Title page

Prasugrel metabolites inhibit neutrophil functions

Elisabetta Liverani, Mario C. Rico, Analia E. Garcia, Laurie E. Kilpatrick and Satya P. Kunapuli

Running title page

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Corresponding Author: Elisabetta Liverani, Ph.D.
Sol Sherry Thrombosis Research Center
Temple University
MRB, 3420 N. Broad Street
Philadelphia, Pennsylvania 19140 USA
Phone: 215-707-4407
Fax: 215-707-4003
Email: eliliverani@temple.edu

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Abbreviations:
ANOVA, analysis of variance; APC, allophycocyanin; BSA, Bovine Serum Albumin; ERK, extracellular-signal-regulated kinase; FITC, Fluorescein isothiocyanate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GMFI, Geometric mean of fluorescence intensity; HBSS, Hank’s balanced salt solution; i.p., intraperitoneal; LPS, Lipopolysaccharide; LTE_4, Leukotriene E_4; MPO, Myeloperoxidase peroxidation; PAF, platelet activation factor; PBS, Phosphate buffered saline; PE, Phyco-erythrin; PRP, Platelet-rich plasma; NADPH, nicotinamide adenine dinucleotide phosphate.
Abstract

Clopidogrel and prasugrel belong to a thienopyridine class of oral anti-platelet drugs that, after having been metabolized in the liver, can inhibit platelet function by irreversibly antagonizing the P2Y_{12} receptor. Furthermore, thienopyridines influence numerous inflammatory conditions but their effects on neutrophils have not been evaluated, despite the important role of these cells in inflammation. Therefore, we investigated the effect of prasugrel metabolites on neutrophils to further clarify the role of thienopyridines in inflammation. Interestingly, a prasugrel metabolite mixture, produced \textit{in vitro} using rat liver microsomes, significantly inhibited fMLP- and PAF-induced neutrophil activation. More specifically, prasugrel metabolites inhibited neutrophil transmigration, CD16 surface expression, and neutrophil-platelet aggregation. Moreover, prasugrel metabolite pre-treatment also significantly decreased fMLP- or PAF-induced ERK phosphorylation as well as calcium mobilization. To determine the target of prasugrel in neutrophils, the role of both P2Y_{12} and P2Y_{13} receptors was studied using specific reversible antagonists, AR-C69931MX and MRS2211, respectively. Neither antagonist had any direct effect on the agonist-induced neutrophil functional responses. Our findings indicate that prasugrel metabolites may directly target neutrophil and inhibit their activation, suggesting a possible explanation for their anti-inflammatory effects previously observed. However, these metabolites do not act through either the P2Y_{12} or P2Y_{13} receptor in neutrophils.
Introduction

ADP-induced platelet activation is mediated by both P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors (Kahner et al., 2006; Murugappa and Kunapuli, 2006; Bhavaraju et al., 2010; Kim and Kunapuli, 2011), although the P2Y\textsubscript{12} receptor also plays a crucial role when platelets are activated by other agonists. Anti-thrombotic drugs targeting the P2Y\textsubscript{12} receptor, such as clopidogrel and prasugrel, have been successfully used to prevent thrombotic events (Bhavaraju et al., 2010; Kim and Kunapuli, 2011). Previous studies have shown that Gi signaling, mediated by the P2Y\textsubscript{12} receptor, is dependent on the cholesterol rich lipid rafts (Quinton et al., 2005) and that high fat diet enhances platelet activation induced by other agonists (Nagy et al., 2011). In addition to chronic hypercholesterolemia, other pathologic conditions ranging from diabetes (Morel et al., 2012) to hypertension may increase P2Y\textsubscript{12} receptor functions, and hence the risk of thrombosis. Moreover, polymorphisms in the P2Y\textsubscript{12} gene which enhance receptor activity have been associated with increased risk of thrombotic events (Bura et al., 2006).

Despite their well-known functions in hemostasis, platelets also play a role in the immune response and inflammation (Semple and Freedman, 2010; Semple et al., 2011). They store a number of chemokines, growth factors, and angiogenic factors that, upon stimulation, are released from their granules, activating other blood cells and the endothelium. Platelet granule release is potentiated by P2Y\textsubscript{12} receptor stimulation (Dangelmaier et al., 2001; Garcia et al., 2010). The P2Y\textsubscript{12} receptor gene variants correlate with pulmonary inflammation and asthma (Bunyavanich et al., 2012), and receptor deficiency abrogated dust mite-induced
airway inflammation (Paruchuri et al., 2009), suggesting an important role for P2Y12 receptor in pulmonary inflammation. Treatment with the P2Y12 receptor antagonist clopidogrel significantly attenuated Lipopolysaccharide (LPS)-induced inflammatory responses and lung injury (Hagiwara et al., 2011). In addition, this drug also alleviated the severity of cardiac inflammation and post myocardial infarction in a mouse myocardial infarction model (Liu et al., 2011). In contrast, two recent studies suggested that clopidogrel enhances inflammatory responses in rodent models of rheumatoid arthritis (Boilard et al., 2010; Garcia et al., 2011). The reasons for the pro-inflammatory effects of clopidogrel in this animal model compared to others is not known, although the use of exposed collagen in generating rheumatoid arthritis may activate unique inflammatory pathways which are regulated differently than those observed in other models of inflammation (Diehl et al., 2010; Hagiwara et al., 2011; Liu et al., 2011). Interestingly, previous studies suggest that the P2Y12 receptor may be expressed in other cells types than platelets, such as lymphocytes (Wang et al., 2004; Diehl et al., 2010), monocytes (Wang et al., 2004) and dendritic cells (Ben Addi et al., 2010) indicating that P2Y12 antagonists might also target the non-platelet P2Y12 receptor.

Despite the well-known role of neutrophils during the early stages of inflammation (Wagner and Roth, 1999) and their direct interaction with platelets (Weiss et al., 1998), the effect of P2Y12 antagonism on neutrophil activation has not been addressed. Therefore, we investigated whether P2Y12 is expressed in neutrophils and whether a P2Y12 receptor antagonist, prasugrel, could target this cell and alter
their functions. Since prasugrel is a pro-drug that needs to be metabolized in the liver to elicit its effects on the P2Y\textsubscript{12} receptor (Kim and Kunapuli, 2011), to perform an \textit{in vitro} study we used rat liver microsomes to generate the active metabolite of prasugrel (Cavin et al., 2001; Brandon et al., 2003). We also investigated possible \textit{in vivo} effects of this drug in a LPS-induced model of systemic inflammation. Our results demonstrate that prasugrel metabolites inhibit neutrophil functional responses and these effects are not mediated through P2Y\textsubscript{12} or P2Y\textsubscript{13} receptors, suggesting off-target effects of this drug on neutrophils.

**Materials and methods**

**Materials**

All reagents, analytical grade, were obtained from Thermo Fisher Scientific (Waltham, MA) unless stated otherwise. AR-C69931MX was a gift from AstraZeneca (Wilmington, DE), while MRS2211 was purchased from Tocris Bioscience (Minneapolis, MN). Prasugrel hydrochloride (Effient\textsuperscript{R} 10mg tablets) was obtained from the Internal Pharmacy of Temple University (Philadelphia, PA). Rat liver microsomes were from Sigma (St. Louis, MO). Ficoll-Paque was purchased from GE healthcare Bio-science AB (Uppsala, SE); Phosphate buffered saline (PBS) and Hank’s balanced salt solution (HBSS) was purchased from Mediatech Inc, Manassa VA and platelet activation factor (PAF) from Calbiochem Corp. (San Diego, CA); N-formyl-methionyl-leucyl-phenylalanine (fMLP) was obtained from Sigma-Aldrich (St. Louis, MO) and Leukotriene E\textsubscript{4}
(LTE₄) from Cayman Chemical (Michigan, USA). Antibodies against human CD41 (Phycocerythrin (PE)-conjugated; clone HIP8), human CD16 (allophycocyanin (APC)-conjugated; clone eBioCD16) and mouse CD11b (PE-conjugated; clone M1/70) were obtained from eBioscience (San Diego, CA); phospho-extracellular-signal-regulated kinases (ERK)1/2 (Thr202/Tyr204) and total ERK2 antibodies were purchased from Cell Signaling Technology (Danvers, MA); DyLight™ 800-conjugated goat anti-rabbit IgG and DyLight™ 680-conjugated goat anti-mouse IgG were obtained from Thermo Scientific (Waltham, MA). Mouse-CD41 (Fluorescein isothiocyanate (FITC)-conjugated; clone MWReg30) antibody and BD FACSTM lysing solution were obtained from BD Pharmingen™ (Franklin Lakes, NJ, USA Nitrocellulose membranes were purchased from Whatman Protran® (Dassel, Germany), while Odyssey blocking buffer was from LI-COR Bioscience (Lincoln, NE) and fura-2 from Invitrogen (Grand Island, NY).

Human platelet isolation

Human blood was obtained from healthy adult donors, following informed consent, in accordance with Institutional Review Board protocols at Temple University School of Medicine. Total blood was diluted with one-sixth volume of acid-citrate-dextrose (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of deionized water). Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 230×g for 20 min at room temperature. The PRP obtained was then centrifuged at 980×g for 10 min at room temperature and the platelet pellet
resuspended in Tyrode's buffer (138mM NaCl, 2.7mM KCl, 2mM MgCl₂, 0.42mM Na₂HPO₄, 5mM glucose, 10mM HEPES, pH 7.4) containing 0.2 units/ml apyrase. Cells were counted using the Hemavet® Multispecies Hematology System (Drew Scientific, Inc. Oxford, CT)

**Human neutrophil isolation**

After removing PRP to isolate platelets, the original volume was restored by adding HBSS. Cells were then incubated in 6% Dextran in 0.9% NaCl for 40 minutes at 25°C. The leukocyte-rich plasma was collected and centrifuged for 5 minutes at room temperature at 350 g. The pellet containing cells was re-suspended in 3 ml of HBSS/0.2% Bovine Serum Albumin (BSA), cell suspension was added to 3 ml of Ficoll-Paque and centrifuged at 300 g for 30 minutes at 25°C. Neutrophils were collected at the bottom of the tube. Red blood cells lysis was achieved by adding 7.5 ml of ice-cold 0.2% NaCl for 90 seconds, followed by 7.5 ml of 1.6% NaCl. Cells were re-suspended in HBSS and counted using the Hemavet® System. Only samples with a cell purity of 95% or higher were used for the experiments.

**Platelet aggregation**

Agonist-induced platelet aggregation was analyzed using a Chrono-Log model 440-VS aggregometer (Havertown, PA) with sample volumes of 0.5 ml in a cuvette holder, equipped with a thermostat at 37°C and set at constant stirring (900 rpm). Aggregometer output was recorded using a Kipp & Zonen type BD
12E flatbed chart recorder (Kipp&Zoen, Bohemia, NY) set at 0.2 mm/s. Platelets at 2 \times 10^8 \text{ cells/ml} \text{ concentration were pre-incubated with AR-C69931MX (100 nM and 1\mu M), MRS2211 (10-50 \mu M) or prasugrel metabolites (2,3 and 5 \mu M) for 2 minutes when appropriate and aggregation was induced by addition of 2MeSADP (100 nM).

**In vitro preparation of prasugrel metabolite mixture**

Pooled male rat liver microsomes were adjusted to 2 mg of protein/ml in 100 nM potassium phosphate buffer, pH 7.4 and incubated with 2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 5 mM glutathione and 0.1 mM prasugrel dissolved in methanol (pro-drug solution). As a control, the same concentration of prasugrel vehicle (methanol) was incubated with rat microsomes, NADPH and glutathione in potassium buffer (all at the same concentration as prasugrel solution). Following incubation for 1 hour at 37\degree C under continuous stirring at 120 rpm and light protection, the solutions were aliquoted and stored at -20\degree C (Brandon et al., 2003).

**Cell viability**

Neutrophils (10^6 \text{ cells/tube in HBSS}) were pre-incubated with prasugrel metabolites at different concentrations (5, 10 and 15 \mu M) or negative control for 2 minutes at 37\degree C, in order to study cell viability after exposure to the mixture generated. Triton-X (0.1% in PBS) was added as a positive control to permeabilize cell membrane. Cells were then incubated with propidium iodide (final concentration of 2 \mu g/ml) on ice for 5 minutes and analyzed on flow
cytometry using a FACSCalibur analyzer (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR).

**Chemotaxis assay**

Chemotaxis was performed using a 96-well Chemotaxis chamber (ChemoTx, Neuroprobe, Gaithersburg, MD) as previously described (Frevert et al., 1998). Briefly, the chemo-attractants fMLP (10 μM), PAF (10 nM) or LTE4 (1 μM), were added to the well in the microplate (bottom chamber), while cells (1 x 10^5 cells/well) were seeded on the filter sites (top chamber). The chamber was then incubated for 1 hour at 37°C, 5% CO₂. Cells were then removed from the top of the membrane and each well washed with HBSS to remove remaining cells. Plate and membrane were then centrifuged for 1 minute at 312 g and cell pellet re-suspended. Neutrophils that have migrated to the bottom chamber were quantified by diluting in Turk’s dye and cells were counted as previously described (Cooper et al., 2010).

**Platelet-neutrophil aggregate formation**

We studied the formation of platelet-leukocyte aggregates by pre-incubating neutrophils (1 x 10^6 cells/tube) and platelets (1 x 10^6 cells/tube) with antibodies (diluted 1:50) against human CD41 (PE-conjugated) and CD16 (APC-conjugated) and either prasugrel or control for 2 minutes at 25° C. Aggregate formation was then induced by adding either fMLP (10 μM), 2MeSADP (100 nM) or PAF (10 nM) and incubated for further 20 minutes at 25° C. The reaction was
stopped by adding 2% paraformaldehyde solution in PBS and samples were kept at 4°C until analysis (Rinder et al., 1991; Kohler et al., 2012). In some cases, prasugrel was added to either the platelet or the neutrophils preparation separately. To this end, both PRP and neutrophils were pretreated with either prasugrel (5 µM) or control for 5 min at 25°C. Following a washing step, platelets and neutrophils were re-suspended in HEPES buffer (NaCl 150 mM, KCl 5 mM, MgSO₄ 1 mM, HEPES 10 mM) and added to tubes in equal volume. After addition of PE-conjugated anti-human CD41 and APC-conjugated anti-human CD16, samples were stimulated with either fMLP (10 µM), PAF (10 nM) or LTE₄ (1 µM) and incubated for 20 minutes at 25°C then fixed using 2% paraformaldehyde and stored at 4°C prior to analysis. Flow cytometry was performed using a FACSCalibur analyzer and data were analyzed with FlowJo software. Platelets and neutrophils were discriminated by forward and side light scatter and identified by their positive staining for PE-CD41 or APC-CD16, respectively. Events double positive for APC and PE identified platelet–neutrophil aggregates and were recorded as a percentage of a total of 10,000 gated neutrophils (Supplemental figure 1).

**Western blotting**

Neutrophils (1 x 10⁷ cells/sample) were pre-incubated with 5 µM of Prasugrel for 2 minutes at 37°C, prior to stimulation with 10 µM fMLP or 10 nM PAF for 30 minutes at 37°C. The cells were centrifuged for 5 minutes at 10,000 g at 4°C and lysed with lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA,
1 mM EDTA and 0.2% NP-40) by vortexing them for 20 minutes at 4 °C and microfuged for 5 minutes at 4 °C at 10,000 g. Sample buffer was added to supernatant and samples were boiled for 5 minutes. Cell lysate was electrophoresed on 12% SDS-PAGE gels. Proteins were electrophoretically transferred to nitrocellulose membrane at 100V for 1 hour. After completion of transfer, the membrane was incubated for 1 hour at room temperature with Odyssey blocking buffer. To determine ERK1/2 phosphorylation, the membrane was incubated simultaneously with phospho-ERK1/2 (Thr202/Tyr204) and total ERK2 antibodies (dilutions 1:1000 for both) in Odyssey blocking buffer with gentle agitation overnight at 4 °C. Membrane was then washed before addition of secondary antibody conjugated to a fluorescent entity: DyLight™ 800-conjugated goat anti-rabbit IgG and DyLight™ 680-conjugated goat anti-mouse IgG (dilution 1:6,000) in Odyssey blocking buffer with gentle agitation for 1 hour at room temperature. At the end of the incubation period, the membrane was washed, then dried, visualized, and analyzed on the Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE).

**Intracellular calcium release**

Neutrophils (6 x 10^6 cells/ml) were incubated with 1 μM fura-2 for 45 minutes at 37° C. Cells were then washed and resuspended in HBSS. Changes in fluorescence were measured using an Aminco-Bowman Series 2 luminescence spectrometer with a water-jacketed cuvette holder, set at a 37° C with constant stirring. Samples were analyzed using an excitation wavelength of 340 nm and
an emission wavelength of 510 nm. After pre-incubation with either the negative control or prasugrel metabolites (2 min – 5 μM), calcium release was induced by addition of fMLP (10 μM) or PAF (10 nM). Fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz et al. (Grynkiewicz et al., 1985) where $F_{\text{min}}$ and $F_{\text{max}}$ were determined with each respective platelet preparation.

**Animals and treatments**

Eight pathogen-free C57BL/6 male mice (weight, 25-30 g) were obtained from Taconic (Rockville, MA). The animals had unlimited access to food and water before treatment and were randomly assigned to one of the four groups considered (2 animals per group): untreated, only prasugrel-treated, LPS-treated and Prasugrel- and LPS-treated mice. Prasugrel was orally administrated at a loading dose of 10mg/kg and a maintenance dose of 3mg/kg. LPS-treated animals received an intra-peritoneal (i.p.) dose of LPS (5mg/kg). After 24 hours, mice were anesthetized, and blood samples were collected by cardiac puncture (10:1 ratio of blood in 3.8% sodium citrate) for hematology studies that were performed using the Hemavet® Multispecies Hematology System. The experimental protocol followed in this study was fully approved by The Institutional Animal Care and Use Committee of Temple University School of Medicine.

**Platelet/neutrophil aggregate formation and CD11b expression**
Murine blood samples were incubated with FITC-conjugated anti-mouse CD11b and PE-conjugated anti-mouse CD41 or only anti-mouse CD11b for 10 minutes. Then BD FACS™ lysing solution was added to fix samples and lysate red blood cells. Samples were kept at 4 °C up to analysis. Flow cytometry was performed on a FACSCalibur analyzer and data were analyzed with FlowJo software.

**Lung Histopathology and Myeloperoxidase peroxidation (MPO)**

Lungs were fixed in 10% formalin and embedded into paraffin blocks. Sections (5 µm) were cut on a microtome and stained with hematoxylin and eosin (H&E). Slides were analysed and compared based on cell infiltration. Part of the lungs were also homogenized and sonicated in cold PBS (pH 7.4). After centrifugation at 10,000 for 10 minutes at 4 °C, MPO levels were detected using a MPO assay kit (Cayman, USA).

**Statistical analysis**

Experiments were conducted in duplicate or triplicate and repeated at least 3 times. Data are reported as mean ± SEM. Statistical differences were analyzed by analysis of variance (ANOVA) followed by Dunnett’s test or by Student’s t test, as appropriate. A p value less than 0.05 was taken as significant.
Results

Prasugrel metabolites generated in vitro abolish platelet aggregation but do not alter neutrophil viability

In order to evaluate the efficacy of the metabolite mixture generated, we tested it on the platelet P2Y<sub>12</sub> receptor, by pre-incubating platelets with the metabolite mixture and then stimulating them with 2MeSADP (100 nM). Rat liver microsomes, enzymes and methanol, without prasugrel were added as negative control. As shown in Figure 1A, 2-MeSADP-induced aggregation was dramatically reduced following the treatment with the metabolite mixture in a concentration-dependent manner. An approximate concentration of 5 μM (based on the concentration of prasugrel originally added to a certain volume of microsomes) was able to abolish aggregation, but not shape change, as expected (negative control: 100% aggregation, prasugrel metabolites 2 μM: 7 ± 2%; prasugrel metabolites 3 μM: 4 ± 1%; prasugrel metabolites 5 μM: 0%). Hence, 5 μM prasugrel metabolite mixture was used for the experiments with neutrophils.

The effect of these metabolites on neutrophil viability was determined using propidium iodide exclusion as an indicator of membrane integrity. Neutrophils were incubated with varying concentrations of metabolites (5, 10 and 15 μM) and compared with the negative control In Figure 1B representative images of flow cytometry histograms indicate that when neutrophils were permeabilized, geometric mean of fluorescence intensity (GMFI) was 852 ± 20, compared with
25 ± 3 values for negative control cells. No difference was noted between negative control values and prasugrel metabolite pre-treated cells at all the concentrations analyzed (GMFI: 21 ± 7 for 5μM; 29 ± 9 for 10 μM and 32 ± 4 for 15μM), indicating that prasugrel metabolites were not toxic for neutrophils.

**Prasugrel metabolites significantly inhibited human neutrophil transmigration and CD16 surface expression**

We analyzed the effect of these compounds on neutrophil chemotaxis in response to the chemo-attractants fMLP (10μM) or PAF (10nM) for 1 hour as described in Methods. As expected, both fMLP and PAF caused significant transmigration of neutrophils (Fig. 2A). When the negative control mixture was incubated with the cells in the top chamber, transmigration in response to either fMLP or PAF was not affected. In contrast, neutrophils incubated with the prasugrel metabolite mixture had significantly decreased chemotaxis in response to either fMLP or PAF (P<0.05, Fig. 2A). Interestingly, when prasugrel mixture was added with the chemo-attractant (in the bottom chamber) no difference in cell migration was observed: (number of cells transmigrated with fMLP alone: 3.9 ± 0.4 x 10^4; negative control: 4.9 ± 0.3 x 10^4; fMLP plus prasugrel metabolites: 5.4 ± 0.3 x 10^4). These data suggest that neutrophils need to be pre-incubated with the drug before activation, in order to be affected by prasugrel mixture. Figure 2B shows flow cytometry data investigating fMLP-induced CD16 surface expression, as an indication of neutrophil stimulation. A significant increase in CD16 surface expression was noted following fMLP
exposure compared to unstimulated cells, as expected. Interestingly, a significant decrease \((p \leq 0.01)\) in geometric mean of fluorescence intensity (GMFI) was observed following pre-incubation (5 \(\mu\)M - 2 min) with prasugrel metabolite mixture versus negative control treated samples, suggesting that prasugrel metabolites could also affect CD16 surface expression. These data clearly show that prasugrel metabolites affect neutrophil stimulation.

**Prasugrel metabolite pre-incubation decreases ERK-phosphorylation in agonist-induced neutrophil activation**

In order to evaluate the mechanism of action of prasugrel metabolites on neutrophil activation, we investigated whether intracellular signaling events were also altered. When cells were pre-incubated with prasugrel metabolites (5 \(\mu\)M - 2 min), fMLP (10 \(\mu\)M)- induced ERK-phosphorylation was diminished (Fig. 3A). Quantitation of the results of Fig. 3A shows a significant decrease \((p \leq 0.01)\) of phosphorylated ERK when samples were pre-incubated with prasugrel metabolites (Fig. 3B). Furthermore, when cells were activated with PAF (10 nM), prasugrel metabolites also decreased PAF-induced ERK phosphorylation \((p \leq 0.05)\) (Fig. 3C & D), suggesting that the effects of prasugrel metabolites on neutrophil signaling are agonist-independent.

**Prasugrel metabolites inhibit agonist-induced calcium mobilization**

Since both fMLP and PAF cause increases in intracellular calcium in neutrophils, we analyzed the effects of pre-treatment with prasugrel metabolites
(5 μM – 2 min) on intracellular calcium levels following stimulation (Fig. 4). Representative curves in Figure 4 show a significant decrease in both fMLP- (Fig. 4A) and PAF- (Fig. 4B) induced calcium mobilization, suggesting that prasugrel effects on these cells are not agonist specific.

**Prasugrel metabolites reduce neutrophil/platelet aggregate formation**

We further investigated the effect of prasugrel mixture on neutrophil-platelet interaction, by investigating neutrophil-platelet aggregate formation by flow cytometry. Neutrophils were gated based on CD16 expression and cell shape (Supplemental Figure 1). Data were analyzed as a percentage of aggregates expressing both platelet and neutrophil receptor markers (CD41 and CD16 respectively). The percentage of aggregates for unstimulated neutrophil-platelets was 10.3 ± 3. The addition of fMLP or PAF resulted in increased percentage of aggregate formation (57 ± 5 and 37 ± 5 for fMLP- and PAF-activated cells respectively). A significant decrease ($p \leq 0.01$) in neutrophil-platelet aggregates was observed in samples pre-incubated with prasugrel metabolites (2 minutes - 5μM) for both fMLP (10 μM) and PAF (10 nM) stimulated samples (Fig. 5A). There was a reduction from 100% (aggregates when cells were stimulated with agonist upon pre-incubation with negative control) to 25 ± 4% (fMLP) and 22 ± 6% (PAF). In order to ascertain whether neutrophils are a direct target for these metabolites or the effects observed are platelet-mediated, both cell types were incubated separately with the metabolite mixture prior to fMLP activation (Fig. 5B). As these metabolites affect platelet
functions (Scott et al., 2009), it was not surprising to observe a decrease in aggregate formation when the metabolite mixture was pre-incubated with platelets alone (Fig. 5B). However, a significant decrease \((p \leq 0.05)\) in aggregate formation was noted (from 100% to 56 ± 1%) when only neutrophils were pre-incubated with the metabolites, suggesting a direct effect of the prasugrel metabolites on this cell type. Considering that CD16 expression was diminished after prasugrel metabolite exposure, we also investigated another neutrophil marker, CD11a, which was not altered by either stimulation or prasugrel metabolite exposure (Supplemental Figure 2A and B). Data show that, when CD11a was used as neutrophil marker, the percentage of platelet/neutrophil aggregate formation was significantly inhibited (Supplemental Figure 2C).

**LTE\(_4\)**-induced neutrophil activation is also inhibited by prasugrel metabolites.

Previous studies have shown a decrease in LTE\(_4\)-induced inflammation in P2Y\(_{12}\) knock out mice (Paruchuri et al., 2009). In order to determine whether LTE\(_4\) signaling in neutrophils is also altered in response to prasugrel metabolites, we investigated the effect of prasugrel mixture pre-treatment (5\(\mu\)M – 2 minutes) (Fig. 6) when neutrophils were activated with LTE\(_4\) (1 \(\mu\)M). Similarly to the results obtained with other stimuli, cell transmigration and CD16 surface expression were significantly inhibited \((p \leq 0.05)\) by these metabolites compared with negative control (Fig. 6 A and B). Also platelet and neutrophil interaction
was decreased ($p \leq 0.05$) (Fig. 6 C).

**The pro-drug form of prasugrel does not inhibit neutrophil functions**

To determine whether the observed inhibition of neutrophil activation was the result of un-metabolized forms of prasugrel, we pre-incubated the neutrophils with prasugrel pro-drug in methanol (5 μM) (Fig. 7), then we stimulated the cells with either fMLP (10 μM) or PAF (10nM). The negative control received the same amount of methanol. No changes were observed in CD16 expression (Fig. 7A) and cell transmigration (Fig. 7B), when neutrophils were pre-exposed to the pro-drug compared with the control. Also interaction with platelets was not affected, as the amount of aggregated formed was not significantly different from the control (Fig. 7C). These data suggest that the pro-drug form of prasugrel requires further metabolization in order to affect neutrophil functions.

**AR-C69931MX, a reversible P2Y$_{12}$ antagonist, did not inhibit fMLP-induced neutrophil transmigration, CD16 expression and neutrophil/platelet interaction**

The target of the active metabolite of prasugrel, the P2Y$_{12}$ receptor, was predicted to be expressed in leukocytes (Diehl et al., 2010) and dendritic cells (Ben Addi et al., 2010). As our data indicate the possibility of a target for prasugrel metabolite on the neutrophil membrane, we determined whether the P2Y$_{12}$ receptor is expressed in neutrophils and if it is inhibited by the prasugrel metabolites. In order to test this possibility, we first analyzed P2Y$_{12}$ receptor
expression through Western blotting (Supplemental Figure 3) and neutrophils did not seem to express this protein. Secondly, we used a reversible P2Y\textsubscript{12} antagonist, AR-C69931MX (Kim and Kunapuli, 2011) which was first tested on platelets (Fig. 8A). This antagonist was able to abolish aggregation at both of the concentrations selected (100nM and 1\(\mu\)M). In contrast, when neutrophils were pre-treated with AR-C69931MX (100nM and 1\(\mu\)M), prior to stimulation with either fMLP (10 \(\mu\)M) or PAF (10 nM), there was no effect of the antagonist on chemotaxis or CD16 expression (Fig 8B and 8C). This data suggests that the P2Y\textsubscript{12} receptor is unlikely to be the target of prasugrel metabolites on neutrophils. Neutrophil and platelet interaction was also evaluated. Pretreatment of both neutrophils and platelets with AR-C69931MX (100 nM and 1 \(\mu\)M) prior fMLP or PAF stimulation did not have any significant effect on neutrophil-platelet aggregate formation (Fig. 8D). We also tested aggregate formation when only neutrophils were pre-incubated with AR-C69931MX (1\(\mu\)M) and found no difference in the % of fMLP-induced aggregate formation as compared to untreated control cells (48 \(\pm\) 8% and 57 \(\pm\) 5%, respectively). This antagonist did not influence neutrophil-platelet aggregate formation, further excluding the possibility of P2Y\textsubscript{12} expression on neutrophils as the target of prasugrel metabolites.

MRS2211, a reversible P2Y\textsubscript{13} antagonist, did not inhibit neutrophil functions

P2Y\textsubscript{13} receptor, another member of the P2Y family that shares similar pharmacological profile with P2Y\textsubscript{12}, might be the target of prasugrel metabolites.
on the neutrophil surface. Therefore, we investigated this possibility by using a reversible P2Y_{13} antagonist, MRS2211 (Marteau et al., 2003; von Kugelgen, 2006) that we first tested on platelet aggregation. Figure 9A shows that 2MeSADP-induced platelet aggregation was not significantly inhibited when the antagonist was used at a concentration of 10 \( \mu \)M, which has been shown effective in other cell types (Marteau et al., 2003). However, aggregation was significantly inhibited when samples were pre-incubated with higher concentrations of MRS2211 (25 \( \mu \)M & 50 \( \mu \)M). As platelets are not known to express the P2Y_{13} receptor, the effects on aggregation at higher concentrations of MRS2211 are probably due to non-specific effects on P2Y_{12} receptor. That considered, we investigated the effects of MRS2211 in a variety of neutrophil functional assays upon fMLP stimulation. No changes were observed in CD16 surface expression (Fig. 9B) and cell transmigration (Fig. 9C), in response to MRS2211 at either 25 or 50 \( \mu \)M, implying that the P2Y_{13} receptor is not likely the target of prasugrel metabolites in neutrophils. On the contrary, platelet-neutrophil aggregates were inhibited (\( p \leq 0.05 \)) when cells were pre-incubated with MRS2211 (Fig. 9D) at the highest concentration tested, probably due to its effect on platelets (shown in Figure 7A) rather than any direct effect on neutrophils. In order to exclude this possibility, aggregate formation was also investigated when only neutrophils were pre-treated with MRS2211 and then reconstituted with platelets. We noted that the % of fMLP-induced aggregates was 60 \( \pm \) 7 when neutrophils were pre-treated with MRS2211 (50\( \mu \)M) compared with untreated samples % of 57 \( \pm \) 5. These results confirmed that MRS2211 has no direct effects on neutrophils.
effects on neutrophil interaction with platelets and the aggregate inhibition observed in Figure 9D was platelet-mediated.

**Prasugrel treatments decreased the level of LPS-induced systemic inflammation**

Mice were pre-treated with prasugrel then challenged with an *i.p.* dose of LPS for 24 hours (Fig. 10). An expected, circulating neutrophils and lymphocytes increased in response to LPS treatment (5mg/kg) while no change was noted in red blood cells suggesting that prasugrel treatment did not cause any bleeding (Supplemental Figure 4). Neutrophil and platelet aggregate formation was investigated by flow cytometry. Neutrophils were gated, based on cell shape and CD11b expression. Percentage of aggregates positive to platelet (CD41) and neutrophil (CD11b) marker was analyzed and aggregate formation was found to be increased in samples from LPS-treated mice as compared to untreated animals (Fig. 10A and 10B). Interestingly, when LPS-treated animals were pre-treated with prasugrel, the circulating aggregates appeared to be less than when they did not receive the drug (Fig.10 A and B). However, aggregate levels were already decreased by the prasugrel alone, suggesting a protective role for this drug in platelet and neutrophil interaction. We also investigated CD11b expression in neutrophils during LPS-induced inflammation (Fig. 10C) through flow cytometry. The GMFI values indicate that CD11b expression was lower in LPS-challenged mice when pre-treated with prasugrel. No change was noted between untreated and prasugrel pre-exposed animals (Fig 10C).
Considering that neutrophil infiltration in the lungs is an early event during inflammation (Nathan, 2006), we studied lung histopathology (Fig. 10D) and myeloperoxidase (MPO) activity (Fig. 10E). Representative images indicate that cell infiltration is diminished in LPS-treated mice when pre-exposed to prasugrel, compared with the LPS-treated animals (Fig. 10D). This data is confirmed by the analysis of MPO activity (rfu/min/mg), which demonstrated a reduction in animals pretreated with prasugrel prior to administration of LPS (Fig. 10E).

Discussion

The thienopyridines class of anti-platelet drugs, such as clopidogrel or prasugrel, were designed to prevent platelet activation by antagonizing P2Y\textsubscript{12} receptor and thereby inhibiting ADP-induced platelet aggregation (Kim and Kunapuli, 2011). Recent studies show that the P2Y\textsubscript{12} receptor is not only expressed in platelets and brain, but also in other cell types, such as monocytes (Wang et al., 2004), dendritic cells (Ben Addi et al., 2010) and lymphocytes (Wang et al., 2004). As a result, these drugs could have wider effects on the immune system than expected. Recent studies have shown that these thienopyridines were able to affect inflammatory responses in a number of diseases (Paruchuri et al., 2009; Bhavaraju et al., 2010; Boilard et al., 2010; Garcia et al., 2011; Liu et al., 2011; Winning et al., 2011). However, despite the important role of neutrophils in inflammation (Nathan, 2006), these drugs have not been evaluated in neutrophil functions. Hence the aim of this study was to understand whether prasugrel metabolites could influence neutrophil activation.
Prasugrel is orally administrated as a pro-drug and requires processing in the liver to an active metabolite (also known as R-138727) that irreversibly inactivates the P2Y$_{12}$ receptor (Scott et al., 2009). However, this active metabolite is highly unstable and is not available commercially to be used in in vitro experiments. Hence, to study in vitro effect of these metabolites, we developed a system of rat liver microsomes and enzymes to generate a mixture of prasugrel metabolites and used them on ex vivo human platelets and neutrophils. We used rat liver microsomes as in the literature rat and human microsomes have shown to have similar functions in metabolizing drugs (Cavin et al., 2001; Brandon et al., 2003) and also thienopyridines have been successfully administrated in a variety of rat models (Garcia et al., 2011; Hagiwara et al., 2011). Previous studies on human plasma have shown that the metabolism of the pro-drug results in vivo in the generation of other metabolites (Brandon et al., 2003; Farid et al., 2007), hence the mixture we prepared may contain other forms rather than the active one alone. After having been generated, this metabolite mixture was tested in a platelet aggregation assay, to verify its efficacy. The metabolites produced were able to inhibit platelet aggregation and the concentration of 5 µM was sufficient to completely abolish aggregation.

When tested on neutrophils, the prasugrel metabolite mixture inhibited several neutrophil functions including transmigration and CD16 surface expression indicating that prasugrel metabolites could inhibit neutrophil function directly. The ability of prasugrel metabolites to prevent neutrophil transmigration, which
is an important event during the early stages of inflammation (Ley et al., 2007), by directly inhibiting neutrophil activation, suggest a new feature for this drug. Previous studies showing that P2Y\textsubscript{12} receptor can be expressed by other cells of the immune system indicate that all the thienopyridine effects were not only platelet-mediated (Diehl et al., 2010), however no previous publications have shown the direct effects of these drugs on neutrophils.

Our next step was to investigate whether these drugs could affect neutrophil-platelet aggregate formation, considering the importance of the interaction between platelets and neutrophils (Ley et al., 2007). Neutrophil-platelet interaction was inhibited following pre-incubation with prasugrel metabolites. However, in order to clarify whether these effects were mediated solely through direct interaction with platelets or if neutrophils were also a direct target, we pre-incubated only neutrophils with the metabolite mixture prior to stimulation. Also in this case, the metabolites were able to decrease aggregate formation, suggesting for the first time that these drugs affect neutrophil-platelet interaction by directly inhibiting both cell types. These effects are not agonist-specific, as fMLP, PAF, and LTE\textsubscript{4}-induced activation was inhibited by prasugrel metabolites. It is important to notice that previous studies on human plasma following prasugrel intake have shown that other metabolites may be produced along with R-138727 (Farid et al., 2007). As a result, it is a possibility that one of these metabolites, and not the so far considered active form, is responsible for these effects on neutrophils.
Hence we sought a possible target receptor on neutrophils that could interact with prasugrel metabolites. First, we evaluated P2Y$_{12}$ receptor expression in neutrophils using western blotting, as this receptor is the target of prasugrel in platelets, and is known to be expressed in other cell types, such as leukocytes (Diehl et al., 2010) and dendritic cells (Ben Addi et al., 2010). Interestingly, we could not detect any receptor expression, suggesting that either the receptor is not expressed or it is expressed below level of detection. Therefore, we decided to study whether a selective reversible P2Y$_{12}$ receptor antagonist, AR-C69931MX (Kim and Kunapuli, 2011), could inhibit neutrophil functions, but it did not elicit the same effects on neutrophils as the prasugrel metabolite mixture, indicating that the mechanism by which these metabolites alter neutrophil function is not through the P2Y$_{12}$ receptor. Secondly, we considered the P2Y$_{13}$ receptor, that is more ubiquitously expressed than the P2Y$_{12}$ receptor (Marteau et al., 2003), but pharmacologically very similar (Marteau et al., 2003; von Kugelgen, 2006; Kim and Kunapuli, 2011). Considering that no reliable antibody for this receptor is commercially available and mRNA studies do not always reflect protein expression, we selected a receptor antagonist previously studied in other cell types. The P2Y$_{13}$ receptor antagonist was also unable to affect neutrophil activation, excluding this receptor as a possible target of prasugrel metabolites in neutrophils.

In order to understand the molecular mechanism behind prasugrel effects, we investigated whether these metabolites affect intracellular signaling events in neutrophils. Both ERK-phosphorylation and calcium mobilization induced by
fMLP or PAF (Coelho et al., 2001; Hauser et al., 2001) were inhibited by prasugrel metabolites. It is possible that prasugrel metabolites antagonize fMLP, PAF, or LTE₄ receptors and prevent neutrophil activation or that these metabolites inhibit Gᵢₒ, the G protein responsible for ERK activation and calcium mobilization (Meshki et al., 2006). Alternatively, prasugrel metabolites could activate a different receptor on neutrophils, which elevates intracellular cAMP levels and inhibits neutrophil functions. We believe that this is unlikely as prasugrel metabolites also failed to cause VASP phosphorylation (downstream of cAMP signaling (Eckert and Jones, 2007)) in neutrophils (data not shown). Hence, further investigations are required in order to evaluate these possibilities.

In order to support our in vitro findings, we also investigated the effects of prasugrel on in a LPS-induced model of systemic inflammation. Previous studies have demonstrated an anti-inflammatory effect of these anti-platelet drugs in different animal models (Hagiwara et al., 2011; Liu et al., 2011). In these studies, we specifically investigated the in vivo effect of prasugrel on neutrophil infiltration and in vivo interaction of neutrophils with platelets. Our data confirms our in vitro findings, indicating a lower cell infiltration and MPO activity in the lungs of prasugrel treated animals, compared with untreated ones. Also the interaction between platelets and neutrophils was diminished when mice were exposed to the drug, suggesting a protective role for prasugrel during inflammation.

In conclusion, neutrophils activation could be directly inhibited by prasugrel metabolites indicating that neutrophils are also a target for the metabolites of
this anti-platelet drug. Some of the in vivo anti-inflammatory effects of the thienopyridines could be due to off-target effect of these metabolites on neutrophils. However, these metabolites do not act through either the P2Y_{12} or P2Y_{13} receptor in neutrophils.

Author contribution

Participated in research design: Elisabetta Liverani, Laurie E. Kilpatrick and Satya P. Kunapuli.

Conducted experiments: Elisabetta Liverani, Mario C. Rico and Analia E. Garcia.

Performed data analysis: Elisabetta Liverani and Satya P. Kunapuli.

Wrote or contributed to the writing of the manuscript: Elisabetta Liverani, Laurie E. Kilpatrick and Satya P. Kunapuli.

Conflicts of interest

The authors have no conflicts of interest to declare.
References


Footnotes

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Legends for Figures:

Figure 1:
Prasugrel metabolites generated *in vitro* abolish platelet aggregation and do not alter neutrophil viability

A) Human platelets were pretreated at 37° C with either prasugrel metabolites (PRAS - 2, 3 and 5 μM) or negative control (Ctr - 5 μM) for 2 minutes and then stimulated with 2MeSADP (100 nM) as noted. All tracings are representative of at least three experiments from different donors. (B) Representative histograms showing propidium iodide exclusion in neutrophils untreated, permeabilized and exposed to different concentrations of prasugrel metabolites (5, 10 and 15 μM) for 2 minutes. Histograms are representative of at least three experiments from different donors.

Figure 2: Prasugrel metabolites attenuate neutrophil transmigration and CD16 surface expression. (A) Effects of prasugrel metabolites (PRAS – 5 μM) and the negative control (Ctr) on cell transmigration, when added directly to the cells with chemo-attractant (fMLP - 10 μM; PAF - 10nM). The number of neutrophils that transmigrated was measured after 1 hour incubation at 37° C (n= 4, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr). (B) CD16 surface expression was measured following PRAS exposure (2 min – 5 μM) using flow cytometry (data shown as Geometric mean of fluorescence intensity (GMFI), mean ± S.E.M., n= 5, **p ≤ 0.01 PRAS versus Ctr).
Figure 3: Prasugrel metabolites inhibit agonist-induced ERK phosphorylation in neutrophils. Isolated human neutrophils were pre-incubated with prasugrel metabolites or control for 2 minutes and stimulated with fMLP (A) or PAF (C) for 30 minutes at 37° C. ERK phosphorylation was measured by Western blot analysis using phospho-specific antibodies against ERK (n= 3, mean ± S.E.M., **p ≤ 0.01 PRAS versus Ctr). Representative images (A and C) and ratio of arbitrary densitometry units of phosphorylated protein values compared with total proteins (B and D) are shown.

Figure 4: Prasugrel metabolites decrease calcium mobilization following fMLP and PAF stimulation in neutrophils. Calcium mobilization was measured after pre-treatment with either Ctr (square) or PRAS (triangle) following stimulation with fMLP (A - 10 μM) or PAF (B - 10 nM). Figure shows the concentration of Ca²⁺ (nM) mobilized in time (seconds) (n= 4, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr).

Figure 5: Prasugrel metabolites reduce neutrophil and platelet interaction. Formation of platelet/neutrophil aggregates was determined using flow cytometry. (A) Both neutrophils and platelets were pre-incubated with prasugrel metabolites (5 μM, 2 minutes) before stimulation with fMLP (10 μM, white bar) or PAF (10 nM, black bar) (n= 3, mean ± S.E.M., **p ≤ 0.01 PRAS versus Ctr). (B) Either neutrophils or platelets were pre-incubated with PRAS before being
reconstituted. Aggregates were measured after stimulation with fMLP (n= 4, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr).

**Figure 6: LTE₄-induced neutrophil activation is also inhibited by prasugrel metabolites.**

(A) CD16 surface expression was determined by flow cytometry following pre-exposure to prasugrel metabolites at the concentration of 5 μM for 2 minutes (n= 4, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr) following stimulation with LTE₄ (μM). (B) Neutrophil transmigration was evaluated after LTE₄ (1 μM) -induced activation (1hr at 37⁰ C) with cells pretreated with prasugrel metabolites or negative control (n= 3, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr). (C) Platelet-neutrophil aggregate formation was evaluated in the presence of prasugrel metabolites (5 μM), following activation with LTE₄ (1 μM) (n= 3, mean ± S.E.M., *p ≤ 0.05 PRAS versus Ctr).

**Figure 7: Neutrophil activation is not altered by prasugrel in the pro-drug form.**

(A) CD16 surface expression was determined by flow cytometry following pre-exposure to prasugrel pro-drug at the concentration of 5 μM for 2 minutes (n= 3, mean ± S.E.M.) following stimulation with either fMLP (10 μM – black bars) or PAF (10nM – white bars). (B) Neutrophil transmigration was evaluated after fMLP (10 μM)-or PAF (10nM)-induced activation (1hr at 37⁰ C) with cells incubated with prasugrel pro-drug or negative control (n= 3, mean ± S.E.M.). (C)
Platelet-neutrophil aggregate formation was evaluated in the presence of prasugrel pro-drug (5 μM), following activation with fMLP (10 μM) or PAF (10nM) (n= 3, mean ± S.E.M.).

**Figure 8:** Neutrophil chemotaxis and CD16 expression were not inhibited by AR-C69931MX, a reversible P2Y<sub>12</sub> antagonist. (A) Human platelets were pretreated at 37° C with AR-C69931MX (100nM and 1 μM) for 2 minutes and then stimulated with 2MeSADP (100 nM). All tracings are representative of at least three experiments from different donors. (B) Neutrophils were incubated with either fMLP (10 μM – black bars) or PAF (10nM – white bars) for 1 hour at 37° C and transmigration was measured as number of cells counted in the bottom chamber. Cells were pre-treated with AR-C69931MX (100 nM and 1 μM), (n= 4, mean ± S.E.M.). (C) CD16 expression was determined in the presence or absence of AR-C69931MX (100nM and 1 μM) by flow cytometry (n= 4, mean ± S.E.M.). (D) Platelet/neutrophil aggregate formation induced by fMLP or PAF was evaluated in the presence or absence of ARC-C69931MX (100nM and 1 μM) (n= 4, mean ± S.E.M.).

**Figure 9:** Neutrophil functions were not affected by MRS2211, a reversible P2Y<sub>13</sub> antagonist. (A) Human platelets were pretreated at 37° C with MRS2211 (10, 25 and 50 μM) for 2 minutes and then stimulated with 2MeSADP (100 nM). All tracings are representative of at least three experiments from different donors. (B) CD16 surface expression was determined through flow cytometry in
the absence or presence of MRS2211 at the concentration of 25 μM (black bars) and 50 μM (gray bars) (n= 4, mean ± S.E.M.) following stimulation with fMLP (10 μM). (C) Neutrophil transmigration was evaluated in the absence or presence of the P2Y<sub>13</sub> antagonist at different concentrations (n= 4, mean ± S.E.M.) after fMLP (10 μM)-induced activation. (D) Platelet-neutrophil aggregate formation in response to fMLP (10 μM) was evaluated in the presence of MRS2211 (25 μM and 50 μM), (n= 4, mean ± S.E.M., *p ≤ 0.05 PRAS compared with negative control).

**Figure 10: In vivo Prasugrel treatment decreased LPS-induced systemic inflammation.** (A) Representative dot plot images of percentage of neutrophil-platelets aggregates in blood samples of untreated animals (Untreated), prasugrel pre-exposed (PRAS), 24 hour LPS-induced inflammation (LPS) and both LPS-challenged and prasugrel treated mice (PRAS+LPS). Aggregates were identified as positive to both platelet (CD41, FITC-labeled) and neutrophils (CD11b, PE-labeled). (B) Graph showing the percentage of aggregate formation of the data represented in A. (n=2, mean ± S.E.M.). (C) CD11b expression indicated as GMFI values detected in gated neutrophils in whole blood samples (n=2, mean ± S.E.M.). (D) H&E staining of lung sections obtained from mice. Representative histomicrographs of lung sections from untreated animals (Untreated), prasugrel pre-exposed (PRAS), LPS-induced inflammation (LPS) and both LPS-challenged and prasugrel treated mice (PRAS+LPS)
(Magnification 10x and 20x). (D) MPO analysis were performed in lung samples (n=2, mean ± S.E.M.).
Figure 1

A

2MeSADP  100nM

Ctr  2µM PRAS  3µM PRAS  5µM PRAS

Light transmission

B

Negative Control  Permeabilized cells

5µM PRAS  10µM PRAS  15µM PRAS
Figure 2

A. Bar chart showing the number of cells (10^4) treated with fMLP and PAF.

B. Bar chart showing the CD16 expression levels in cells treated with fMLP and PRAS.

Legend:
- fMLP
- PAF
- Ctr
- PRAS

* p < 0.05
** p < 0.01
Figure 3

**Figure 3**

A. fMLP

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B. Ratio of P-ERK/Total ERK

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C. PAF

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D. Ratio of P-ERK/Total ERK

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<td>Ratio of P-ERK/Total ERK</td>
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Figure 4

A

B

**[Ca^{2+}]_i (nM)**

- Ctr
- PRAS

Time (s)

- fMLP
- PAF

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Figure 5

A

% of PMN-platelet aggregates

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B

% of PMN-platelet aggregates

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* * *
Figure 6

A

Number of cells (10^4)

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CD16 expression

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C

% of PMN-platelet aggregates

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* indicates significant difference.
Figure 7

A

CD16 expression

fMLP  PAF

B

Number of cells (10^4)

fMLP  PAF  Ctr  Pro-drug

C

% of PMN-platelet aggregates

Ctr  Pro-drug

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Figure 8 (part 1 of 2)

A

Light transmission

2MeSADP 100nM

Ctr 100nM ARC 1µM ARC

B

Number of cells (10^4)

0 2 4 6 8 10

fMLP - + + + - - -
PAF - - - - + + +
ARC (100nM) - - + - - + -
ARC (1µM) - - - + - - +
Figure 8 (part 2 of 2)

C

CD16 expression

fMLP  -  +  +  +  -  -  -
PAF  -  -  -  -  +  +  +
ARC (100nM)  -  -  +  -  -  +  -
ARC (1µM)  -  -  -  +  -  -  +

D

% of PMN-platelet aggregates

Ctr  fMLP  PAF

ARC (100nM)  
ARC (1µM)  

fMLP  PAF
Figure 9

A

2MeSADP 100nM

Light transmission

10μM MRS2211
25μM MRS2211
50μM MRS2211

B

CD16 expression

MRS 2211 - 25μM 50μM

C

Number of cells (10^4)

MRS 2211 - 25μM 50μM

D

% of PMN-platelet aggregates

MRS 2211 - 25μM 50μM
Figure 10 (part 2 of 2)

D

Untreated

PRAS

LPS

PRAS + LPS

E

MPO (rfu/min/mg)

LPS

PRAS

-  +  +

+  -  +
Supplemental Figure 1

Prasugrel metabolites inhibit neutrophil functions

Elisabetta Liverani, Mario C. Rico, Analia E. Garcia, Laurie E. Kilpatrick and Satya P. Kunapuli

Journal of Pharmacology and Experimental Therapeutics
Supplemental Figure 3

The image shows a gel with lanes labeled PBMC, PMN, Mono, and Platelets. The gel is stained with an antibody against P2Y_12.
Prasugrel metabolites inhibit neutrophil functions
Elisabetta Liverani, Mario C. Rico, Analia E. Garcia, Laurie E. Kilpatrick and Satya P. Kunapuli
Journal of Pharmacology and Experimental Therapeutics

Supplemental data

Supplemental Figure 1: Formation of neutrophil/platelet aggregates was investigated using flow cytometry. Top left panel: scatter plot indicating the neutrophil population (gate). Top right panel, plot for CD41 and CD16 in unstimulated identifies the quadrant for double positive events. Bottom right and left blot after fMLP (10μM) and PAF (10nM) stimulation respectively.

Supplemental Figure 2: Prasugrel metabolites did not alter CD11a surface expression, but they could still inhibit neutrophil/platelet aggregate formation. Neutrophils were labeled with FITC-Conjugated anti-human CD11a (Clone HI111; eBioscience, San Diego, CA). (A) Representative histogram of unlabelled neutrophils (negative control - gray line) compared with unstimulated cells labeled with CD11a (unstimulated – black line) and fMLP (10μM)-stimulated cells (fMLP-stimulated – red line) (left panel). GMFI values of fMLP and PAF (10nM) are shown (right panel; Mean ± SEM, n = 3). (B) Representative histogram of fMLP-activated cells previously pre-incubated with either negative control (green line) or prasugrel metabolite (2 min – 5 μM; purple line) (left
panel). GMFI values of fMLP and PAF activated cells (right panel; Mean ± SEM, n = 3). (C) Both neutrophils and platelets were pre-incubated with prasugrel metabolites (5 μM, 2 minutes) before stimulation with fMLP (10 μM, white bar) or PAF (10 nM, black bar) (n= 3, mean ± S.E.M., **p ≤ 0.01 PRAS versus Ctr).

**Supplemental Figure 3: P2Y_{12} expression in a variety of human cells.** Western blotting image is showing P2Y_{12} expression in a variety of human cells such as neutrophils (PMN), peripheral blood mononuclear cells (PBMC), Monocytes (Mono) and platelets as a positive control. Goat anti-human antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Supplemental Figure 4: Hematology cell counts following prasugrel treatment in LPS-induced systemic inflammation.** Graphs show murine blood cell counts of neutrophils (A), lymphocytes (B), platelets (C) and red blood cells (D). Values are expressed as 10^3/μL, mean ± SEM, (n = 2).