2001). A possible

JPET#184143

mechanism involved in maintenance of the intracellular receptor pool in platelets was described for the thrombin receptor where tonic internalization, mediated by a different mechanism than agonist-induced receptor endocytosis, might be important in sustaining the intracellular pool (Shapiro et al., 1996). Agonist-induced regulation, trafficking and cellular distribution of the P2Y₁₂ receptor have been studied. Initial studies did not demonstrate desensitization of P2Y₁₂-mediated inhibition of cAMP, or colocalization with the recycling vesicle marker transferrin in astrocytoma cells transfected with GFP-tagged P2Y₁₂ (Baurand et al., 2005). However, subsequent studies showed that P2Y₁₂ rapidly desensitizes and internalizes in platelets and when expressed in the 1321N1 cell line, internalizes mainly via GRK2 and PKC (novel)-dependent processes (Hardy et al., 2005). In addition it has been demonstrated that after internalization P2Y₁₂ recycles back to the plasma membrane allowing for rapid resensitization to occur (Mundell et al., 2008). Although there is an intracellular distribution of P2Y₁₂ receptors in resting platelets (Baurand et al., 2005), it is not known whether P2Y₁₂ redistributes to the platelet surface upon stimulation and whether this inducible pool contributes to functional response.

In this study, we determined whether human and mouse platelets have a functional, inducible (intracellular) pool of P2Y₁₂ that is biologically active and could account for clopidogrel sub-optimal antithrombotic activity.

JPET#184143

Methods

In vivo thrombosis model

Thrombosis on mouse mesenteric arteries ($1000\text{-}1300\text{ s}^{-1}$) 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% FeCl₃ solution. After 5 minutes, the filter paper was removed and mesenteric arteries rinsed with 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. C57 Bl6 J mice or P2Y₁₂^{-/-} mice (Andre et al., 2003b) were orally gavaged 48, 24 and 2 hours prior to injury with either vehicle control (0.5% methylcellulose, WT mice and P2Y₁₂^{-/-}), clopidogrel (WT mice; 50mg/kg q.d.), elinogrel (WT mice; 60mg/kg twice a day for 3 consecutive days) (Ueno et al., 2010) or ticagrelor (100 mg/kg twice a day for 3 consecutive days). Finally, in some experiments, elinogrel was injected intravenously (1mg/kg) in clopidogrel-dosed mice (50mg/kg, 3 days, q.d.) after initiation of the thrombotic process. All procedures conformed to institutional guidelines and to the Guide for Care and Use of Laboratory Animals (NIH, Bethesda, Maryland, USA).

Mouse platelet rich plasma (PRP) aggregation

Aggregations using PRP (3.8% citrated mouse blood centrifugated at 200g 10min) were performed at 37° using a Chronolog aggregometer (Chrono-Log

JPET#184143

Corp., Havertown, PA) set at a stirring speed of 1200 rpm. PRP (3×10^8 platelets/ml) was prepared from vehicle control and clopidogrel (0.5 – 50mg/kg) dosed mice (see above). Platelet poor plasma (PPP) was used for calibration. Changes in light transmittance were determined over a 6 min time period after addition of either ADP (10 μ M) (Sigma-Aldrich, Atlanta, GA), murine PAR4 TRAP (AYPGKF) peptide (mTRAP) (1.6mM) (Bachem, Torrance, CA) or collagen (20 μ g/ml) (Chrono-Log Corp., Havertown, PA).

Preparation of mouse washed platelets

Washed platelets were prepared as previously described (Jantzen et al., 2001). Briefly, 0.7ml of mouse blood was collected by cardiac puncture into 0.14ml of acid-citrate-dextrose (ACD - 38mM citric acid, 75mM trisodium citrate, 100mM dextrose) and 0.56ml saline containing PGE1 (0.1 μ M final concentration). Binding and flow cytometry assays were performed with washed platelets (3×10^8 platelets/ml) resuspended in HEPES-Tyrodes buffer (10mM HEPES, 138mM NaCl, 5.5mM glucose, 2.9mM KCl, 12mM NaHCO₃, pH 7.4) containing 1mM CaCl₂, 1mM MgCl₂, and 0.1% BSA.

Determination of cAMP in mouse platelets

Platelet cAMP levels were measured using the cAMP ELISA System (Assay Design, Ann Arbor, MI) in washed mouse platelets (2.5×10^8 /ml) isolated from mice treated with either vehicle or clopidogrel (1.5–50 mg/kg) for 3 days. Following incubation with 100 μ M 3-isobutyl-1-methylxanthine (IBMX) (Sigma-

JPET#184143

Aldrich, Atlanta, GA), platelets were treated with ADP (0.01 – 20 μ M) in the presence of 1 μ M forskolin (Sigma-Aldrich, Atlanta, GA) for 10 min. Reactions were terminated, processed and quantified according to manufacturer's instructions.

Platelet activation as measured by P-selectin activation

Washed platelets (2.5x10⁸/ml) from vehicle control, P2Y₁₂^{-/-} (Andre et al., 2003a) or clopidogrel treated mice (0.5 – 50mg/kg) were incubated for 5 min at RT with either saline, thrombin (0.5 or 5nM) or convulxin (0.5 μ g/ml). In order to detect surface P-selectin expression, platelets were incubated with FITC-CD62 antibodies (BD Bioscience, Pasadena, CA) for 20 min in the dark at RT. Analysis of platelet-bound FITC-CD62 was performed using a FACSort flow cytometer following collection of 10,000 events.

Radioligand binding studies

Saturation binding studies were performed using a range of [³H]-2MeSADP (Perkin Elmer, Boston, MA) concentrations (0.1 – 100nM) on human and mouse platelets. In initial experiments the number of total binding sites (P2Y₁₂+P2Y₁) was established using unlabeled 2MeSADP (20 μ M), while specific P2Y₁₂ or P2Y₁ binding sites were defined by using selective P2Y₁₂ (elinogrel 30 μ M) or P2Y₁ (MRS2179 100 μ M) antagonists. Elinogrel is a competitive P2Y₁₂ receptor antagonist and can displace [³H]-2MeSADP in radioligand binding studies (see supplemental figure 1). Specific binding was calculated as the difference

JPET#184143

between total binding determined in the presence of [³H]-2MeSADP and nonspecific binding measured in the presence of either excess elinogrel (P2Y₁₂), MRS2179 (P2Y₁) or 2MeSADP (P2Y₁₂+ P2Y₁) (Sigma-Aldrich, Atlanta, GA). On mouse platelets ~82% of sites labeled by [³H]-2MeSADP represent P2Y₁₂ receptors. (Total binding sites P2Y₁₂+P2Y₁ = 914±24; P2Y₁₂ = 745±50; P2Y₁ = 160±11; N=3). On human platelets ~95% of sites labeled by [³H]-2MeSADP can be attributed to P2Y₁₂ receptors and the remainder attributed to P2Y₁. In further experiments the number of P2Y₁₂ receptors on mouse platelets isolated from vehicle- or clopidogrel-treated mice before and after stimulation with convulxin (Pentapharm, Norwalk, CT) (0.5µg/ml; 5min RT) or thrombin (Haematologic Technologies Inc., Essex Junction, VT) (5nM; 5min RT) was assessed by saturation binding, where specific P2Y₁₂ binding was determined and B_{max} and K_d were calculated using GraphPad Prism software. Binding experiments were performed using murine PRP with and without convulxin treatment and using murine washed platelets with and without thrombin treatment. The same approach was used to examine the number of P2Y₁₂ receptors on resting and TRAP (5µM)-stimulated human platelets using PRP. Duplicate binding reactions were carried out in 0.11 mL (2x10⁸ platelets/ml) for 20 min at room temperature and were terminated by rapid filtration through Whatman GF/C glass fiber filters under vacuum using a Micro96 Harvester (Molecular Devices, Sunnyvale, CA). Radioactivity bound to the filters was measured by scintillation counting in a MicroBeta2 counter (Perkin Elmer, Boston, MA). In some experiments platelets isolated from vehicle control and

JPET#184143

clopidogrel-treated mice were fixed after stimulation with strong agonist by continuous rotation for 25 min in the presence of 4% formaldehyde. Platelets were isolated by centrifugation (10min 1000g) and resuspended in binding buffer (20nM Hepes and 1mM MgCl₂) to a density of 2x10⁸ platelets/ml and binding reactions were carried out as described above.

JPET#184143

Results

The effect of clopidogrel on blocking ADP-induced platelet aggregation, surface P2Y₁₂ receptors and P2Y₁₂-mediated signaling on resting platelets

We studied the effect of different doses of clopidogrel (1.5 – 50mg/kg; 3 days dosing, po) on multiple outcomes mediated by P2Y₁₂. First, we examined *ex vivo* platelet aggregation induced by 10μM ADP, relative to vehicle-treated mice. Clopidogrel treatment dose-dependently inhibited aggregation, reaching near complete inhibition (98%) at the highest dose of 50mg/kg (clopidogrel 1.5mg/kg p<0.01 vs vehicle control, doses 5-50mg/kg p<0.001 vs vehicle control) (Figure 1). Platelets isolated from clopidogrel-treated mice (50mg/kg) were functional as they aggregated in response to high concentrations of collagen (20μg/ml) or PAR-4 agonist mTRAP (1.6mM) to a similar level as platelets isolated from vehicle-treated mice (data not shown).

In addition, oral administration of increasing doses of clopidogrel significantly and dose-dependently inhibited binding of [³H]-2MeSADP to P2Y₁₂ receptors (B_{max}), reaching maximal inhibition (100%) of P2Y₁₂ at the highest dose (50mg/kg) in mouse PRP (Figure 2A) and mouse washed platelets (Figure 2B).

Although the B_{max} value for P2Y₁₂ receptors decreased with increasing clopidogrel concentrations, the dissociation constant, K_d of [³H]-2MeSADP, was not significantly affected (vehicle control K_d=0.57±0.31nM; clopidogrel (5mg/kg)-treated K_d=0.35±0.24nM) as expected for an irreversible antagonist.

As P2Y₁₂ couples through G_{o*i*} and represses cAMP, we next assessed the effect of clopidogrel doses on cAMP signaling induced by ADP. In vehicle control

JPET#184143

mice, significant inhibition of forskolin-stimulated cAMP by ADP was detected ($EC_{50}=0.24\pm 0.09\mu\text{M}$). In clopidogrel-treated mice, at the lower dose (1.5mg/kg) we observed a rightward shift of the ADP dose-response curve for inhibition of cAMP ($EC_{50}=3.04\pm 0.9\mu\text{M}$). The ADP-induced inhibitory effect on forskolin-stimulated cAMP was blocked by higher doses of clopidogrel (15mg/kg, forskolin vs forskolin+ADP $p=0.06$; 50mg/kg, forskolin vs forskolin+ADP $p=0.88$) confirming maximal blockade of all $P2Y_{12}$ (Figure 3).

Our results demonstrated maximal inhibition of ADP-mediated platelet function and signaling as a consequence of maximal blockade of surface $P2Y_{12}$ in 50mg/kg clopidogrel-dosed mice. These data are in agreement with previous studies from $P2Y_{12}^{-/-}$ mice showing complete inhibition of ADP-induced platelet aggregation and cAMP-mediated signaling (Andre et al., 2003a).

Residual thrombosis *in vivo* observed in 50mg/kg clopidogrel-treated mice can be blocked by elinogrel

The antithrombotic activity of clopidogrel (50mg/kg) was next assessed *in vivo* using a ferric chloride-induced vascular injury model as previously described (Andre et al., 2003a) and directly compared to the phenotype of $P2Y_{12}^{-/-}$ mice. Although clopidogrel (50mg/kg) prevented vascular occlusion due to an effect on thrombus stability during the 40 min observation period, similar to $P2Y_{12}^{-/-}$ mice (Andre et al., 2011), there were significant differences in the thrombotic profiles in that with clopidogrel-treated animals larger platelet aggregates were observed after clopidogrel treatment (Figure 4A, right panel), with a total

JPET#184143

fluorescence intensity (which reflects the amount of fluorescently labeled platelets at the site of vascular injury accumulated over 40 min observation period) significantly higher compared to P2Y₁₂^{-/-} mice (p=0.0023) (Figure 4B). In contrast, elinogrel (60mg/kg) provided a superior level of inhibition of thrombosis compared to clopidogrel (p=0.02), reproducing the phenotype of P2Y₁₂^{-/-} mice (Figure 4A, right panel, Figure 4B). A similar level of inhibition was achieved by ticagrelor (100 mg/kg), another direct-acting P2Y₁₂ antagonist, (Figure 4A, right panel). Since these data suggested that P2Y₁₂-mediated signaling occurred *in vivo* through unblocked P2Y₁₂ we next determined whether elinogrel treatment on top of clopidogrel could provide additive activity. In a second set of experiments, injury was initiated in clopidogrel (50mg/kg, 3 days po)-treated mice and elinogrel was subsequently injected as a 1 mg/kg IV bolus via the tail vein. The residual thrombotic process observed in animals treated with clopidogrel was eliminated after IV injection of elinogrel (Figure 4C).

Since 50mg/kg clopidogrel completely blocked i) all surface P2Y₁₂ receptors on resting platelets, ii) signaling through cAMP and iii) ADP-induced aggregation to the level observed on platelets from P2Y₁₂^{-/-} mice (Andre et al., 2003a), we hypothesized that residual thrombosis observed following treatment with 50mg/kg clopidogrel could be mediated by an intracellular pool of P2Y₁₂ exposed on the platelet surface upon stimulation by strong agonists generated during the thrombotic process.

JPET#184143

A new pool of P2Y₁₂ can be expressed on mouse platelets upon strong agonist stimulation

In order to examine whether an inducible (intracellular) pool of P2Y₁₂ receptors can be exposed on mouse platelets upon activation, we established conditions of maximal platelet activation as assessed by P-selectin expression (an α granule marker upregulated following platelet stimulation with strong agonists). Convulxin (0.5 μ g/ml) or thrombin (5nM) induced significant and comparable increase in the expression of P-selectin on mouse platelets (Figure 5A). Using agonist treatments (convulxin or thrombin) demonstrated to fully activate mouse platelets, we determined the number of surface P2Y₁₂ receptors present following strong agonist stimulation. A significant increase in P2Y₁₂ receptor number (B_{max}) was observed upon stimulation with convulxin (PRP) or thrombin (washed platelets) with no significant change in K_d observed in control vs stimulated condition (control $K_d=1.00\pm 0.97$ nM; thrombin stimulated $K_d=1.17\pm 1.00$ nM) (Figure 5B,C).

Clopidogrel does not completely block the inducible pool of P2Y₁₂

We next examined whether an increase in P2Y₁₂ receptor number could be detected following thrombin stimulation using platelets isolated from clopidogrel-treated mice (5-50mg/kg). First we established the concentration of thrombin that caused maximal stimulation of platelets isolated from clopidogrel-treated mice by measuring the level of P-selectin expression on the platelet surface. Unlike platelets from control mice where both concentrations of thrombin

JPET#184143

(0.5nM and 5nM) induced comparable increases in P-selectin expression (data not shown), the higher concentration of thrombin (5nM) was required to achieve the same level of P-selectin expression on platelets from clopidogrel (50 mg/kg)-treated mice. This was confirmed in P2Y₁₂^{-/-} mice where only the higher thrombin concentration (5nM) caused maximal P-selectin expression (data not shown).

To determine whether clopidogrel blocked this inducible pool of P2Y₁₂ receptors, we determined the number of P2Y₁₂ receptors on platelets from mice dosed with clopidogrel (using saturation binding studies with [³H]-2MeSADP) with and without treatment by 5nM thrombin. Surprisingly, we observed an increase in P2Y₁₂ receptor number following thrombin treatment on platelets isolated from clopidogrel-treated mice, as was previously seen with platelets from clopidogrel-naïve animals (Table I) (since 50mg/kg clopidogrel dose induced complete inhibition of [³H]-2MeSADP binding to P2Y₁₂, we could not determine B_{max} for non-treated condition). We also confirmed this result using formaldehyde-fixed platelets, which minimizes possible internalization of P2Y₁₂ following activation, an approach validated previously (Mundell et al., 2006). In this method, platelets were fixed after stimulation with agonist and before performing radioligand binding studies using a single [³H]-2MeSADP concentration (50nM) and conditions allowing assessment of P2Y₁₂-specific binding. Using fixed conditions, we also observed an increase in surface expression of P2Y₁₂ in platelets from vehicle control and clopidogrel-treated mice (5, 15, 50mg/kg) (Figure 6) (*p<0.05; **p<0.01; ***p<0.001 thrombin

JPET#184143

stimulated vs corresponding unstimulated), demonstrating that clopidogrel does not completely block this inducible pool (Figure 6).

Human platelets express an inducible pool of P2Y₁₂ upon strong agonist stimulation

We next assessed whether an inducible pool of P2Y₁₂ exists in human platelets. Stimulation of human platelets with 5 μ M or 20 μ M TRAP-peptide induced comparable and significant increase in the expression of P-selectin measured by flow cytometry (Figure 7A). We then addressed whether newly exposed P2Y₁₂ receptors can be detected using saturation radioligand binding to determine P2Y₁₂ receptor number on unstimulated (control) platelets versus those stimulated with TRAP (5 μ M). A representative saturation binding experiment with and without treatment with 5 μ M TRAP is shown in Figure 7B. The increase in the P2Y₁₂ receptor number observed upon TRAP stimulation was significant (untreated 423 \pm 28; TRAP-stimulated 529 \pm 28 p=0.02; N=3) (Figure 7C), with no significant change in K_d observed in control vs stimulated conditions (control K_d=3.6 \pm 1.7 nM; TRAP stimulated K_d=5 \pm 1.5 nM). These data confirm that, similar to mouse platelets, an inducible pool of P2Y₁₂ receptors exists on human platelets, and these internal receptors can be mobilized to the platelet surface following strong agonist stimulation.

JPET#184143

Discussion

Dosing of mice with clopidogrel (50mg/kg) completely inhibited ADP-induced *ex vivo* platelet aggregation and signaling (cAMP), and blocked all surface P2Y₁₂ receptors on resting platelets. Interestingly, the same high dose of clopidogrel (50 mg/kg) prevented vascular occlusion but did not achieve the thrombotic profile associated with P2Y₁₂^{-/-} deficiency, while the direct acting inhibitor elinogrel did recapitulate the profile of P2Y₁₂^{-/-} -deficient mice. Addition of elinogrel (IV 1mg/kg), to this clopidogrel regimen (50 mg/kg for 3 days) completely blocked the residual thrombosis, confirming that this process was mediated by P2Y₁₂ receptors. Based on this data we hypothesized that the residual thrombosis observed in clopidogrel-dosed mice might be due to an unblocked (intracellular) pool of P2Y₁₂ exposed on the platelet surface following platelet activation *in vivo*. In this study we demonstrated that an inducible pool of P2Y₁₂ receptors exists in human and mouse platelets and becomes exposed following activation with strong platelet agonists (e.g. TRAP-peptide, convulxin or thrombin). This inducible pool of P2Y₁₂ that contributed to thrombosis could be blocked by elinogrel but not by clopidogrel.

A number of platelet receptors have been shown to be distributed as an extracellular pool at the plasma membrane and a second intracellular pool on the α granule membrane and/or in the open canalicular system (OCS) (Nurden et al., 1997; Nurden et al., 2003). The existence of receptors in two populations, extracellular and intracellular, needs to be taken into account when evaluating drug efficacy as this could lead to improper dosing (Quinn et al., 2001).

JPET#184143

Although the P2Y₁₂ receptor has been shown to be present at the plasma membrane and an intracellular localization was detected in resting platelets (Baurand et al., 2005), the precise intracellular distribution and possible redistribution to the plasma membrane has not been studied. Agonist-induced regulation of P2Y₁₂ and post-endocytic trafficking in platelets has recently been studied. These studies demonstrated that P2Y₁₂ desensitizes in platelets by a GRK-dependent mechanism and undergoes agonist induced phosphorylation and internalization (Mundell et al., 2006). The same authors showed that P2Y₁₂ recycles back to the surface following agonist-induced internalization and that any disruption in P2Y₁₂ trafficking (either internalization or recycling) blocks resensitization of P2Y₁₂ (Mundell et al., 2008). In the present study we detected an increase in the number of P2Y₁₂ receptors expressed on human platelets in response to TRAP-peptide stimulation (25%) and on mouse platelets after stimulation with convulxin (27%) or thrombin (33%). This is the first demonstration of P2Y₁₂ up-regulation suggesting the existence of an inducible (intracellular) pool of P2Y₁₂. In a previously published study (Judge et al., 2008) an intracellular pool of P2Y₁₂ receptors was not detected. A possible explanation for this discrepancy might be due to differences in methodologies between the two studies. In Judge et al, there was a greater time interval between platelet stimulation and radioligand binding studies (due to a centrifugation step), which may have resulted in receptor trafficking (internalization) that possibly prevented detection of the newly exposed pool of P2Y₁₂. To avoid receptor trafficking formaldehyde-fixed platelets can be used,

JPET#184143

an approach described previously (Mundell et al., 2006). One important observation in our study is the incomplete blockage of the inducible pool of P2Y₁₂ by clopidogrel. Thrombin induced the expression of intracellular P2Y₁₂ receptors despite treatment with clopidogrel (50 mg/kg) at a dose that completely blocked surface expressed P2Y₁₂ on resting platelets. In addition, these data also demonstrate that the inducible pool of P2Y₁₂ can be exposed on platelets isolated from mice treated with sub-optimal doses of clopidogrel. This observation might be important in the context of clopidogrel treatment in human patients where different levels of P2Y₁₂ receptor occupancy as well as variability in clopidogrel-induced inhibition of ADP-mediated aggregation have been demonstrated (Bal Dit Sollier et al., 2009). Interestingly, our data presented herein demonstrated a discrepancy between inhibition of the P2Y₁₂ receptor and platelet aggregation. Indeed, while sub-maximal doses of clopidogrel (0.5-5 mg/kg) inhibited P2Y₁₂ receptor binding by 65-86% on resting platelets, it only inhibited ADP-mediated aggregation by 32-57%. Thus, a limited number of functional receptors is sufficient to provide a sustained aggregation response, suggesting that exposure of a new pool of P2Y₁₂ (25-35% increase) could have a significant impact on thrombosis, as shown in our *in vivo* experiments. Our *in vivo* data in mice also demonstrated that when the entire surface P2Y₁₂ is blocked following chronic clopidogrel dosing, there is an inducible pool of P2Y₁₂ expressed following platelet stimulation that is not inhibited by the clopidogrel active metabolite but is fully functional and capable of mediating platelet thrombosis. This inducible pool seems to preferentially impact the initial growth

JPET#184143

of the thrombus triggered by the highly thrombogenic materials exposed and/or released at the site of vascular injury, as illustrated by the greater slope of thrombus growth immediately following injury in clopidogrel-treated animals, relative to elinogrel-treated animals (see Figure 4A).

Data presented here may have significant implications in the clinical setting. Following daily oral dosing of clopidogrel in humans, platelets are transiently exposed to a "pulse" (1-2 hr) of active metabolite, which inactivates a percentage of the total platelet population. If a plaque rupture occurs and initiates thrombosis after this "pulse" of active metabolite has passed, the newly-mobilized P2Y₁₂ receptors on the platelet surface which are unblocked by clopidogrel would be "active" and could contribute to the thrombotic process. Hence, intraday repetitive dosing of clopidogrel could be warranted for optimal protection. Another alternative is the use of a direct-acting P2Y₁₂ antagonist that does not require metabolic conversion to an active metabolite, as this class of inhibitor is present throughout the daily dosing cycle and may contribute to superior clinical outcomes.

In conclusion, results from this study show that 1) an inducible pool of P2Y₁₂ exists on platelets and can be exposed upon platelet activation by strong agonists, 2) this inducible pool is not completely blocked by clopidogrel and 3) this pool contributes to thrombosis in vivo. In addition, different properties and mechanism of action between irreversible prodrugs (thienopyridines) and direct-acting, reversible P2Y₁₂ antagonists may provide insight into the differences observed in clinical trials between these two classes of P2Y₁₂ antagonists.

JPET#184143

Acknowledgements

We would like to thank Dr. S. Delaney for providing helpful scientific suggestions and recommendations, Dr. Yvonne Pak for preparing the dosing solutions.

JPET#184143

Authorship Contributions

Participated in research design: Helena Haberstock-Debic, Pamela B. Conley, Patrick Andre, and David R. Phillips.

Conducted experiments: Helena Haberstock-Debic, Patrick Andre, and Scott Mills.

Performed data analysis: Helena Haberstock-Debic, and Patrick Andre.

Wrote or contributed to the writing of the manuscript: Helena Haberstock-Debic, Patrick Andre, and Pamela B. Conley.

JPET#184143

References

- Andre P, Deguzman F, Haberstock-Debic H, Mills S, Pak Y, Inagaki M, Pandey A, Hollenbach S, Phillips D and Conley P (2011) Thienopyridines, but Not Elinogrel, Result in Off-Target Effects at the Vessel Wall That Contribute to Bleeding. *J Pharmacol Exp Ther* **338**:22-30.
- Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR and Conley PB (2003a) P2Y₁₂ regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest* **112**:398-406.
- Andre P, LaRocca T, Delaney SM, Lin PH, Vincent D, Sinha U, Conley PB and Phillips DR (2003b) Anticoagulants (thrombin inhibitors) and aspirin synergize with P2Y₁₂ receptor antagonism in thrombosis. *Circulation* **108**:2697-2703.
- Bal Dit Sollier C, Berge N, Boval B, Hovsepian L and Drouet L (2009) Functional variability of platelet response to clopidogrel correlates with P2Y₁₂ receptor occupancy. *Thromb Haemost* **101**:116-122.
- Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, Cazenave JP, Leon C and Gachet C (2005) Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? *Mol Pharmacol* **67**:721-733.
- Campo G, Parrinello G, Ferraresi P, Lunghi B, Tebaldi M, Miccoli M, Marchesini J, Bernardi F, Ferrari R, Valgimigli M. (2011) Prospective evaluation of on-clopidogrel platelet reactivity over time in patients treated with percutaneous

JPET#184143

coronary intervention relationship with gene polymorphisms and clinical outcome. *J Am Coll Cardiol.* **57**:2474-2483.

Gachet C (2001) ADP receptors of platelets and their inhibition. *Thromb Haemost* **86**:222-232.

Gurbel PA, Bliden KP, Antonino MJ, Stephens G, Gretler DD, Jurek MM, Pakyz RE, Shuldiner AR, Conley PB and Tantry US (2010) The effect of elinogrel on high platelet reactivity during dual antiplatelet therapy and the relation to CYP2C19*2 genotype: first experience in patients. *J Thromb Haemost* **8**:43-53.

Gurbel PA, Bliden KP, Hiatt BL and O'Connor CM (2003) Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation* **107**:2908-2913.

Gurbel PA and Tantry US (2006) Drug insight: Clopidogrel nonresponsiveness. *Nat Clin Pract Cardiovasc Med* **3**:387-395.

Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW and Mundell SJ (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**:3552-3560.

Jantzen HM, Milstone DS, Gousset L, Conley PB and Mortensen RM (2001) Impaired activation of murine platelets lacking G α_{i2} . *J Clin Invest* **108**:477-483.

Judge HM, Buckland RJ, Sugidachi A, Jakubowski JA and Storey RF (2008) The active metabolite of prasugrel effectively blocks the platelet P2Y12 receptor and inhibits procoagulant and pro-inflammatory platelet responses. *Platelets* **19**:125-133.

JPET#184143

Leonardi S, Rao SV, Harrington RA, Bhatt DL, Gibson CM, Roe MT, Kochman J, Huber K, Zeymer U, Madan M, Gretler DD, McClure MW, Paynter GE, Thompson V and Welsh RC (2010) Rationale and design of the randomized, double-blind trial testing INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y(12)-receptor inhibitor, versus clopidogrel to eVALuate Tolerability and Efficacy in nonurgent Percutaneous Coronary Interventions patients (INNOVATE-PCI). *Am Heart J* **160**:65-72.

Lieu H, Conley P, Andre P, Leese P, Oromanko K, Phillips D, Jurek M, Meloni A, Hutchaleelaha A and Gretler D (2007) Initial intravenous experience with PRT060128 (PRT128), an orally-available, direct-acting, and reversible P2Y12 inhibitor. *J Thromb Haemost* **5**:P-T-292 (abstract).

Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H, Zhao F, Chrolavicius S, Copland I and Fox KA (2001) Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet* **358**:527-533.

Mundell SJ, Barton JF, Mayo-Martin MB, Hardy AR and Poole AW (2008) Rapid resensitization of purinergic receptor function in human platelets. *J Thromb Haemost* **6**:1393-1404.

Mundell SJ, Jones ML, Hardy AR, Barton JF, Beaucourt SM, Conley PB and Poole AW (2006) Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. *Mol Pharmacol* **70**:1132-1142.

JPET#184143

- Nurden P, Poujol C and Nurden AT (1997) The evolution of megakaryocytes to platelets. *Baillieres Clin Haematol* **10**:1-27.
- Nurden P, Poujol C, Winckler J, Combrie R, Pousseau N, Conley PB, Levy-Toledano S, Habib A and Nurden AT (2003) Immunolocalization of P2Y1 and TPalpha receptors in platelets showed a major pool associated with the membranes of alpha -granules and the open canalicular system. *Blood* **101**:1400-1408.
- Oestreich JH (2010) Elinogrel, a reversible P2Y12 receptor antagonist for the treatment of acute coronary syndrome and prevention of secondary thrombotic events. *Curr Opin Investig Drugs* **11**:340-348.
- Price MJ, Berger PB, Teirstein PS, Tanguay JF, Angiolillo DJ, Spriggs D, Puri S, Robbins M, Garratt KN, Bertrand OF, Stillablower ME, Aragon JR, Kandzari DE, Stinis CT, Lee MS, Manoukian SV, Cannon CP, Schork NJ and Topol EJ (2011) Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. *JAMA* **305**:1097-1105.
- Quinn MJ, Murphy RT, Dooley M, Foley JB and Fitzgerald DJ (2001) Occupancy of the internal and external pools of glycoprotein IIb/IIIa following abciximab bolus and infusion. *J Pharmacol Exp Ther* **297**:496-500.
- Savi P, Labouret C, Delesque N, Guette F, Lupker J and Herbert JM (2001) P2Y₁₂, a new platelet ADP receptor, target of clopidogrel. *Biochem Biophys Res Comm* **283**:379-383.

JPET#184143

- Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M and Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* **84**:891-896.
- Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL and Topol EJ (2005) Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol* **45**:246-251.
- Shapiro MJ, Trejo J, Zeng D and Coughlin SR (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J Biol Chem* **271**:32874-32880.
- Ueno M, Rao SV and Angiolillo DJ (2010) Elinogrel: pharmacological principles, preclinical and early phase clinical testing. *Future Cardiol* **6**:445-453.
- Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA, Freij A and Thorsen M (2009) Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med* **361**:1045-1057.
- Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G and Fox KK (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med* **345**:494-502.

JPET#184143

Footnotes

Disclosure:

All co-authors are present or former 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

JPET#184143

Legends for Figures

Figure 1:

Platelet aggregation measurements in response to 10 μ M ADP in PRP obtained from vehicle- and clopidogrel-treated mice (1.5mg/kg – 50mg/kg; 3 days dosing po). Data are expressed as % of maximal aggregation measured over a period of 6 minutes. Clopidogrel dose-dependently inhibited platelet aggregation compared to vehicle control (** p<0.01; *** p<0.001). Values are expressed as mean \pm sem; N=4-12 per group. ‡ The percentage shown above each bar indicates the percentage of inhibition of aggregation compared to vehicle control.

Figure 2:

Clopidogrel (0.5 – 50 mg/kg; 3 days dosing po) blocks surface P2Y₁₂ receptors on resting mouse platelets. Administration of increasing doses of clopidogrel alters the number of surface P2Y₁₂ (B_{max}) detected in saturation radioligand binding ([³H]-2MeSADP radioligand, unlabeled 30 μ M elinogrel). P2Y₁₂ receptor inhibition was established in mouse PRP **(A)** and mouse washed platelets **(B)**. Values are expressed as mean \pm sem of the P2Y₁₂ receptor number; N=3-8 per group. † The percentage shown above each bar represents P2Y₁₂ receptor occupancy by clopidogrel. Clopidogrel in all doses significantly blocked the surface P2Y₁₂ compared to vehicle-treated mice (**p<0.01, ***p<0.001).

JPET#184143

Figure 3:

Effect of different doses of clopidogrel (1.5 - 50mg/kg; 3 days dosing po) on ADP-induced inhibition of forskolin-stimulated cAMP. Data are expressed as the mean \pm sem (N=3-4) and normalized to the cAMP level in the presence of forskolin (100%). Clopidogrel (50mg/kg) blocked ADP-induced inhibitory effect on forskolin-stimulated cAMP (forskolin vs forskolin+ADP, $p=0.88$).

Figure 4:

Elinogrel (1mg/kg, IV) blocks residual thrombotic activity observed in clopidogrel (50mg/kg; 3 days dosing po)-treated mice in ferric chloride-induced vascular injury model. **A)** *In vivo* arterial thrombotic profiles of control (left panel), clopidogrel- (50 mg/kg), elinogrel- (60 mg/kg) and ticagrelor (100 mg/kg)-treated WT animals (right panel). One frame was captured every 2 seconds. **B)** Platelet accumulation at the site of vascular injury over the 40 min observation period, as determined by fluorescence intensity. **C)** *In vivo* thrombotic profiles for clopidogrel-dosed mice (50mg/kg; 3 days dosing po) with or without addition of elinogrel (IV 1mg/kg). Data are expressed as fluorescence intensity over 15 min observation period. Each point represents the mean calculated from 5 experiments.

Figure 5:

A new pool of P2Y₁₂ receptors is expressed on mouse platelets after stimulation with thrombin or convulxin. **A)** Flow cytometry analysis of P-selectin expression

JPET#184143

on mouse resting platelets (control), or on platelets stimulated with convulxin (0.5 μ g/ml) or thrombin (5nM). Values are expressed as the mean \pm sem, a representative experiment out of three experiments is shown. **B)**

Representative saturation binding experiment measuring P2Y₁₂ specific binding (0.01-100nM [³H]-2MeSADP radioligand; unlabeled 30 μ M elinogrel) performed on untreated (control) and convulxin (0.5 μ g/ml) or thrombin (5nM) stimulated platelets. **C)** Significant increase in the surface P2Y₁₂ receptor number was observed upon platelet stimulation with convulxin (untreated 798 \pm 56; convulxin-stimulated 1014 \pm 67, p=0.03; N=3) or thrombin (untreated 872 \pm 87; thrombin-stimulated 1162 \pm 98, p=0.001; N=5). Values are expressed as mean \pm sem.

Figure 6:

The P2Y₁₂ receptor up-regulation on platelets isolated from clopidogrel-treated mice (5 – 50mg/kg; 3 days dosing po) upon thrombin stimulation.

Radioligand binding was performed (50nM [³H]-2MeSADP, unlabeled ligand 30 μ M elinogrel) in formaldehyde-fixed mouse platelets. An increase in specific P2Y₁₂ binding was observed on platelets from vehicle- and clopidogrel-treated mice after treatment with 5nM thrombin. Data are expressed as mean \pm sem (N=3-6) (thrombin stimulated vs respective unstimulated controls *p<0.05; **p<0.01; ***p<0.001).

Figure 7:

JPET#184143

New P2Y₁₂ receptors are exposed on human platelets after stimulation with TRAP-peptide. **A)** P-selectin expression on human platelets was measured by flow-cytometry analysis of FITC-CD62P antibody binding to resting (control) or TRAP (5μM and 20 μM) stimulated platelets. Values are expressed as mean fluorescence + sem, representative experiment is shown). **B)** Representative experiments of saturation binding (radioligand 0.01-100nM [³H]-2MeSADP; competition with unlabeled 30μM elinogrel) performed on untreated (control) or TRAP (5μM) stimulated platelets. **C)** Significant increase in the surface P2Y₁₂ receptor number was observed upon platelet stimulation with TRAP (p=0.02, N=3).

JPET#184143

Table 1: Increase in P2Y₁₂ receptor number on mouse platelets upon stimulation with thrombin

Saturation radioligand binding (radioligand [³H]-2MeSADP; unlabeled ligand 30μM elinogrel) was performed to detect the P2Y₁₂ receptor number on resting and thrombin (5nM)-stimulated platelets isolated from clopidogrel-treated mice (0.5 - 50mg/kg). Increase in P2Y₁₂ receptor number was significant (p values for nonstimulated vs thrombin-stimulated for vehicle control p=0.001; 1.5mg/kg clopidogrel p=0.014; 5mg/kg clopidogrel p=0.002; 10mg/kg clopidogrel p=0.045; 50mg/kg clopidogrel. * B_{max} could not be calculated for non-treated condition due to complete inhibition of [³H]-2MeSADP binding to P2Y₁₂; see text in the result section.

P2Y ₁₂ receptor number per platelet ± sem	n		
	Untreated	5nM Thrombin	
Vehicle Control	872 ± 87	1162 ± 98	5
Clopidogrel 1.5mg/kg	226 ± 18	326 ± 23	4
Clopidogrel 5mg/kg	77 ± 5	143 ± 2	3
Clopidogrel 10mg/kg	58 ± 12	140 ± 3	3
Clopidogrel 50mg/kg	*		3

Figure 1:

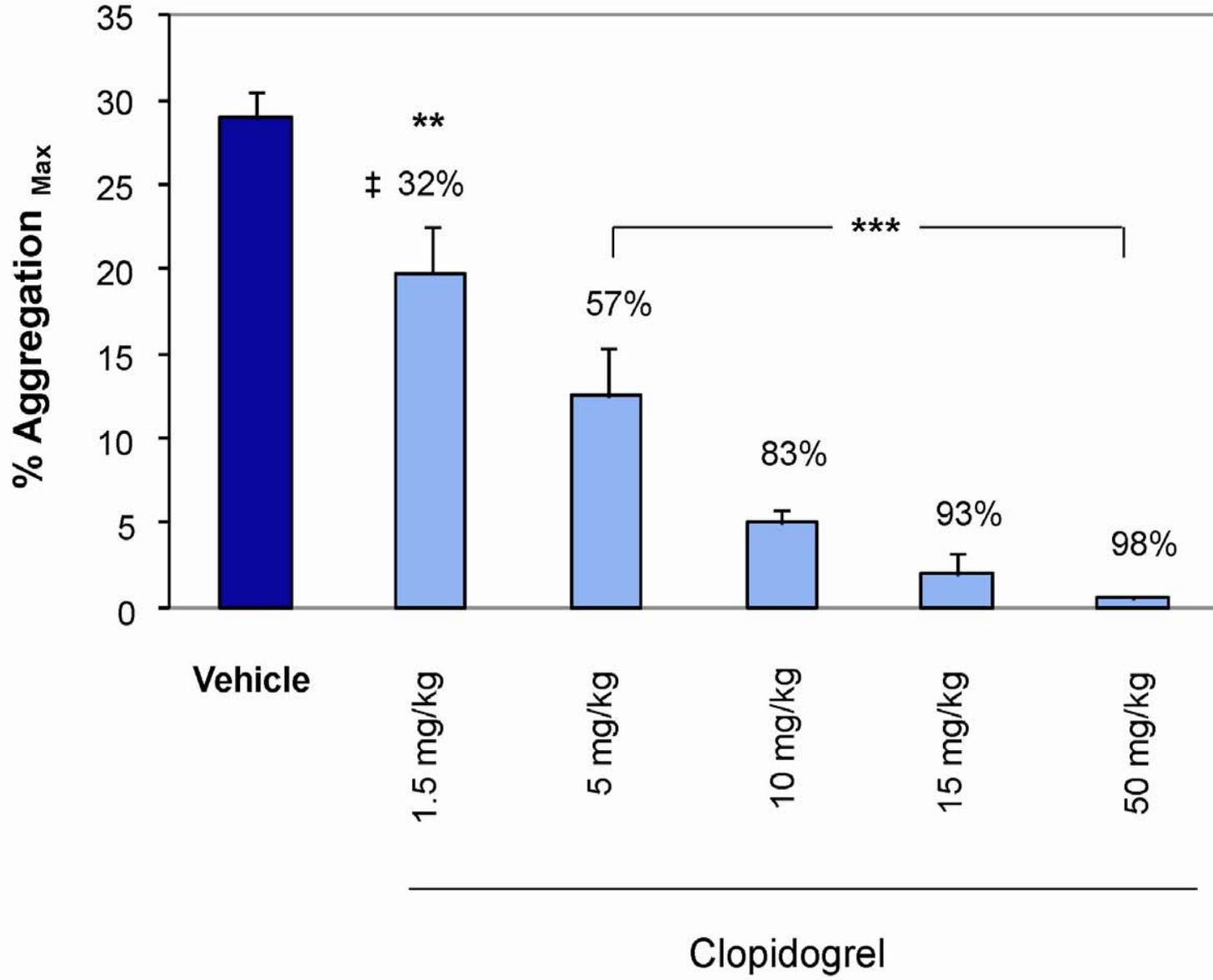
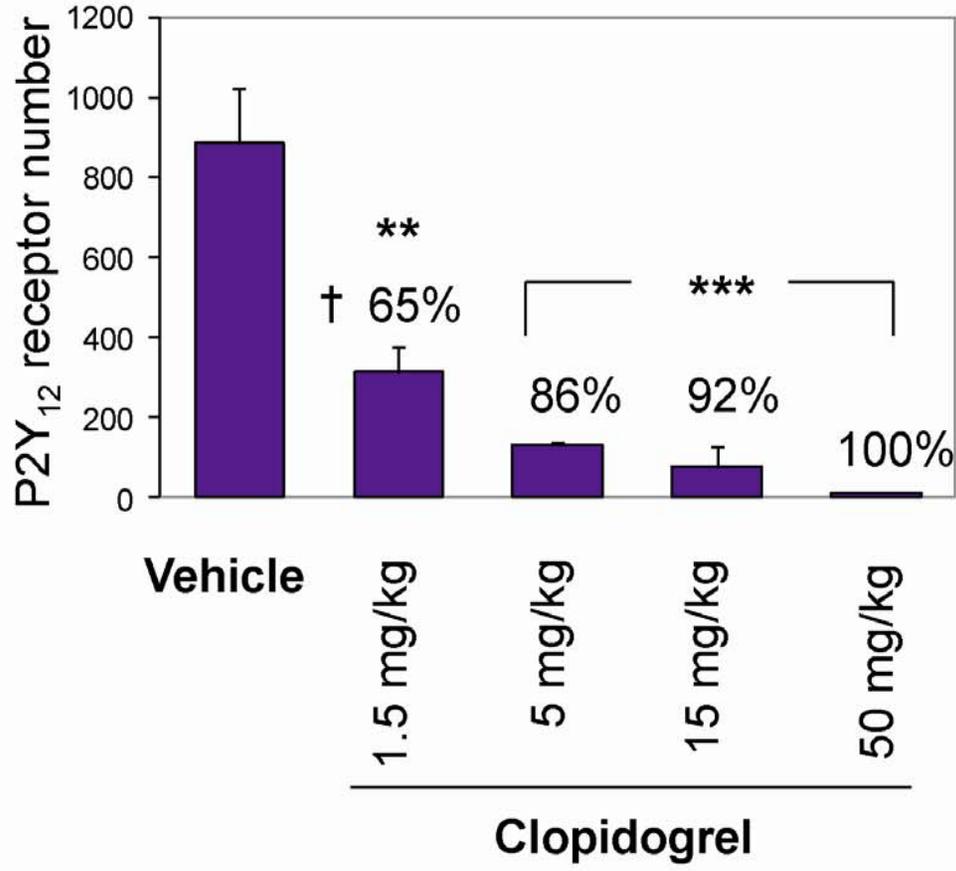


Figure 2:

A)



B)

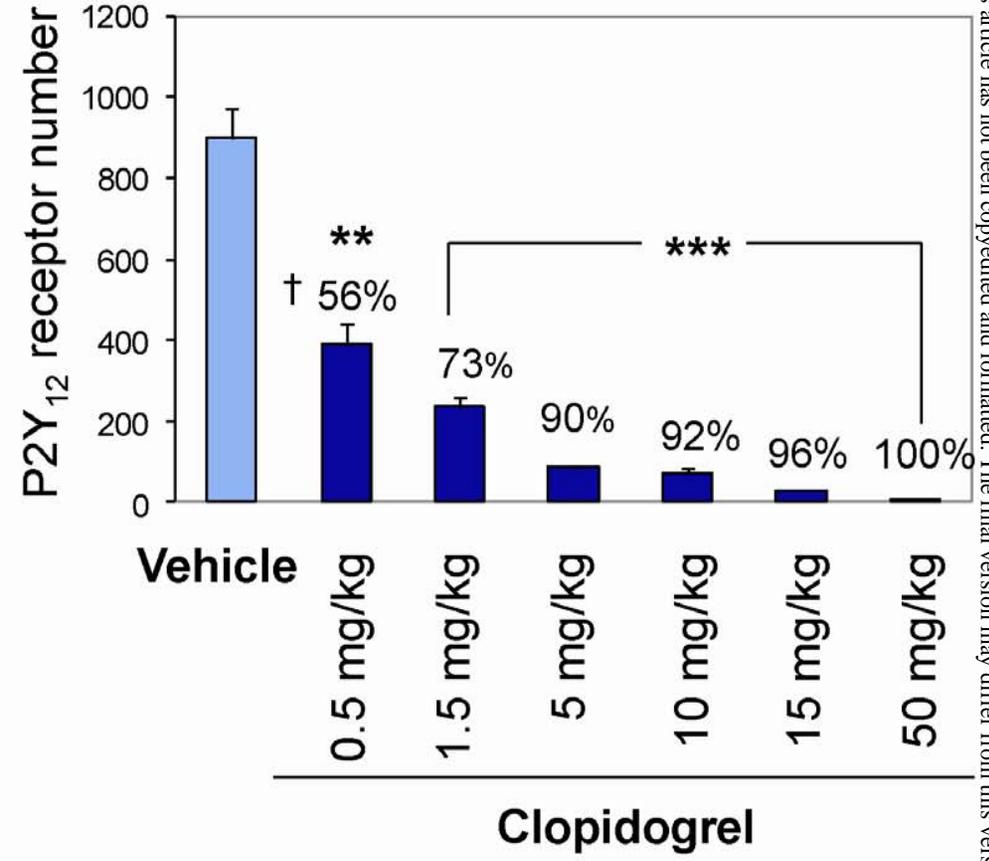


Figure 3:

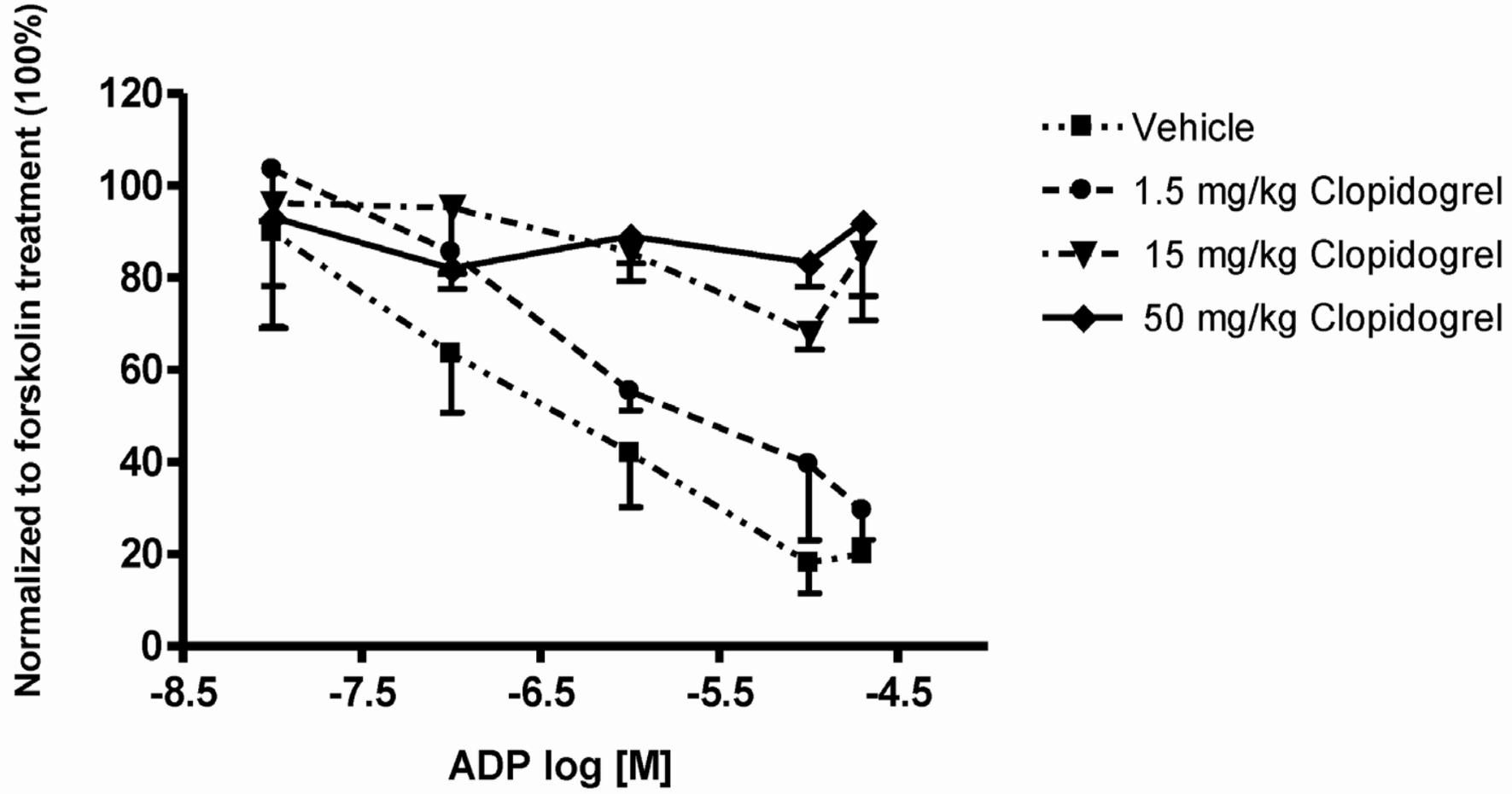


Figure 4:

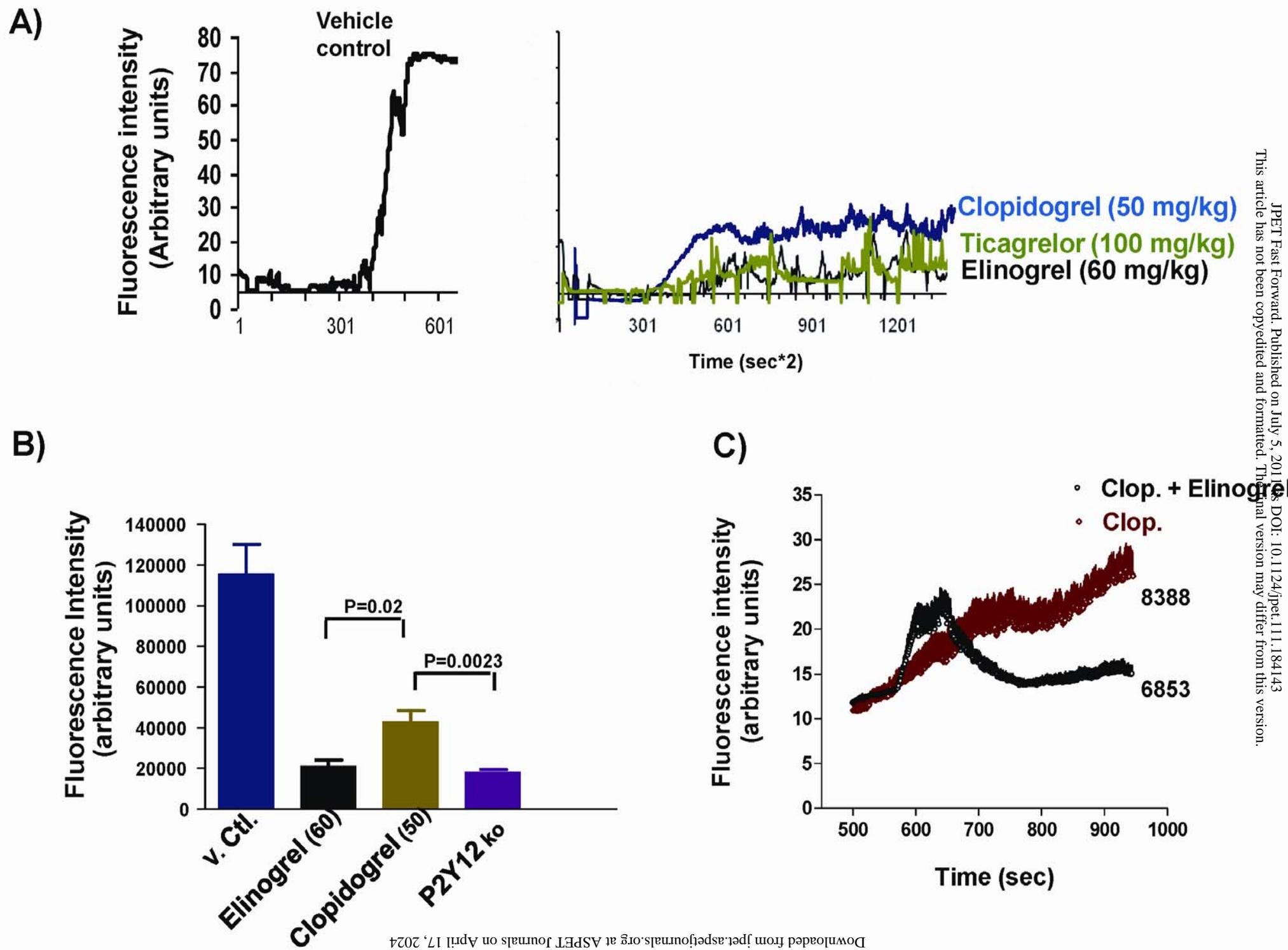


Figure 5:

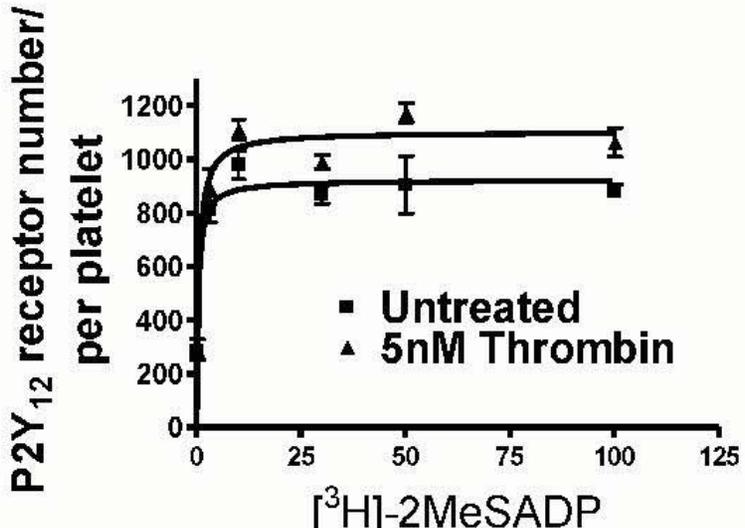
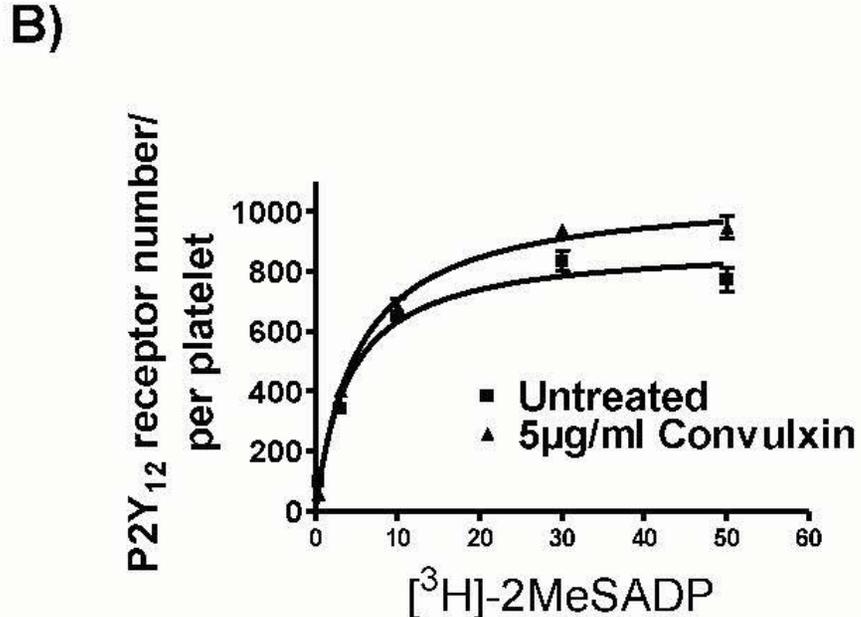
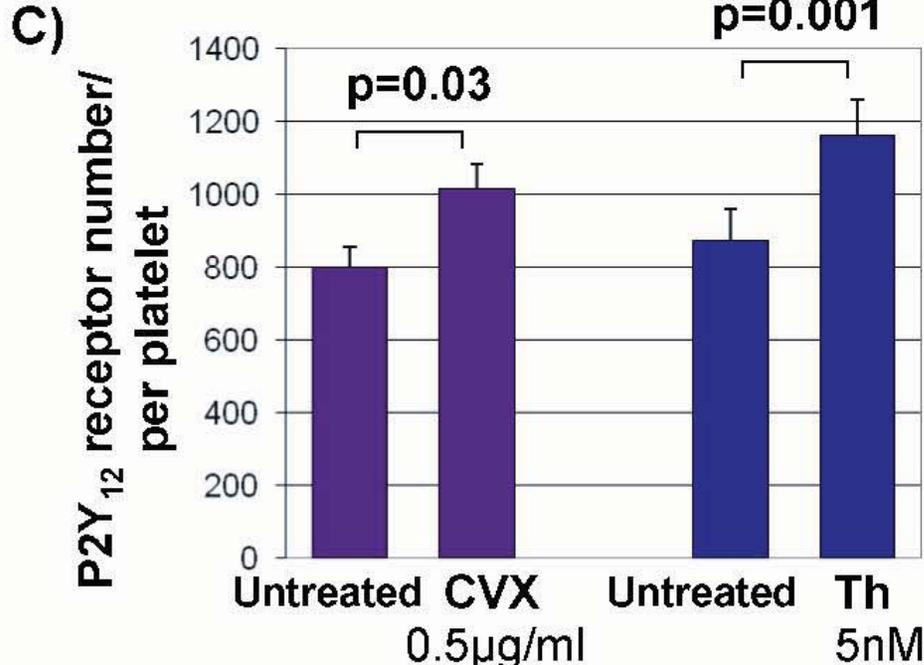
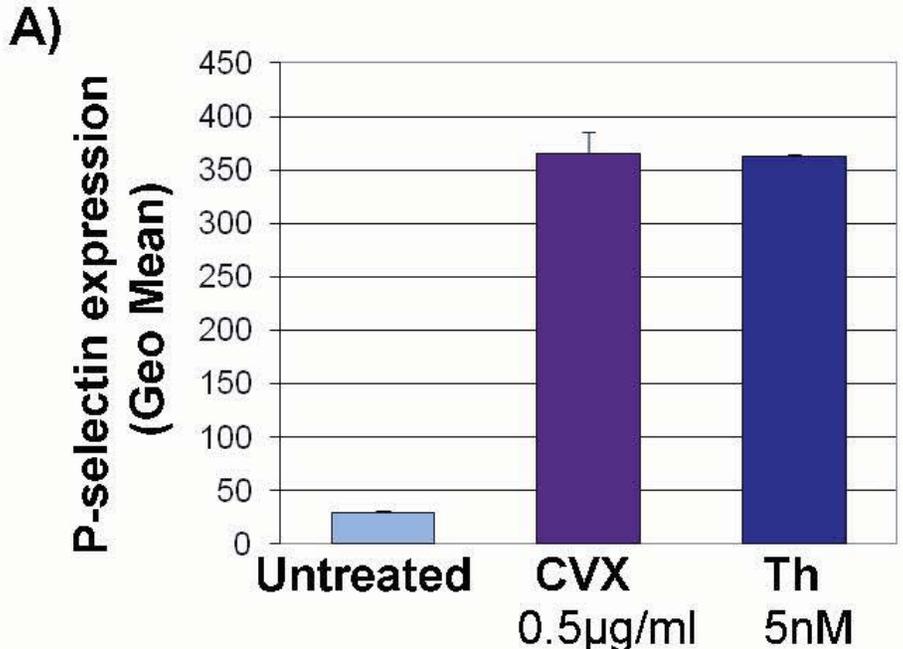


Figure 6:

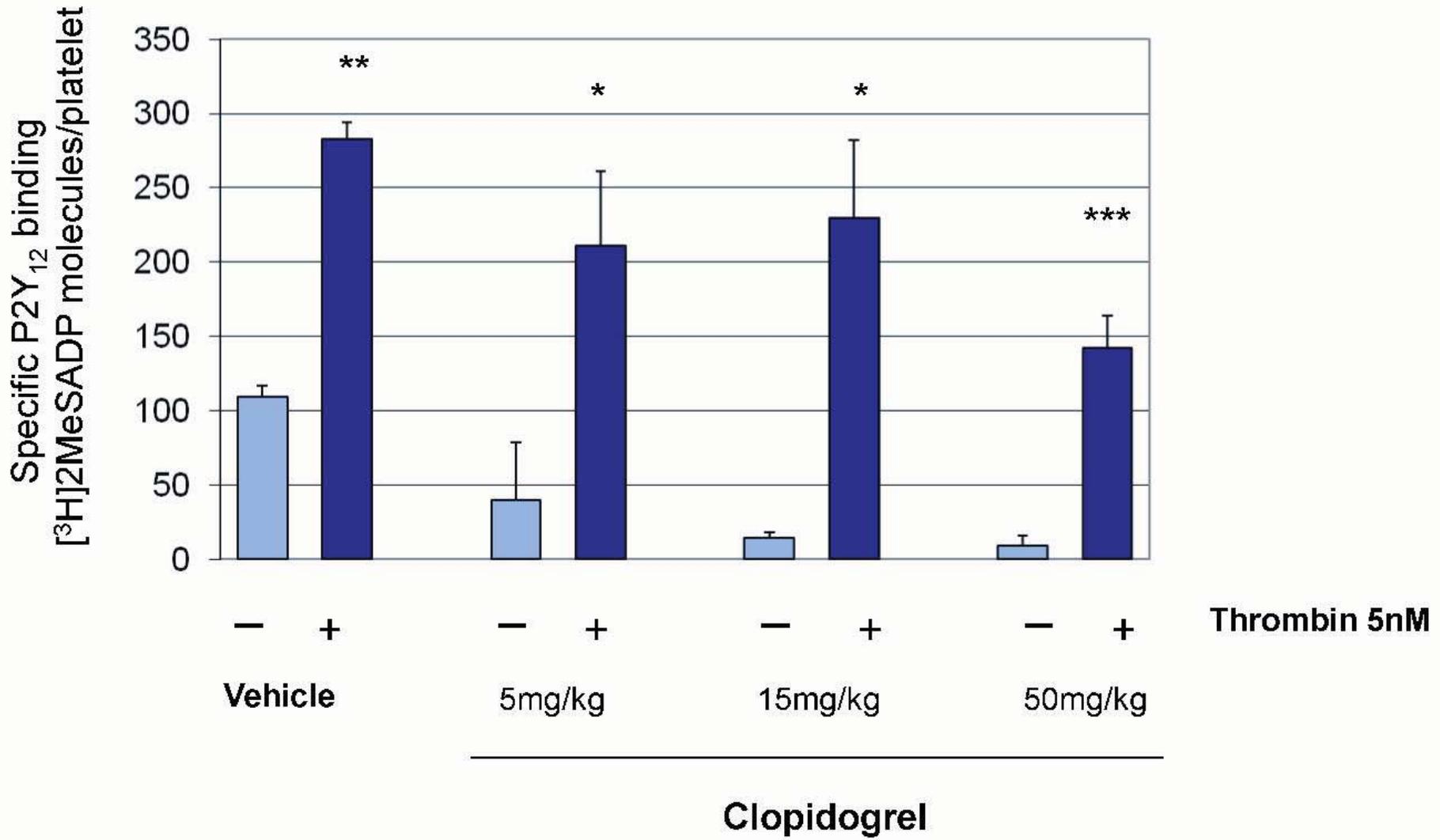
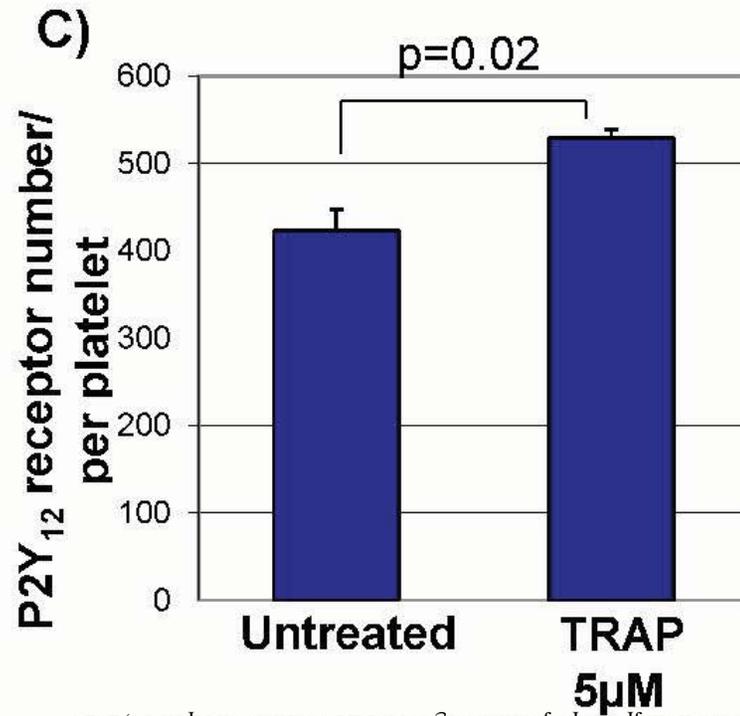
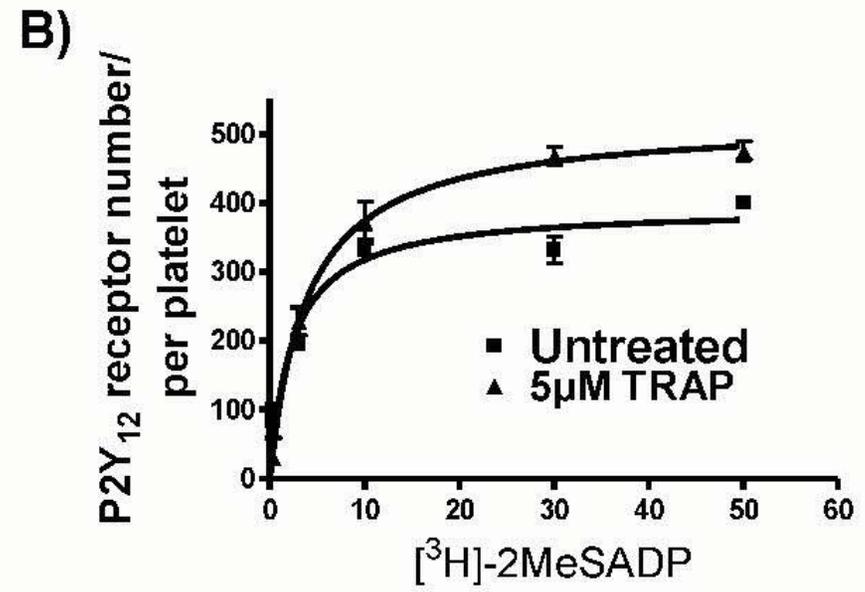
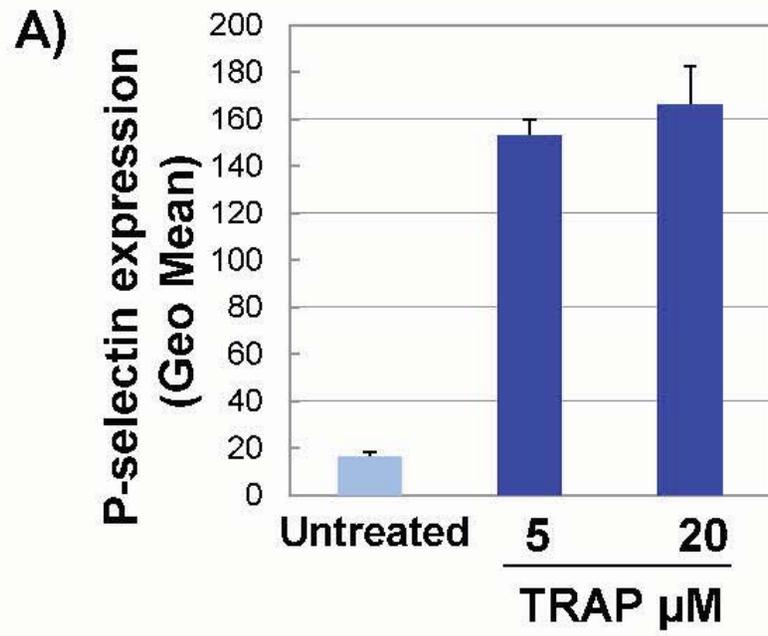


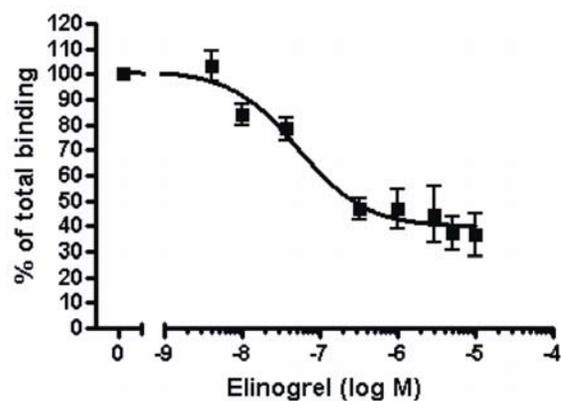
Figure 7:



A clopidogrel-insensitive inducible pool of P2Y₁₂ receptors contributes to thrombus formation: Inhibition by elinogrel, a direct –acting reversible P2Y₁₂ antagonist

Helena Haberstock-Debic, Patrick Andre, Scott Mills, David R. Phillips, Pamela B. Conley

Journal of Pharmacology and Experimental Therapeutics



EC50 | 5.208e-008

Supplemental Figure 1

Competitive binding experiments on mouse washed platelets. Data are expressed as % of total binding. Elinogrel dose-dependently inhibited 10 nM [³H]2MeSADP binding to platelets P2Y₁₂ receptors with the IC₅₀ value of 52nM. Data are expressed as mean ± sem; n=2.