Combination of cyclic nucleotide modulators with P2Y\textsubscript{12} receptor antagonists as anti-platelet therapy

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ESSENTIALS

1. A synergistic relationship exists between cyclic nucleotides and P2Y\textsubscript{12} receptor inhibition.
2. Approved drugs that modulate cyclic nucleotide tone in platelets produce numerous side effects including headache.
3. Low dose GC activators synergise with P2Y\textsubscript{12} inhibition to produce a powerful anti-platelet effect without altering blood flow.
4. This novel combination can provide a strong and focused anti-thrombotic regimen.

ABSTRACT

Background: Endothelium-derived prostacyclin and nitric oxide elevate platelet cyclic nucleotide levels and maintain quiescence. We previously demonstrated a synergistic relationship exists between cyclic nucleotides and P2Y\textsubscript{12} receptor inhibition. A number of clinically approved drug classes can modulate cyclic nucleotide tone in platelets including activators of NO-sensitive guanylyl cyclase (GC) and phosphodiesterase (PDE) inhibitors. However, the doses required to inhibit platelets produce numerous side effects including headache.

Objective: We investigated using GC-activators in combination with P2Y\textsubscript{12} receptor antagonists as a way to selectively amplify the anti-thrombotic effect of both drugs.

Methods: \textit{In vitro} light transmission aggregation and platelet adhesion under flow were performed on washed platelets and platelet rich plasma. Aggregation in whole blood and a ferric chloride-induced arterial thrombosis model were also performed.

Results: The GC-activator BAY-70 potentiated the action of the P2Y\textsubscript{12} receptor inhibitor prasugrel active metabolite in aggregation and adhesion studies and was associated with raised intra-platelet cyclic nucleotide levels. Furthermore, mice administered sub-maximal doses of the GC activator cinaciguat together with the PDE inhibitor dipyridamole and prasugrel, showed significant inhibition of \textit{ex vivo} platelet aggregation and significantly reduced \textit{in vivo} arterial thrombosis in response to injury without alteration in basal carotid artery blood flow.
Conclusions: Using *in vitro*, *ex vivo* and *in vivo* functional studies, we show that low dose GC activators synergise with P2Y\(_{12}\) inhibition to produce powerful anti-platelet effects without altering blood flow. Therefore modulation of intra-platelet cyclic nucleotide levels alongside P2Y\(_{12}\) inhibition can provide a strong, focused anti-thrombotic regimen whilst minimising vasodilator side effects.

**INTRODUCTION**

Platelets play a central role in cardiovascular disease, as they are integral to the development of acute thrombotic events. For this reason, anti-platelet therapy is prescribed for the secondary prevention of atherothrombotic events in patients with acute coronary syndromes or following percutaneous coronary intervention [1, 2]. Aspirin, which irreversibly inhibits the cyclooxygenase enzyme and downstream thromboxane (Tx)A\(_2\) production [3, 4], is often coadministered with a P2Y\(_{12}\) receptor antagonist, such as clopidogrel or prasugrel, to produce dual anti-platelet therapy (DAPT). P2Y\(_{12}\) receptor antagonists inhibit platelet aggregation by blocking the amplifying effects of adenosine diphosphate (ADP) [5, 6]. Whilst such therapy is effective, recurrent events still occur [7, 8] and alternative ways to prevent thrombosis continue to be required.

Vascular endothelial cells produce the short-lived autacoids prostaglandin I\(_2\) (prostacyclin; PGI\(_2\)) and nitric oxide (NO) that relax blood vessels and inhibit platelets. PGI\(_2\) binds to platelet PGI\(_2\) (IP) receptors that in turn activate adenylyl cyclase (AC) to increase intracellular cyclic adenosine monophosphate (cAMP) levels [9]. In contrast, NO diffuses freely into platelets activating the \(\alpha_1\beta_1\) isoform of guanylyl cyclase (GC-1, formerly known as soluble GC) [10] to increase intracellular cyclic guanosine monophosphate (cGMP) levels [11]. This intra-platelet elevation of levels of individual cyclic nucleotides is synergistic in maintaining basal platelet quiescence and preventing inappropriate platelet activation [12]. Drugs targeting the NO-cGMP pathway, such as organic nitrates are long established clinically for treatment of heart
failure and angina pectoris [13]. In recent years, drugs which directly activate or stimulate GC have been developed as potential vasodilators and have been clinically approved for the treatment of pulmonary vascular disease [14]. Similarly, drugs which modulate the cAMP pathway such as PDE inhibitors and PGI₂ analogues are approved for the treatment of peripheral and pulmonary vascular disease. However, the doses of cyclic nucleotide elevating drugs that produce anti-platelet effects are associated with side effects such as headache, nausea and hypotension [15, 16]. This is consistent with the doses required to inhibit platelets being the same as those that produce vasodilatation.

We have recently demonstrated that blockade of platelet P2Y₁₂ receptor further synergises with PGI₂ and NO [17, 18] to produce profound platelet inhibition. We therefore hypothesised that the actions of pharmacological agents acting upon cyclic nucleotides could be selectively amplified in platelets by combination with P2Y₁₂ receptor antagonists, thereby producing an enhanced anti-platelet effect of both drugs at doses which do not produced systemic vasodilator side effects. Here we report in vitro, ex vivo and in vivo studies that support this hypothesis.

METHODS

Blood collection and isolation of human platelets

Use of human blood samples was approved by St Thomas’s Hospital Research Ethics Committee (Ref. 07/Q0702/24) and all studies were conducted in accordance with the Declaration of Helsinki. Blood was obtained by venepuncture from the median cubital vein using a 19G butterfly needle into tri-sodium citrate (0.32% w/v final; Sigma, UK). Blood from healthy volunteers free of antiplatelet drugs was centrifuged at 180 x g for 15 min to obtain platelet-rich-plasma (PRP). Where appropriate, washed platelets (WP) were isolated from PRP by further centrifugation (1000 x g, 10 min) in the presence of PGI₂ (1 μg/ml; Tocris, UK) and apyrase (0.02 U/ml; Sigma). The resulting pellet was washed in modified Tyrode’s (MTH) buffer (containing 134 mM NaCl, 2.9
mM KCl, 0.34 mM Na$_2$HPO$_4$, 12 mM NaHCO$_3$ and 1 mM MgCl$_2$; pH 7.4) containing HEPES (20 mM; Sigma) and 0.02 U/ml apyrase (Sigma) and re-suspended in MTH buffer to a concentration of 3 x10$^8$ platelets/ml.

Washed platelets, PRP or whole blood were treated either with vehicle (0.5% DMSO), P2Y$_{12}$ receptor antagonist prasugrel active metabolite (PAM; kind gift from AstraZeneca, Sweden), ARC66096 tetrasodium (Tocris, UK) and/or GC-1 activator BAY 60-2770 (BAY-70; kind gift from Dr. Johannes-Peter Stasch, Bayer AG, Germany) for 30 min at 37°C.

**Mouse strains**

C57Bl/6 wild-type (WT) mice were purchased from Charles River UK. All mice were 8-12 weeks old (20-25 g) and housed for a minimum of 7 days before commencement of experiments. They were housed on a 12-hour light-dark cycle, at a temperature of 22 to 24°C with access to water and food *ad libitum*. Animal procedures were conducted under UK Home Office project licence authority (PPL/8422) in accordance with “The Animals (Scientific Procedures) Act 1986”, EU directive 2010/63/EU, and were subject to local approval from Queen Mary University of London and Imperial College London Ethical Review Panels.

**Mouse dosing and obtaining blood**

To maximise clinical relevance *in vivo* we replaced PAM with prasugrel and BAY-70 with cinaciguat (BAY 58-2667, kind gift from Bayer AG, Germany), as both are approved for human administration. However, given the short half-life of intra-platelet cyclic nucleotides we co-administered the clinically used PDE inhibitor dipyridamole so as to maximise the detection of anti-platelet effects *ex vivo*. In total four test groups were conducted, 1) vehicle alone, 2) prasugrel alone, 3) cinaciguat & dipyridamole, or 4) prasugrel, cinaciguat & dipyridamole together (combined therapy).

Prasugrel (0.3mg/kg, Sigma) or vehicle (0.6% DMSO) *i.v.* plus dipyridamole (2 mg/kg; Sigma) or vehicle (0.03% v/v HCl) *i.p.* was administered 2 hours prior to blood collection.
or arterial injury. Mice were subsequently anaesthetised with ketamine (Narketan, 100 mg/kg; Vetoquinol, UK) and xylazine (Rompun, 10 mg/kg; Bayer, Germany) i.p. Cinaciguat (0.3mg/kg) or vehicle (2% DMSO) i.v. was administered 10 min prior to blood collection or arterial injury. Blood was collected from the inferior vena cava into tri-sodium citrate (0.32%).

**Light transmission aggregometry**
Aggregation in response to collagen (10 µg/ml, Horm collagen; Nycomed, Austria), thrombin (1 U/ml, Sigma), or thrombin receptor activating peptide SFLLRN (30 µM, TRAP-6, Bachem, Switzerland) was measured by light transmission aggregometry (LTA) in a Bio/Data PAP-8E turbidometric aggregometer (Alpha Laboratories, UK). Percent final aggregation, or percent inhibition of final aggregation values after 5 min are reported.

**Platelet adhesion under physiological flow**
Flow chamber slides (VI0.1 µ-slide, Ibidi, Germany) were coated with Horm collagen (100 µg/ml) followed by blocking with bovine serum albumin (BSA, 4%, Sigma). Whole blood, treated with mepacrine (10 µM, Sigma) to label platelets, was perfused across the coated surface at 1000s⁻¹ for 5 min. Post-flow images were taken (4 per experiment) at x40 magnification using a TE-2000S, Nikon Eclipse inverted microscope connected to a RT slider CCD camera (Diagnostic Instruments Inc., USA). Images were analysed using Image J (NIH, USA).

**Whole blood aggregation**
Aggregation was conducted as we have previously described [19]. Half-area 96-well microtiter plates (Greiner Bio-One, UK) were pre-coated with hydrogenated gelatin (0.75% w/v; Sigma) in phosphate-buffered saline to block nonspecific activation of blood. Horm collagen (10 µg/ml), TRAP-6 (30 µM), the PAR-4 activating peptide AYPGKF amide (PAR4-amide, 30 µM; Bachem), or the TxA₂ mimetic U46619 (3 µM;
Cayman Chemical Company, USA) were freeze-dried onto the plate and the plates vacuum sealed until needed.

At the time of experiment whole blood was placed into each well and aggregation stimulated by placing the plate on a heated plate shaker (Bioshake IQ, Q Instruments, Germany) at 37°C, mixing at 1200rpm, for 5 min. The single platelet counts of each well were determined by flow cytometry. Human platelets were labelled with allophycocyanin (APC) conjugated anti-CD61 monoclonal antibody (clone VI-PL2, Life Technologies, Hatfield, UK) for 30 min. Alternatively, mouse platelets were labelled using APC-conjugated anti-CD41 monoclonal antibody (clone MWReg30, Life Technologies) for 30 min. Samples were then diluted in phosphate buffered saline containing 0.1% formalin (Sigma), 0.1% dextrose (Sigma) and 0.2% BSA before addition of 10^4 CountBright™ absolute counting beads (Life Technologies). Labelled, diluted blood was then analysed using a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK).

**Mouse ferric chloride arterial injury**

In mice anaesthetised with ketamine and xylazine, the carotid artery was exposed and isolated from surrounding tissues to permit a ferric chloride (FeCl3, 10% solution, Sigma) soaked filter paper to be applied for 3 min. The carotid artery was then flooded with saline and the filter paper removed. A Doppler flow probe (Transonic, USA) was then placed around the artery and flow monitored for up to 30 min. The time to stable occlusion (defined as flow 0.0 ± 0.2ml/min for 1 min) was recorded.

**Mouse tail bleeding assay**

Anaesthetised mice were maintained at 37°C and their tail was transected with a scalpel blade at 1 mm from its end. The tail was immersed immediately in warm saline (37°C) and time recorded until it stopped bleeding for 30 sec.
Statistics and data analysis

Parametric data presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 6.0 (GraphPad software, USA). Significance was determined by one-way analysis of variance (ANOVA) with Dunnett’s post-hoc test unless otherwise stated, and data sets considered different if p<0.05. For non-parametric data significance was determined by Mantel-Cox test and with Holm-Sidak correction for multiple comparisons where necessary. Flow cytometry data was analysed using FlowJo v7.4 (Tree Star, USA). For analysis, the “single platelet” population was gated based on side scatter and anti-platelet immunoreactivity (fluorescence intensity).

RESULTS

BAY-70 potentiates the inhibitory action of PAM in washed platelets and PRP in a concentration-dependent manner.

Aggregation responses to thrombin were first established in WP (Figure 1a, 1b). A threshold concentration of PAM (3μM) that had little inhibitory effect when used alone was determined (64 ± 1% vehicle vs. 59 ± 1% PAM, p>0.05). Increasing concentrations of the GC-1 activator BAY-70 produced concentration-dependant potentiation of the inhibitory effect of PAM. At the maximum tested concentration of BAY-70 (10μM) in presence of PAM, aggregation was reduced by 54 ± 4% (p<0.05). The same concentration of BAY-70 in the absence of PAM had little inhibitory action, with aggregation being reduced by 7 ± 3% (p>0.05). A similar pattern was seen in experiments examining collagen-induced aggregation (Figure 1b). Interestingly, in these conditions BAY-70 achieved a 65 ± 4% decrease at the far lower concentration of 10nM in the presence of PAM, compared to a 17 ± 5% decrease without PAM (p<0.05).

In experiments using PRP, thrombin was replaced by the activator peptide TRAP-6. As in WP, PAM 3μM had little effect on TRAP-6 induced aggregation (77 ± 10%
vehicle; 70 ± 8% PAM; Figure 1c and 1d). Similarly, BAY-70 potentiated the effect of PAM against both TRAP-6 and collagen. Aggregation (Figure 1d) conducted in the presence of 10μM BAY-70 plus PAM were inhibited by 55 ± 10% and 76 ± 11% when induced by TRAP-6 and collagen respectively, compared to 15 ± 3% and 28 ± 5% respectively when PAM was not present (p<0.05 v BAY-70 plus PAM). In a separate mechanistic study (Suppl. Figure S1) we found that PAR4, as compared to PAR1, agonism was more sensitive to P2Y₁₂ antagonism in presence of BAY-70.

**BAY-70 potentiates the anti-thrombotic action of PAM in whole blood.**

Similar data were obtained in whole blood assays. In studies of whole blood platelet adhesion under flow, BAY-70 and PAM when used individually produced similar reductions in platelet coverage from 9.8 ± 2.6% (vehicle) to 5.4 ± 1.1% and 5.5 ± 0.9% respectively. Combination of BAY-70 and PAM together caused a further reduction of coverage to 2.3 ± 0.5% (p<0.05 v vehicle) (Figure 2a, 2b).

Aggregation in whole blood stimulated by TRAP-6 was determined by flow cytometry. In this assay, neither BAY-70 nor PAM alone had any inhibitory effect (final aggregations of 76 ± 8% vehicle versus 84 ± 4% and 76 ± 4% respectively, p>0.05). In contrast BAY-70 and PAM strongly combined to reduce aggregation by 71 ± 7% (p<0.05 v vehicle).

**Cyclic nucleotide modulators potentiate the inhibitory action of prasugrel in vivo.**

Having established that the GC-1 activator BAY-70 potentiates the anti-platelet effect of PAM we next sought to investigate if this can occur and is relevant in vivo.

Following in vivo drug administration, ex vivo platelet aggregation studies demonstrated no significant inhibitory effects of either prasugrel alone or the combination of two cyclic nucleotide elevating drugs – the GC-1 activator cinaciguat plus the PDE inhibitor dipyridamole (Figure 3a-c) at the selected doses. In contrast, platelets in blood from mice that had received the combination prasugrel with
cinaciguat plus dipyridamole demonstrated significantly lower aggregatory responses than those from vehicle treated animals (collagen, 22 ± 9% vs. 53 ± 9%; PAR-4 peptide, 7 ± 4% vs. 37 ± 9%; U46619, 13 ± 6% vs. 48 ± 11%; p<0.05 for all).

In the FeCl3-induced arterial thrombosis injury model prasugrel alone (time to occlusion, 438 ± 87 secs) or cinaciguat plus dipyridamole (482 ± 110 secs) had no effect relative to vehicle (432 ± 50 secs). In contrast, the combination of all three caused a significant increase in the time to occlusion (p<0.01) with only 2 of 6 mice fully occluding within 30mins. Consistent with these results we also observed a significantly extended bleeding time (p<0.01) in a tail transection model. No differences in blood flow in the carotid artery immediately prior to injury were noted (vehicle, 0.7 ± 0.1ml/min; prasugrel alone, 0.8 ± 0.1ml/min; cinaciguat plus dipyridamole, 0.8 ± 0.1ml/min; combination therapy, 0.9 ± 0.1ml/min) demonstrating that at the doses used the drugs did not cause systemic vasodilation alone or in combination.

**DISCUSSION**

Here we demonstrate that cyclic nucleotide level modulators can preferentially target platelets through combination with P2Y12 receptor antagonists. In all experiments we observed potentiation of the effects of PAM or prasugrel using direct GC-1 activation. Importantly, this combination of multiple drugs achieved anti-platelet protection using lower concentrations or doses that were ineffective individually and which did not cause systemic vasodilation *in vivo*.

In our *in vitro* experiments we observed this combinatorial effect against both adhesion and aggregation of platelets. Moreover, we established that this effect is maintained across a range of environments, from washed platelet preparations to platelet rich plasma or anti-coagulated whole blood. Finally, we translated our findings towards the clinic by demonstrating the combinatorial effect was displayed in *ex vivo* whole blood aggregation and in *in vivo* thrombosis models in mice that had received clinical formulations of these therapeutics.
Currently patients at risk of coronary thrombotic events receive a dual anti-platelet regimen consisting of aspirin plus a P2Y\textsubscript{12}-receptor antagonist such as clopidogrel or prasugrel [20, 21]. However, research efforts continue to identify the optimal combination of existing medications. One aspect, assessed in studies such as GLOBAL LEADERS and TWILIGHT [22, 23], is whether aspirin is essential as a baseline therapy. We have previously described how in the presence of strong P2Y\textsubscript{12} receptor blockade, addition of aspirin could produce a net pro-thrombotic effect and so potentially a reduction in clinical efficacy [24-26]. An alternative therefore is to use P2Y\textsubscript{12} antagonists as a baseline therapy with the addition of anti-thrombotic drugs acting upon other pathways. Since the effects of P2Y\textsubscript{12} receptor antagonists in vivo may be, at least partly, dependent upon the presence of the endothelial autacoids NO and PGI\textsubscript{2} [27], their clinical effectiveness could well be reduced in patients with endothelial dysfunction, which is an early event in the pathophysiology of cardiovascular disease [28]. During endothelial dysfunction NO and PGI\textsubscript{2} production will be reduced, leading to reduced intra-platelet cyclic nucleotide tone and so increased platelet reactivity. Therefore, it may well be those patients with the greatest level of endothelial dysfunction that get the smallest benefit from adequate P2Y\textsubscript{12} inhibition. Indeed we recently reported that the P2Y\textsubscript{12} inhibitor ticagrelor attenuated platelet function more potently in samples from well-trained middle-aged men with a superior vascular function compared to matched untrained men with a reduced vascular function [29].

A logical extension of the argument above is that drug therapy to increase intra-platelet cyclic nucleotides would boost the anti-platelet effect of P2Y\textsubscript{12} antagonists. There exists a number of agents available to modulate cyclic nucleotide levels, however we chose to focus upon the relatively recently developed direct GC-1 activators [30] in combination with standard drugs already used for anti-thrombotic prophylaxis. These GC-1 activators directly act upon NO-sensitive GC to stimulate cGMP production.
without the requirement of NO or the haem moiety [31], the separation of which can occur during endothelial dysfunction and oxidative stress [14].

Our initial in vitro experiments were completed using the compound BAY-70 as a pharmacological tool which is effective at raising cGMP levels [32]. Notably it has also previously been reported that BAY-70, in the micro-molar range, can inhibit washed platelet activation [33]. In our experiments in washed platelet preparations, nano-molar concentrations were sufficient to inhibit platelet activation in the presence of PAM.

For the in vivo studies we chose to use the related compound cinaciguat. Cinaciguat has previously been studied in a phase IIb clinical trial in patients with acute decompensated heart failure [15], and therefore has potentially more clinical relevance. We also opted to include dipyridamole as a PDE inhibitor with the intention to prolong cyclic nucleotide tone and thus detect their influence ex vivo. Dipyridamole has historically been prescribed combined with aspirin as anti-thrombotic therapy for patients who have had an ischaemic stroke. Its use however, like that of other drugs targeting cyclic nucleotides modulation, is associated with localised or systemic vasodilation [16] due to effects on the vascular smooth muscle. In our study, we administered lower doses of cinaciguat and dipyridamole as we hypothesised that in the presence of platelet P2Y12 receptor blockade there would be a synergistic focus of effects upon platelet function and away from the vasculature (Figure 4). Indeed we did not observe any significant change to arterial blood flow in our in vivo studies in any of our treatment groups whilst observing clear anti-thrombotic effects only in our combined treatment group.

Whilst our study primarily centred upon directly activating NO-sensitive GC, the physiology of the synergy resulting in increased P2Y12 efficacy means that this could realistically be achieved using alternative clinically available agents targeting a number of pathways to equally modulate cyclic nucleotide tone. For example the related compound riociguat, approved for use in pulmonary hypertension, acts by a related
mechanism of stimulating, rather than activating, GC-1. Alternatively, selexipag, approved for use in pulmonary arterial hypertension [34], acts as an agonist of the PGI₂ IP receptor and stimulates AC-dependant cAMP production [35]. Equally, selective PDE isoform inhibitors, such as cilostazol that targets PDE3 and is used in the management of intermittent claudication, may be effective for the targeted prolongation of platelet cyclic nucleotide half-life.

Another important component of this study is that the concentrations or doses of BAY-70 or cinaciguat, PAM or prasugrel, and dipyridamole used had little functional effect when used on their own. This means that it may be possible to achieve therapeutic effectiveness using lower doses than those currently prescribed for individual use and so reducing drug-associated side effects.

In conclusion, our study builds upon our previous observations of a synergistic relationship between P2Y₁₂ receptor inhibition and platelet cyclic nucleotide levels to identify a novel potential anti-platelet drug regimen. We demonstrate the principle of a combination of low doses of approved drugs targeted at cyclic nucleotide modulation, combined with P2Y₁₂ inhibition, as a realistic and powerful therapeutic regimen. Whilst more work and optimisation will be required to clinically translate this in human studies, such combined pharmacological approaches represent a focused anti-platelet regimen whilst potentially sparing associated off-target side effects.

**AUTHOR CONTRIBUTIONS**

P Armstrong designed the research, conducted experiments, interpreted the data, and wrote the manuscript. P Ferreira, M Chan, M Lundberg Slingsby, C Shih and M Crescente conducted experiments and wrote the manuscript. A Hobbs interpreted the data and wrote the manuscript. N Kirkby and T Warner designed the research interpreted the data and wrote the manuscript.
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CONFLICT OF INTEREST

TDW has received research grants and consultancy fees from AstraZeneca. AJH is a scientific advisory board member/consultant for Novo Nordisk and Palatin Technologies Inc., and has received research support from Palatin Technologies Inc. All other authors have no conflict of interests to declare.

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**FIGURE LEGENDS**

**Figure 1:** GC-1 activator BAY-70 potentiates anti-platelet actions of the P2Y₁₂ inhibitor PAM *in vitro*. (A) Representative LTA trace of aggregation in response to thrombin (1 U/ml) of washed platelets treated with BAY-70 (1µM) and/or PAM (3µM). (B) Concentration inhibitor curves (as % of final aggregation after 5 minutes) for BAY-70 in presence of vehicle or PAM against aggregation induced by thrombin or collagen (10 µg/ml). (C) Representative LTA trace of aggregation to TRAP-6 (30 µM) of PRP treated with BAY-70 (10µM) and/or PAM (3µM). (D) Concentration inhibitor curves (as % of final aggregation after 5 minutes) for BAY-70 in presence of vehicle or PAM against aggregation induced by TRAP-6 or collagen (10 µg/ml). Data presented as mean ± SEM. *p<0.05 by two-way ANOVA, n=4 for all.

**Figure 2:** GC-1 activator BAY-70 potentiates inhibition by PAM of platelet adhesion and aggregation in whole blood. (A) Representative images of platelet (green) adhesion to collagen (100 µg/ml) following perfusion (1000 s⁻¹ for 5 minutes). Images acquired at x40 magnification using a Nikon TE-2000S inverted microscope. (B) Quantification of area covered (%), n=6. (C) Aggregation (%), n=7) in whole blood in response to TRAP-6 (30 µM) using flow cytometry. Data presented as mean ± SEM. **p<0.01 vs. vehicle by paired ANOVA.

**Figure 3:** GC-1 activator cinaciguat, in combination with dipyridamole, potentiates the inhibitory effects of prasugrel against *ex vivo* aggregation and *in vivo* thrombosis in
mice. (A) Aggregation in whole blood in response to collagen (10 µg/ml), PAR-4 amide (30 µM) and TxA₂ mimetic U46619 (3 µM). *p<0.05 vs. vehicle, n=5-7 per group. (B) Kaplan Meier plot for occlusion against time (min) following FeCl₃-induced arterial injury in mice. **p<0.01 vs vehicle by Mantel-Cox test with Holm-Sidak correction, n=6 per group. (C) Kaplan Meier plot of bleeding against time for vehicle and combined treatment groups. **p<0.01 vs vehicle by Mantel-Cox test, n=6 per group.

**Figure 4:** Summary of the patho-physiological rationale for the efficacy of combined cyclic nucleotide modulators with P2Y₁₂ receptor antagonists as anti-platelet therapy. (A) In the healthy circulation endothelial-derived mediators NO and PGI₂ act upon platelets to raise cyclic nucleotide (cAMP and cGMP) levels that in turn maintain platelets in a quiescent state. (B) During established cardiovascular disease, concurrent endothelial dysfunction results in reduced production of NO and PGI₂, lowering intra-platelet cyclic nucleotide tone and decreasing the threshold for activation. (C) A synergic relationship exists between intra-platelet cyclic nucleotides and P2Y₁₂ receptor blockade such that pharmacological modulators of cyclic nucleotides, to compensate for reduced endothelial cell function, combined with P2Y₁₂ receptor antagonist produces a focused anti-platelet effects at low-doses of each associated with reduced drug side effects.