P2Y12 Receptor Blockade Synergises Strongly with Nitric Oxide and Prostacyclin to Inhibit Platelet Activation

Melissa V Chan, PhD1; Rebecca B M Knowles, MD1; Martina H Lundberg, PhD1; Arthur T Tucker, PhD1; Nura A Mohamed, MRes2,3; Nicholas S Kirkby, PhD1,3; Paul C J Armstrong, PhD1; Jane A Mitchell, PhD3; Timothy D Warner, PhD1

1 The William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom.
2 Qatar Foundation Research and Development Division, Doha, Qatar
3 National Heart & Lung Institute, Imperial College London, London, United Kingdom

*These authors contributed equally to the manuscript and their names appear in alphabetical order

Address for Correspondence:
Timothy D. Warner, PhD
The William Harvey Research Institute
Barts and the London School of Medicine and Dentistry
Charterhouse Square
London
EC1M 6BQ, UK
Telephone: + 44 20 7882 2100
Fax: + 44 20 7882 8252
Email: t.d.warner@qmul.ac.uk

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ABSTRACT

Aim - In vivo platelet function is a product of intrinsic platelet reactivity, modifiable by dual antiplatelet therapy (DAPT), and the extrinsic inhibitory endothelial mediators, nitric oxide (NO) and prostacyclin (PGI₂), that are powerfully potentiated by P2Y₁₂ receptor blockade. This implies that for individual patients endothelial mediator production is an important determinant of DAPT effectiveness. Here, we have investigated this idea using platelets taken from healthy volunteers treated with anti-platelet drugs.

Methods - Three groups of male volunteers (n=8) received either prasugrel (10 mg), aspirin (75 mg) or DAPT (prasugrel + aspirin) once daily for 7 days. Platelet reactivity in the presence of DEA/NONOate and PGI₂ was studied before and following treatment.

Results - Ex vivo, PGI₂ and/or DEA/NONOate had little inhibitory effect on TRAP-6-induced platelet reactivity in control conditions. However, in the presence of DAPT, combination of DEA/NONOate+PGI₂ reduced platelet aggregation (74±3% to 19±6%, p<0.05). In vitro studies showed even partial (25%) P2Y₁₂ receptor blockade produced a significant (67±2% to 39±10%, p<0.05) inhibition when DEA/NONOate+PGI₂ was present.

Conclusions - We demonstrate that PGI₂ and NO synergise with P2Y₁₂ receptor antagonists to produce powerful platelet inhibition. Furthermore, even with submaximal P2Y₁₂ blockade the presence of PGI₂ and NO greatly enhances platelet inhibition. Our findings highlight the importance of endothelial mediator in vivo modulation of P2Y₁₂ inhibition and introduces the concept of refining ex vivo platelet function testing by incorporating an assessment of endothelial function to better predict thrombotic outcomes and adjust therapy to prevent adverse outcomes in individual patients.
What is already known about this subject

- Platelet function is a product of intrinsic platelet reactivity.
- This can be modified by dual anti-platelet therapy (DAPT), but also by the influence of the endothelial mediators, nitric oxide (NO) and prostacyclin (PGI₂).
- NO and PGI₂ also independently amplify each other’s effects.

What this study adds

- Three-way synergy between PGI₂, NO and P2Y₁₂ receptor antagonism produces powerful platelet inhibition.
- Even with submaximal (25%) P2Y₁₂ blockade, the presence of PGI₂ and NO greatly enhances platelet inhibition.
- Assessing endothelial mediator production and associations to platelet cyclic nucleotides in vivo could improve thrombotic outcomes in individual patients.
INTRODUCTION

Compromise in the integrity of the vascular endothelium precipitates rapid platelet activation as platelets become exposed to sub-endothelial collagen and tissue factor. This activation is driven by a cascade of complex intracellular signaling pathways leading to the production of secondary platelet agonists, notably thromboxane (TX) A₂ and ADP [1, 2]. Dual anti-platelet therapy (DAPT) is recommended for the secondary prevention of atherothrombotic events in patients with acute coronary syndromes or following percutaneous coronary intervention [3, 4] and targets these two pathways with a P2Y₁₂ receptor antagonist, such as clopidogrel, prasugrel or ticagrelor, and aspirin. The P2Y₁₂ receptor blockers inhibit platelet aggregation by blocking the amplifying effects of ADP acting on platelet P2Y₁₂ receptors [5], while aspirin targets the TXA₂-dependent pathway by inhibiting the cyclooxygenase (COX) enzyme within platelets [6]. Whilst DAPT is associated with an improvement in patient outcomes, thrombotic events do still occur. An often explored hypothesis is that the risk of experiencing a thrombotic event is associated with the level of platelet blockade: i.e. those individuals with less effective blockade provided by aspirin and, particularly, P2Y₁₂ receptor blockers are more at risk of thrombotic events. However, studies have failed to show any benefits from ex vivo monitoring of platelet function and subsequent tailoring of treatment in patients receiving dual anti-platelet therapy [7–10]. This failure is possibly because the ex vivo platelet tests used in these trials do not consider the environment in which platelets reside in vivo. Namely that within the circulation endothelium-derived autacoids, nitric oxide (NO) and prostacyclin (PGI₂), reduce platelet reactivity and prevent inappropriate platelet activation [11, 12]. Indeed, within the circulation each platelet is balanced by approximately 50 endothelial cells (e.g. 1.25 trillion platelets vs. 60 trillion endothelial cells in a 70 kg man) [13].

NO diffuses freely into platelets activating guanylyl cyclase (GC) to increase intracellular cGMP levels [14], while PGI₂ binds to IP receptors activating adenylyl cyclase (AC) to
increase intracellular cAMP levels [15]. Elevations in the intracellular levels of individual cyclic nucleotides promotes a generalised inhibition of platelet function [16] and the two pathways synergise to produce particularly strong inhibition [12]. NO and PGI₂ also individually synergise with P2Y₁₂ inhibition producing robust anti-aggregatory platelet effects [17, 18].

Taking account of the above observations we hypothesised that within the circulation the levels of endothelium-derived mediators are an important determinant of the efficacy of DAPT. Therefore, for individual patients *in vitro* measures of platelet reactivity do not accurately predict the *in vivo* effectiveness of DAPT due to the confounding of differences in endothelial mediator production. To test this hypothesis we added NO and PGI₂ to standard *ex vivo* tests of platelet function in blood taken from healthy volunteers receiving anti-platelet therapies.
METHODS

Study participants

24 healthy, non-smoking male volunteers (aged 18-40 years) were recruited and participated in the study. Health status was determined through medical history and physical examination, including blood pressure, pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study. The study was approved by St Thomas’s Hospital Research Ethics Committee (Ref. 07/Q0702/24) and all volunteers gave written consent before entering the study.

Study protocol

Healthy volunteers abstained from aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) and any other anti-platelet therapy for 14 days before commencing the study. The volunteers were divided into groups of 8. The first group received aspirin (75 mg; Nu-Seals Cardio 75, Alliance Pharmaceuticals Ltd, Chippenham, UK), the second prasugrel (10 mg; Effient®, Eli Lilly, RA Houten, The Netherlands) and the third both aspirin (75 mg) and prasugrel (10 mg) to represent dual anti-platelet therapy (DAPT), for 7 days. Adherence was assessed by interview. Blood samples were collected before and after drug treatment.

Blood collection

Blood for platelet aggregation was collected by venepuncture into tri-sodium citrate (0.32 % final; Sigma, Poole, Dorset, UK). Platelet-rich plasma (PRP) was obtained by centrifugation at 175 x g for 15 min at 25°C. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 15 000 x g for 2 min. All experiments were completed within 2 hr of blood collection.
Incubation with platelet function inhibitors

For \textit{in vitro} incubation experiments, PRP was treated with either vehicle (0.5\% DMSO) or the P2Y\textsubscript{12} receptor blocker prasugrel-active-metabolite (PAM; a kind gift of AstraZeneca) at 1.5 μM, 3 μM or 6 μM, to represent 25\%, 50\% or 100\% of the concentration needed for complete P2Y\textsubscript{12} receptor blockade, respectively, in the absence or presence of aspirin (acetylsalicylic acid; ASA, 30 μM) for 30 min at 37°C.

Light transmission aggregometry (LTA)

Baseline aggregation of PRP to arachidonic acid (AA, final concentration, 1 mM; Sigma), adenosine diphosphate (ADP, 5-20 μM; Labmedics, Salford, Manchester, UK), Horm collagen (0.4 and 10 μg/mL; Nycomed, Linz, Austria) and U46619 (10 μM; Cayman Chemical Company, Ann Arbor, MI, USA) was measured in a Bio/Data PAP-8E turbidimetric aggregometer (1200 rpm, 37°C; Alpha Laboratories, Eastleigh, UK) before and following treatment. Aggregations to TRAP-6 amide specific for PAR1 (TRAP-6, 25 μM; SFLLRN, Bachem, Bubendorf, Switzerland) or Horm collagen (4 μg/mL) after pre-incubation (1 min, 37°C) with the NO donor diethyiammonium (Z)-1-(N,N-diethylamino)diazen-1-iium-1,2-diolate (DEA/NONOate, 100 nM; Sigma) and/or prostacyclin (PGI\textsubscript{2}; 1 nM; R&D systems, Abingdon, UK) or vehicle (NaOH, 10 mM; Sigma) were also recorded. Using an NO measuring system (iNO600, Harvard apparatus) we showed 83 nM NO release at 2 mins and 154 nM at 4 min when 100 nM DEA/NONOate was incubated at pH7.4.
Isobolographic analysis

Inhibitory concentration curves for PGI$_2$ (1-8 nM) or DEA/NONOate (10 nM – 1 µM) against aggregation induced by TRAP-6 (25 µM) or collagen (30 µg/mL) in the presence of vehicle or PAM (6 µM) were constructed with data fitted to a logistic equation using least-squares method (Prism 6.0e, GraphPad Software, La Jolla, CA, USA). Derived data was used to generate isobolograms [18, 19].

ADP+ATP secretion

PRP was pre-incubated for 1 min with DEA/NONOate, PGI$_2$ or vehicle in an optical lum-aggregometer (560 CA; Chronolog, Havertown, PA, USA). ADP+ATP secretion was evaluated by luminescence in the presence of Chrono-Lume reagent (0.2 µM luciferin/luciferase; Chronolog) after stimulation with TRAP-6 (25 µM) or collagen (4 µg/mL).

P-selectin expression and GPIIb/IIIa activation

PRP, pre-incubated with PAM or vehicle, as described earlier, was incubated with PGI$_2$, DEA/NONOate or vehicle and then activated with TRAP-6 (25 µM) with gentle mixing at 37 °C. After 2 min, the reaction was stopped by dilution with a 10-fold excess of cold saline. Platelets were immediately stained with anti–CD61-allophycocyanin (CD61-APC; eBioscience, Hatfield, UK), PAC-1-FITC (BD Bioscience, Oxford, UK), and anti–P-selectin-PE (eBioscience) for 15 min at 4°C and then fixed in 2% (vol/vol) formalin (Sigma). PAC-1-FITC and anti–P-selectin-PE immunoreactivity was measured by flow cytometry using a FACSCalibur instrument (Becton Dickenson, Oxford, UK). Representative histograms are
shown in Supplementary Figure 5A-5D.

**VASP phosphorylation**

PAM or vehicle-treated PRP was stimulated with collagen (4 µg/mL) or TRAP-6 (25 µM) in the presence of PGI₂, DEA/NONOate or vehicle. After 4 min, the reaction was stopped with methanol-free formaldehyde (2% final; Fisher Scientific). Platelets were permeabilised (0.2% Triton X-100; Sigma) and incubated with anti-vasodilator stimulated phosphoprotein (VASP)-P(Ser²³⁹) primary antibody (Enzo Life-sciences, Exeter, UK), Alexa647-conjugated secondary antibody (Invitrogen, Paisley, UK), and FITC-conjugated anti-CD42b (eBioscience, Hatfield, UK), for 30 min each, in turn, before the platelet pellet was resuspended in 0.9% saline. VASP-P(Ser²³⁹) immunoreactivity was measured by flow cytometry, using a FACS-Calibur instrument (Becton Dickenson). Representative histograms are shown in Supplementary Figure 5E-5F.

**cAMP and cGMP measurements**

PAM or vehicle-treated PRP was stimulated with collagen (4 µg/mL) or TRAP-6 (25 µM) in the presence of PGI₂, DEA/NONOate or vehicle. After 4 min, platelets were lysed with Triton-X-100 (0.625%) and treated with iso-butylmethylxanthine (IBMX; 500 µM) and potassium fluoride (0.5 M). cAMP and cGMP concentrations were determined by homogenous time-resolved fluorescence-based competitive immunoassays (Cisbio Bioassays, Codolet, France).

**Statistics and data analysis**
Data were analysed using Prism 6.0e. Summary data (IC\textsubscript{50}, EC\textsubscript{50}) were obtained by fitting of data to a logistic equation and tested by Student's t-test (2 groups) or one-way ANOVA (>2 groups). Flow data were analysed using FlowJo v8.7 (Treestar, Ashland, USA) where the “single platelet” population was gated based on forward scatter and CD61-APC immunoreactivity (FL-4 mean fluorescence intensity). Statistical significance was determined by two-way ANOVA with Dunnett’s post-hoc test unless otherwise stated, and data sets considered different if p<0.05.

P2Y\textsubscript{12} nomenclature conforms to the BJP guidelines.
RESULTS

Light transmission aggregation responses following *in vivo* mono and dual anti-platelet therapy

In individuals taking aspirin, standard LTA responses to AA (1 mM) were strongly inhibited, as were responses to collagen. Responses to ADP (5 µM) were also significantly reduced, although to a lesser degree, while those to U46619 (10 µM) were unaffected (Supplementary Figure 1A). In individuals taking prasugrel, aggregatory responses to AA, collagen, ADP and U46619 were all significantly reduced (Supplementary Figure 1B). Aggregations induced by AA, collagen and ADP were abolished in individuals taking DAPT (Supplementary Figure 1C) while responses to U46619 were strongly reduced.

Platelet aggregation, ATP secretion, P-selectin expression and GPIIb/IIIa activation in the presence of PGI₂ and DEA/NONOate together with DAPT

In blood from individuals before DAPT, PGI₂ (1 nM), DEA/NONOate (100 nM) or DEA/NONOate+PGI₂ had little effect upon platelet aggregation. Following DAPT, collagen (4 µg/ml)-induced aggregation was significantly reduced from 73±2% to 31±2% (p<0.05; Figure 1A & Supplementary Figure 2A), as seen previously with a lower concentration of collagen (0.4 µg/mL; Supplementary Figure 1A). TRAP-6 (25 µM)-induced aggregation was, however, unaffected by DAPT unless DEA/NONOate+PGI₂ was present, when it was substantially reduced (67±3% to 19±6%, p<0.05; Figure 1B). In parallel experiments, we used lumi-aggregometry to quantify ADP+ATP release as a measure of dense granule secretion (Supplementary Figure 2B). These studies indicated that TRAP-6 induced dense granule secretion was unaffected in individuals receiving DAPT administration but was reduced with the further addition of DEA/NONOate+PGI₂ (6.3±1.9 to 3.7±1.3 nM, p<0.05; Figure 1C).
Similar effects were found in individuals receiving prasugrel, in whom aggregation in response to TRAP-6 was significantly reduced (63±3% to 7±3%, p<0.05; Supplementary Figure 3D) by the addition of DEA/NONOate+PGI₂. Treatment with aspirin alone significantly reduced collagen-induced aggregation in the presence of both DEA/NONOate (58±9% to 36±8%, p<0.05; Supplementary Figure 3A) and PGI₂ (66±5% to 35±7, p<0.05; Supplementary Figure 3A). Following treatment with aspirin, TRAP-6-induced aggregation was unaffected by DEA/NONOate or PGI₂, but was reduced by DEA/NONOate+PGI₂ (58±8% to 28±9%, p<0.05; Supplementary Figure 3B). To aid in visualisation of results these data are also expressed as heatmaps (Supplementary Figures 3E-F).

In individuals receiving DAPT, TRAP-6-induced P-selectin expression (geometric mean fluorescence index; MFI, 29.0±7.5 units to 2.4±0.6 units, p<0.05; Supplementary Figure 6A) and PAC-1 binding (12.4±3.4 units to 1.3±0.5 units, p<0.05; Supplementary Figure 6B) were significantly reduced. This pattern was similar with regard to P-selectin expression (25.0±6.1 units to 8.8±3.4 units, p<0.05) and PAC-1 binding (19.2±3.7 units to 1.6±0.3 units, p<0.05) in individuals receiving prasugrel only. Treatment with aspirin alone had no effect on either P-selectin or PAC-1 binding.

Effects of PGI₂ and DEA/NONOate on platelet aggregation and ATP release in the presence of submaximal P2Y₁₂ antagonism is sufficient to produce platelet inhibition

PRP was taken from healthy volunteers and pre-treated in vitro with prasugrel-active metabolite (PAM; 1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% of the concentration of PAM required for total P2Y₁₂ receptor inhibition in the absence (Table 1A) and presence (Table 1B) of aspirin. Aggregation in response to ADP (20 µM) was increasingly inhibited with increasing levels of PAM: control, 73±5%; aspirin, 51±7%;
aspirin+PAM-25%, 33±11%; aspirin+PAM-50%, 23±8%; aspirin+PAM-100%, 7±1%
(p<0.05).

Maximum platelet aggregation to collagen in the presence of PAM-100% was reduced by
the addition of DEA/NONOate (74±4% to 23±9%, p<0.05), PGI₂ (74±4% to 22±6%, p<0.05)
and DEA/NONOate+PGI₂ (50±10% to 4±1%, p<0.05). Similarly, TRAP-induced aggregation
was reduced in the presence of DEA/NONOate (70±2% to 36±6%, p<0.05), PGI₂ (67±2% to
35±3%, p<0.05) and DEA/NONOate+PGI₂ (63±3% to 4±2%, p<0.05). Indeed, even with
submaximal, PAM-50% and PAM-25% P2Y₁₂ receptor inhibition, significant inhibition of
platelet aggregation was found following addition of DEA/NONOate and PGI₂.

Although aspirin alone inhibited collagen-induced platelet aggregation, TRAP-6-induced
aggregation was only significantly inhibited with the further addition of DEA/NONOate+PGI₂
to aspirin+PAM-100% (58±5 to 15±8%, p<0.05), aspirin+PAM-50% (67±6% to 33±15%,
p<0.05), and even aspirin+PAM-25% (70±4% to 36±13%, p<0.05; Table 1B).

Collagen (4 µg/mL)-induced ATP release was significantly inhibited by DEA/NONOate+PGI₂
in the presence of PAM-100% (8.2±2.1 nM to 2.3±0.5 nM), PAM-50% (10.1±2.9 nM to
4.0±1.8 nM, p<0.05) and PAM-25% (11.0±3.0 nM to 4.1±1.7 nM, p<0.05; Table 2A), but not
by DEA/NONOate or PGI₂ alone. Similarly, in the presence of aspirin (Table 2B), collagen-
induced ATP release was significantly reduced by DEA/NONOate+PGI₂ with submaximal
levels of P2Y₁₂ blockade (aspirin+PAM-0%, 8.0±0.5 nM; aspirin+PAM-25%, 1.9±0.3 nM,
p<0.05; aspirin+PAM-50%, 2.1±0.3, p<0.05; aspirin+PAM-100%, 1.8±0.2 nM, p<0.05).

**Synergy between PGI₂, DEA/NONOate and P2Y₁₂ blockade**

Isobolographic analyses indicated strong synergistic inhibition between DEA/NONOate and
PGI₂ against platelet aggregation induced by collagen (30 µg/mL; Figure 2A) or TRAP-6
amide (25 µM; Figure 2B), with isoboles curving strongly towards the axes. P2Y12 blockade caused a further powerful (5-fold and 10-fold, respectively) enhancement in the synergy between DEA/NONOate and PGI2 for the inhibition of aggregations induced by collagen (Figure 2B) and TRAP-6 (Figure 2D).

Involvement of cAMP and cGMP in the synergistic effects of P2Y12 blockade, PGI2 and DEA/NONOate

We found no significant change in cGMP levels in the platelets in response to DEA/NONOate and/or PGI2 after incubation with aspirin, PAM or aspirin+PAM after platelet aggregation stimulated by collagen (Figure 3A) or TRAP-6 (Figure 3B).

In collagen-stimulated platelets, cAMP levels (0.8±0.1 nM) were not altered by DEA/NONOate, but were significantly increased by PGI2 (2.6±0.3 nM, p<0.05) and even more so by the combination of DEA/NONOate+PGI2 (4.9±0.6 nM, p<0.05). Neither PAM, aspirin or PAM+aspirin altered the cAMP response in collagen-stimulated platelets (Figure 3C). In contrast, in TRAP-6-stimulated platelets, DEA/NONOate, PGI2 and the combination did not elevate cAMP levels in vehicle or aspirin groups, but did in the presence of PAM (1.0±0.1 nM to 2.3±0.2 nM, p<0.05) or PAM+aspirin (1.4±0.5 nM to 2.2±0.4 nM, p<0.05), PGI2 increased cAMP levels and this response was further enhanced by addition of DEA/NONOate (PAM: 3.4±0.6 nM, PAM+aspirin: 3.5±0.6 nM, p<0.05; Figure 3D).

Phospho (Ser239)-VASP, a downstream marker of PKG activation, remained unchanged in most conditions studied but was increased following TRAP-6 stimulation in the presence of PAM in all cases and most so in the presence of PGI2 (28±5 to 47±15 units, p<0.05) or DEA/NONOate+PGI2 (27±4 to 46±11 units, p<0.05; Figure 3F).
DISCUSSION

Here we show in healthy individuals receiving standard DAPT leading to consensus levels of platelet inhibition that ex vivo responses to the strong primary platelet activators collagen and TRAP-6 are powerfully influenced by the presence of NO and PGI\(_2\). The strong synergies between P2Y\(_{12}\) inhibitors and the cAMP and cGMP signaling systems mean that the in vivo platelet reactivity in patients receiving DAPT will be a function of the level of P2Y\(_{12}\) receptor blockade and the levels of endothelium-derived NO and PGI\(_2\). This provides an explanation for different thrombotic outcomes in the presence of similar levels of platelet blockade; i.e. individual patients with different levels of endothelial function, or indeed disease-driven endothelial dysfunction, would have different levels of in vivo platelet inhibition for the same level of DAPT activity, as determined by ex vivo testing.

DAPT, aspirin plus a P2Y\(_{12}\) receptor blocker, is the preventative therapy provided to patients at particular risk of coronary thrombosis, notably for the first 12 months following coronary stent implantation or an acute coronary syndrome [20, 21]. Despite this therapeutic approach coronary thrombosis still occurs, and there have been great efforts made to find ex vivo tests that could predict for clinical outcomes [22, 23]. Deductive reasoning leads to the conclusion that less effective platelet blockade would leave individuals at increased risk of thrombosis and so multiple efforts have been made to link levels of platelet reactivity in ex vivo tests to clinical outcomes. Despite the attractive logic of this approach, tailoring anti-platelet therapy to ex vivo platelet responses has failed to provide any improvement in clinical outcomes as noted in large scale studies such as ADRIE [24] and several large-scale prospective, randomised clinical trials, such as GRAVITAS [7], ARCTIC [8], TRIGGER-PCI [9] and TRILOGY [10].

In patients receiving clopidogrel, there are well-characterised metabolic differences that can produce suboptimal levels of its active metabolite and consequently result in suboptimal levels of P2Y\(_{12}\) receptor blockade [25]. There are also some reports of variability in the
effects of prasugrel and ticagrelor, although to a much lesser extent than for clopidogrel [26].

Biochemical resistance to the effects of aspirin are also particularly rare [27]. Allowing for differences dependent upon adherence to therapy, individuals on DAPT may in fact present rather a more homogenous level of platelet inhibition than can be associated to different clinical outcomes. Having recently reported that blockade of P2Y<sub>12</sub> receptors greatly increases the inhibitory effects of NO, and knowing that not only was there a similar interaction with the inhibitory effects of PGI<sub>2</sub> but that NO and PGI<sub>2</sub> powerfully synergise to inhibit platelets, we reasoned that differences in the levels of NO and PGI<sub>2</sub> in the presence of the same levels of P2Y<sub>12</sub> receptor blockade would produce different levels of platelet inhibition. By testing this hypothesis in individuals receiving standard DAPT we show here that strong and synergistic interactions between P2Y<sub>12</sub> receptor blockade and endothelium-derived mediators produce profound inhibitory effects upon platelets. We firstly established that the drug regime given in our studies elicited satisfactory reduction in baseline reactivity, therefore establishing effectiveness of P2Y<sub>12</sub> and/or COX inhibition in accordance with suggested analytical cutoffs [28]. These reductions were against high pre-treatment levels of platelet reactivity (>70% response to 5 µM ADP) [29]. In these studies, and others presented here, we took care to include the standard measures of platelet function as determined in consensus statements [28, 30]. Then to make data readily accessible we have presented results in the form of heat maps that move from red to green, indicating movement from full platelet activation to no platelet activation.

It is well known that NO and PGI<sub>2</sub> synergise to inhibit platelets [12] and it has been demonstrated by ourselves and others that P2Y<sub>12</sub> antagonists potentiate the inhibitory actions of both PGI<sub>2</sub>, dependent upon cAMP [17], and NO, dependent upon cGMP generation [18]. Here, we have shown that this synergy, in the presence of NO and PGI<sub>2</sub> is mostly cAMP dependent, therefore phosphodiesterase 3 (PDE3) inhibitors, such as cilostazol, may have an enhanced effect compared to PDE5 inhibitors, such as sildenafil. Though not widely commented upon, the body contains many more endothelial cells than
platelets, in the order of 50 times more, and the two populations constantly interact. In the circulation DAPT exerts its effects upon platelets in the presence of endothelium-derived mediators while these are absent in ex vivo testing. In the studies presented here we found that the interactions of NO, PGI₂ and P2Y₁₂ receptor blockade in inhibiting platelets were markedly synergistic as noted by isobolographic analysis and measures of aggregation, ATP release, activation of GP IIb/IIIa receptors and P-selectin expression. In volunteers taking DAPT we noted inhibition of responses to ADP and AA that were in keeping with consensus statements of effective DAPT; i.e. in our study the drugs were working to an effective level of clinical efficacy. Despite this level of effective inhibition, high concentrations of the strong primary platelet activators, TRAP-6 or collagen, still caused notable platelet activation.

Addition of low concentrations of NO and PGI₂, to model the environment within the blood vessel, had little effect on their own but led to almost complete inhibition in platelets from individuals treated with DAPT. Similarly, while NO, PGI₂ or DAPT alone had relatively little effect upon platelet granule release, determined as ATP release, when combined they caused more than 50% inhibition. These results indicate that even in the presence of effective DAPT, i.e. within consensus guidelines, the presence of NO and PGI₂ lead to very much higher levels of platelet inhibition.

Next using an in vitro approach we modeled events in the presence of suboptimal levels of P2Y₁₂ receptor blockade by using concentrations of PAM that were 50% and 25% of the effective concentration. Under these conditions we noted that relative to the consensus levels of DAPT we did not achieve significant reduction in platelet aggregation. Notably, however, in the presence of NO and PGI₂ effective levels of inhibition were achieved, even when platelets were exposed to only 25% of the effective concentration of PAM. As we express in heat maps, there is a clear interaction between DAPT and the endothelial mediators that move platelets from reactive (‘red’) to unreactive (‘green’). Interestingly, these comparisons indicate that 25% of the effective concentration of PAM plus NO and PGI₂ produces a stronger inhibition in LTA, the ‘gold standard test’, than 100% of the effective
concentration of PAM in the absence of NO and PGI$_2$ (i.e. the normal conditions for testing
ex vivo platelet responsiveness). This suggests that in individuals in whom suboptimal P2Y$_{12}$
inhibition is achieved, such as poor clopidogrel metabolisers, anti-platelet efficacy may be
particularly sensitive to any changes in endothelial function. Our in vitro data also
demonstrate that the triple synergy between P2Y$_{12}$ blockade, NO and PGI$_2$ can be explained
by changes in cAMP signaling, which is consistent with known interactions between NO and
PGI$_2$ [31] and PGI$_2$ and P2Y$_{12}$ [17].

We show that following standard DAPT the level of platelet reactivity is a function of the level
of P2Y$_{12}$ receptor blockade and the levels of NO and PGI$_2$. While we added NO and PGI$_2$
exogenously they are surrogates for the effects of endogenous NO and PGI$_2$ and other
elevators of platelet cyclic nucleotides such as adenosine. We propose that, since in vivo
platelet function is a product of both internal platelet responsive signaling reactivity and the
external influence of the endothelium, an assessment of endothelial mediator production
could be combined with results from ex vivo platelet testing to better predict thrombotic
outcomes in individual patients. Furthermore, with the emergence of this complex and very
powerful synergy between PGI$_2$, NO and P2Y$_{12}$ inhibitors (Figure 4), we should perhaps
consider optimising the availability and activity of endothelium-derived mediators (such as
PDE inhibitors), or providing mimetic drugs, rather than adding in further anti-platelet
therapies. These findings could, eventually, be applied in a personalised medicine
framework where the endothelial mediator production of individuals is assessed and
appropriate add-on therapy applied. In a more generalised approach these additional
therapies could also be supplied to patient groups with known endothelial dysfunction, such
as diabetics. This approach could provide increased anti-platelet efficacy while avoiding the
increased risk of bleeding events associated with the approach of triple anti-platelet therapy.
Authors’ Contributions

RBMK, ATT, NSK and TDW designed the study and experiments. MVC, RBMK, MHL, NAM, NSK and PCJA collected data. MVC, NAM and RBMK performed data analysis. MVC, RBMK and TDW drafted the manuscript. All authors contributed to the writing of the manuscript. MVC and RBMK contributed equally to this work as first authors.

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Competing Interests

"All authors have completed the Unified Competing Interest form at
http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: RBMK, MVC, MHL and PCJA had support from the British Heart Foundation [FS/12/53/29643 to RBMK, PG/11/75/29105 to MVC and MHL, PG/12/68/29779 to PCJA] and all authors had support from the William Harvey Research Foundation for the submitted work. TDW has received research grants from AstraZeneca relating to clinical development of P2Y$_{12}$ inhibitors in the previous 3 years. There are no other relationships or activities that could appear to have influenced the submitted work."
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Table 1. In vitro effects of aspirin and PAM on platelet aggregation. PRP from healthy volunteers (n = 4) was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total P2Y₁₂ receptor inhibition, respectively. Tables show % final aggregation in response to ADP (20 µM), collagen (4 µg/mL) and TRAP-6 (25 µM) in the presence of vehicle (NaOH, 10 mM), NO (100 nM), PGI₂ (1 nM) or NO + PGI₂ in the (A) absence and (B) presence of aspirin (30 µM). Significance is shown as * p<0.05 vs vehicle, † p<0.05 vs PGI₂.

Table 2. In vitro effects of aspirin and PAM on ATP release. PRP from healthy volunteers (n = 4) was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total P2Y₁₂ receptor inhibition, respectively. Tables show ATP release (nM) in response to collagen (4 µg/mL) and TRAP-6 (25 µM) in the presence of vehicle (NaOH, 10 mM), NO (100 nM), PGI₂ (1 nM) or NO + PGI₂ in the (A) absence and (B) presence of aspirin (30 µM). Significance is shown as * p<0.05 vs vehicle, † p<0.05 vs PGI₂.

Figure 1. Interactions of NO and PGI₂ with DAPT: platelet aggregation & ATP release. Bar graphs and heatmaps of platelet aggregation in response to (A) collagen (4 µg/ml), and (B) TRAP-6 amide (25 µM), and (C) ATP release in response to TRAP-6 amide (25 µM). Aggregometry was conducted before and after 7 days DAPT (aspirin, 75 mg, plus prasugrel, 10 mg). Aggregometry conducted in the presence of vehicle (NaOH, 10 mM), NO (100 nM), PGI₂ (1 nM), or NO + PGI₂. Data are presented as final aggregation (%), mean±SEM or ATP.
release (nM, mean±SEM). Heatmaps indicate maximum aggregation or ATP release with red and minimum aggregation or ATP release with green. N = 8 for all. Significance is shown as * p<0.05 vs non-treated, † p<0.05 vs NaOH DAPT-treated ‡ p<0.05 vs PGI2 DAPT-treated.

Figure 2. Synergism between NO, PGI2 and PAM. IC50 isobolograms were generated by analysing combinations of NO and PGI2 required to produce a 50% inhibition of platelet aggregation stimulated by: collagen (30 µg/mL) in the (A) absence and (B) presence of PAM (6 µM); and, by TRAP-6 (25 µM) in the (C) absence and (D) presence of PAM. The linear relationship is predicted by the arithmetic sum of the effect of either NO or PGI2 alone, as described in the methods, and the experimental line curving towards the axes indicates a strong, synergistic relationship. N = 4 for each point.

Figure 3. In vitro effects of aspirin and PAM on cyclic nucleotide levels. PRP from healthy volunteers (n = 4) was treated with aspirin (30 µM), PAM (6 µM) or both followed by addition of vehicle (NaOH, 10 mM), NO (100 nM), PGI2 (1 nM) or NO + PGI2. cGMP levels were then measured following stimulation by (A) collagen (4 µg/mL) and (B) TRAP-6 (25 µM); as were cAMP levels following (C) collagen and (D) TRAP-6. Phospho (Ser239)-VASP levels in (E) collagen and (F) TRAP-6-stimulated PRP were determined by flow cytometry as a composite of cGMP and cAMP responses. Significance is shown as * p<0.05 vs non-treated and † p<0.05 vs PGI2 with corresponding PAM or aspirin+PAM.

Figure 4. Summary of the interaction between the endothelium and P2Y12 antagonism. In the healthy intact circulation platelets are kept in a non-activated state in part by the action
of endothelium-derived mediators, NO and PGI$_2$. At areas of endothelial damage platelets become activated leading to platelet adherence and activation. This effect is partly driven by stimulation of P2Y$_{12}$ receptors following from platelet release of ADP. This stimulation of P2Y$_{12}$ receptors inhibits the effects of cAMP and cGMP within platelets, making platelets more excitable, and amplifying platelet activation. When P2Y$_{12}$ receptors are blocked, cAMP and cGMP pathways are not inhibited by ADP and the inhibitory effects of NO and PGI$_2$ are sustained. The inhibitory effectiveness of P2Y$_{12}$ receptor blockade and DAPT in vivo is therefore strongly dependent upon the production of NO and PGI$_2$ within the circulation.
Figure 1

A. 

% Aggregation (4 U/mL collagen/mg)

- Pre-treatment
- DAPT

<table>
<thead>
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<td>DAPT</td>
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B. 

% Aggregation (25 µM TRAP-6)

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C. 

25 µM TRAP-6 induced Max ATP release (nMole)

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</table>
Figure 2

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)
Figure 4

[Diagram showing various signaling pathways and their interactions, including P2Y12 inhibition, platelet aggregation, NO, PGI2, and their regulatory mechanisms such as eNOS, COX, and PRASUGREL.]
SUPPORTING MATERIALS

P2Y₁₂ Receptor Blockade Synergises Strongly with Nitric Oxide and Prostacyclin to Inhibit Platelet Activation

Melissa V. Chan, PhD¹; Rebecca B.M. Knowles, MD¹; Martina H. Lundberg, PhD¹; Arthur T. Tucker, PhD¹; Nura A Mohamed, MRes²,³; Nicholas S. Kirkby, PhD¹,³; Paul C.J. Armstrong, PhD¹; Jane A Mitchell, PhD³; Timothy D. Warner, PhD¹

¹ The William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom.
² Qatar Foundation Research and Development Division, Doha, Qatar
³ National Heart & Lung Institute, Imperial College London, London, United Kingdom

*These authors contributed equally to the manuscript and their names appear in alphabetical order

Address for Correspondence:
Timothy D. Warner, PhD
The William Harvey Research Institute
Barts and the London School of Medicine and Dentistry
Charterhouse Square
London
EC1M 6BQ, UK
Telephone: +44 20 7882 2100
Fax: +44 20 7882 8252
Email: t.d.warner@qmul.ac.uk
1 SUPPORTING LEGENDS TO FIGURES

2

3 **Supplementary Figure 1.** Standard platelet aggregation tests. Standard light transmission aggregometry responses to AA (1 mM), ADP (5 µM), collagen (0.4 µg/mL) and U46619 (10 µM) in healthy volunteers before and following treatment with (A) aspirin (75 mg), (B) prasugrel (10 mg), or (C) DAPT (aspirin, 75 mg, plus prasugrel, 10 mg) for 7 days. N=8 for all. Significance is shown as * p<0.05 vs non-treated.

4

5 **Supplementary Figure 2.** The effect of DAPT on platelet aggregation and ATP release. Representative light transmission aggregometry traces of PRP before and after DAPT (aspirin, 75 mg, plus prasugrel, 10 mg) treatment in the presence of vehicle (NaOH, 10 mM), DEA/NONOate (100 nM), PGI₂ (1 nM), or DEA/NONOate+PGI₂ following stimulation with (A) collagen (4 µg/mL) or (B) TRAP-6 amide (25 µM). (C) Representative lumi-aggregometry traces in the same conditions after TRAP-6 amide (25 µM) stimulation, where ATP release is measured as an increase in voltage.

6

7 **Supplementary Figure 3.** The effect of aspirin and prasugrel on platelet aggregation. Healthy volunteers (n = 8) were treated with aspirin (75 mg) or prasugrel (10 mg) for 7 days. Aggregometry was conducted in the presence of vehicle (NaOH, 10 mM), DEA/NONOate (100 nM), PGI₂ (1 nM), or DEA/NONOate + PGI₂ before and after aspirin, using as agonists (A) collagen (4 µg/mL) or (B) TRAP-6 amide (25 µM), and before and after prasugrel, also using (C) collagen (4 µg/mL) or
(D) TRAP-6 amide (25 μM). Data are presented as final aggregation (%,
mean±SEM). Summary heatmaps after stimulation with (E) collagen (4 μg/mL) or (F)
TRAP-6 amide (25 μM) indicate maximum aggregation with red and minimum
aggregation with green, before treatment and after aspirin (75 mg), prasugrel (10
mg), or DAPT for 7 days. N = 8 for all. Significance is shown as * p<0.05 vs non-
treated, † p<0.05 vs NaOH drug-treated ‡ p<0.05 vs PGI₂ drug-treated.

Supplementary Figure 4. The effect of aspirin and prasugrel on platelet ATP
release. Healthy volunteers (n = 8) were treated with aspirin (75 mg) or prasugrel (10
mg) for 7 days. Lumi-aggregometry was conducted in the presence of vehicle
(NaOH, 10 mM), DEA/NONOate (100 nM), PGI₂ (1 nM), or DEA/NONOate + PGI₂
before and after (A) aspirin or (B) prasugrel, using collagen (4 μg/mL) as an agonist.
Data are presented as maximum ATP release (%, mean±SEM). (C) A summary
heatmap after stimulation with collagen (4 μg/mL) indicates maximum ATP release
with red and minimum ATP release with green before treatment and after aspirin (75
mg), prasugrel (10 mg) or DAPT for 7 days. N = 8 for all. Significance is shown as *
p<0.05 vs non-treated, † p<0.05 vs NaOH drug-treated ‡ p<0.05 vs PGI₂ drug-
treated.

Supplementary Figure 5. Representative control data for flow cytometry
experiments. GPIIb/IIIa activation by PAC-1 binding in the (A) absence and (B)
presence of DAPT, P-selectin expression in the (C) absence and (D) presence of
DAPT and VASP phosphorylation (Ser²³⁹) in the (E) absence and (F) presence of
DAPT was measured by flow cytometry in PRP stimulated with TRAP-6 (25 μM) in
the presence of vehicle (NaOH, 10 mM), DEA/NONOate (100 nM), PGI₂ (1 nM), or
DEA/NONOate + PGI₂. Histograms are representative of n = 3.

Supplementary Figure 6. The effect of aspirin, prasugrel and DAPT on P-selectin and glycoprotein IIb/IIIa. Healthy volunteers (n = 8) were treated with aspirin (75 mg), prasugrel (10 mg), or DAPT for 7 days. Heatmaps in response to TRAP-6 amide (25 μM)-stimulated PRP before and after DAPT treatment in the presence of vehicle (NaOH, 10 mM), DEA/NONOate (100 nM), PGI₂ (1 nM) or DEA/NONOate+PGI₂ were generated for (A) CD62P (P-selectin) and (B) PAC-1 (GPIIb/IIIa binding). Red represents maximum expression and green shows minimum expression with each cell representing data from 3 groups of 8 subjects.
Supplementary Figure 5

A

B

C

D

E

F