Combination of cyclic nucleotide modulators with $P2Y_{12}$ receptor antagonists as anti-platelet therapy

Paul C. Armstrong[1], Plinio M. Ferreira[1], Melissa V. Chan[1], Martina H. Lundberg Slingsby[1], Marilena Crescente[1], Chih-Chin Shih[1], Nicholas S. Kirkby[2], Adrian J. Hobbs[3], Timothy D. Warner[1]

[1]Blizard Institute, Barts & the London School of Medicine & Dentistry, Queen Mary University of London, 4 Newark Street, London, E1 2AT, UK. [2]Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London, Dovehouse Street, London, SW3 6LY, UK. [3]William Harvey Research Institute, Barts & the London School of Medicine & Dentistry, Queen Mary University of London, Charterhouse Square, EC1M 6BQ, UK.

Address for correspondence:

Paul Armstrong, PhD

Blizard Institute, Barts & the London School of Medicine & Dentistry, Queen Mary University of London, 4 Newark Street, London, E1 2AT, UK

email: p.c.armstrong@qmul.ac.uk

Word Count: 250 words (Abstract), 3131 words (main body text)

Key words: Blood Platelets; Thrombosis; Nucleotides, Cyclic; Pharmacology; Purinergic P2Y Receptor Antagonists

Running title: cyclic nucleotides potentiate P2Y12 platelet inhibition

ESSENTIALS

1. A synergistic relationship exists between cyclic nucleotides and $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ receptor antagonists as a way to selectively amplify the anti-thrombotic effect of both drugs.

Methods: *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 $P2Y_{12}$ 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 *ex vivo* platelet aggregation and significantly reduced *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 PGI_2 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 $P2Y_{12}$ receptor further synergises with PGI_2 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 $P2Y_{12}$ 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 i*n 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 PGI_2 (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_2HPO_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 37ºC.

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 TxA_2 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$-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 $P2Y_{12}$ 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 $P2Y_{12}$ antagonists as a baseline therapy with the addition of anti-thrombotic drugs acting upon other pathways. Since the effects of $P2Y_{12}$ receptor antagonists *in vivo* may be, at least partly, dependent upon the presence of the endothelial autacoids NO and PGI_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_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 $P2Y_{12}$ inhibition. Indeed we recently reported that the $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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 PGI_2 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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.

ACKNOWLEDGEMENTS

This work was supported by the British Heart Foundation (PG/15/79/31777, PG/15/47/31591, FS/16/1/31699) and FAPESP (São Paulo Research Foundation – 14/04478-5).

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.

REFERENCES

1 Steinhubl SR, Berger PB, Mann JT, 3rd, Fry ET, DeLago A, Wilmer C, Topol EJ, Observation CICftRoED. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. *JAMA*. 2002; **288**: 2411-20.

2 Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial I. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med*. 2001; **345**: 494-502.

3 Warner TD, Nylander S, Whatling C. Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. *Br J Clin Pharmacol*. 2011; **72**: 619-33.

4 Vane JR, Botting RM. The mechanism of action of aspirin. *Thromb Res*. 2003; **110**: 255-8.

5 Michelson AD. P2Y12 antagonism: promises and challenges. *Arterioscler Thromb Vasc Biol*. 2008; **28**: s33-8.

6 Armstrong PC, Dhanji AR, Tucker AT, Mitchell JA, Warner TD. Reduction of platelet thromboxane A2 production ex vivo and in vivo by clopidogrel therapy. *J Thromb Haemost*. 2010; **8**: 613-5.

7 Committee CS. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet*. 1996; **348**: 1329-39.

8 Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA, Investigators P, Freij A, Thorsen M. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009; **361**: 1045-57.

9 Armstrong RA. Platelet prostanoid receptors. *Pharmacol Ther*. 1996; **72**: 171-91.

10 Alexander SP, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA, Collaborators C. THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Enzymes. *Br J Pharmacol*. 2017; **174 Suppl 1**: S272-S359.

11 Moro MA, Russel RJ, Cellek S, Lizasoain I, Su Y, Darley-Usmar VM, Radomski MW, Moncada S. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc Natl Acad Sci U S A*. 1996; **93**: 1480-5.

12 Radomski MW, Palmer RM, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol*. 1987; **92**: 639-46.

13 Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. *Br J Pharmacol*. 2007; **151**: 305-21.

14 Papapetropoulos A, Hobbs AJ, Topouzis S. Extending the translational potential of targeting NO/cGMP-regulated pathways in the CVS. *Br J Pharmacol*. 2015; **172**: 1397-414.

15 Erdmann E, Semigran MJ, Nieminen MS, Gheorghiade M, Agrawal R, Mitrovic V, Mebazaa A. Cinaciguat, a soluble guanylate cyclase activator, unloads the heart but also causes hypotension in acute decompensated heart failure. *Eur Heart J*. 2013; **34**: 57-67.

16 European Stroke Prevention Study 2. Efficacy and safety data. *J Neurol Sci*. 1997; **151 Suppl**: S1-77.

17 Kirkby NS, Lundberg MH, Chan MV, Vojnovic I, Solomon AB, Emerson M, Mitchell JA, Warner TD. Blockade of the purinergic P2Y12 receptor greatly increases

the platelet inhibitory actions of nitric oxide. *Proc Natl Acad Sci U S A*. 2013; **110**: 15782-7.

18 Chan MV, Knowles RB, Lundberg MH, Tucker AT, Mohamed NA, Kirkby NS, Armstrong PC, Mitchell JA, Warner TD. P2Y12 receptor blockade synergizes strongly with nitric oxide and prostacyclin to inhibit platelet activation. *Br J Clin Pharmacol*. 2016; **81**: 621-33.

19 Armstrong PC, Kirkby NS, Chan MV, Finsterbusch M, Hogg N, Nourshargh S, Warner TD. Novel whole blood assay for phenotyping platelet reactivity in mice identifies ICAM-1 as a mediator of platelet-monocyte interaction. *Blood*. 2015; **126**: e11-8.

20 Levine GN, Bates ER, Blankenship JC, Bailey SR, Bittl JA, Cercek B, Chambers CE, Ellis SG, Guyton RA, Hollenberg SM, Khot UN, Lange RA, Mauri L, Mehran R, Moussa ID, Mukherjee D, Nallamothu BK, Ting HH. 2011 ACCF/AHA/SCAI Guideline for Percutaneous Coronary Intervention: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines and the Society for Cardiovascular Angiography and Interventions. *Circulation*. 2011; **124**: e574-651.

21 Amsterdam EA, Wenger NK, Brindis RG, Casey DE, Jr., Ganiats TG, Holmes DR, Jr., Jaffe AS, Jneid H, Kelly RF, Kontos MC, Levine GN, Liebson PR, Mukherjee D, Peterson ED, Sabatine MS, Smalling RW, Zieman SJ. 2014 AHA/ACC Guideline for the Management of Patients with Non-ST-Elevation Acute Coronary Syndromes: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2014; **64**: e139-e228.

22 Vranckx P, Valgimigli M, Windecker S, Steg PG, Hamm C, Juni P, Garcia-Garcia HM, van Es GA, Serruys PW. Long-term ticagrelor monotherapy versus standard dual antiplatelet therapy followed by aspirin monotherapy in patients undergoing biolimus-eluting stent implantation: rationale and design of the GLOBAL LEADERS trial. *EuroIntervention*. 2016; **12**: 1239-45.

23 Mehran R, Baber U, Sharma SK, Cohen DJ, Angiolillo DJ, Briguori C, Cha JY, Collier T, Dangas G, Dudek D, Dzavik V, Escaned J, Gil R, Gurbel P, Hamm CW, Henry T, Huber K, Kastrati A, Kaul U, Kornowski R, Krucoff M, Kunadian V, Marx SO, Mehta SR, Moliterno D, Ohman EM, Oldroyd K, Sardella G, Sartori S, Shlofmitz R, Steg PG, Weisz G, Witzenbichler B, Han YL, Pocock S, Gibson CM. Ticagrelor with or without Aspirin in High-Risk Patients after PCI. *N Engl J Med*. 2019; **381**: 2032-42.

24 Warner TD, Armstrong PC, Curzen NP, Mitchell JA. Dual antiplatelet therapy in cardiovascular disease: does aspirin increase clinical risk in the presence of potent P2Y12 receptor antagonists? *Heart*. 2010; **96**: 1693-4.

25 Armstrong PC, Leadbeater PD, Chan MV, Kirkby NS, Jakubowski JA, Mitchell JA, Warner TD. In the presence of strong P2Y12 receptor blockade, aspirin provides little additional inhibition of platelet aggregation. *J Thromb Haemost*. 2011; **9**: 552-61.

26 Kirkby NS, Leadbeater PD, Chan MV, Nylander S, Mitchell JA, Warner TD. Antiplatelet effects of aspirin vary with level of P2Y(1)(2) receptor blockade supplied by either ticagrelor or prasugrel. *J Thromb Haemost*. 2011; **9**: 2103-5.

27 Vane JR. The Croonian Lecture, 1993. The endothelium: maestro of the blood circulation. *Philos Trans R Soc Lond B Biol Sci*. 1994; **343**: 225-46.

28 Warner TD, Armstrong PC, Chan MV, Knowles RB. The importance of endothelium-derived mediators to the efficacy of dual anti-platelet therapy. *Expert Rev Hematol*. 2016; **9**: 223-5.

29 Lundberg Slingsby MH, Gliemann L, Thrane M, Rytter N, Egelund J, Chan MV, Armstrong PC, Warner TD, Hellsten Y. Platelet responses to pharmacological and physiological interventions in middle-aged men with different habitual physical activity levels. *Acta Physiol (Oxf)*. 2018.

30 Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov*. 2006; **5**: 755-68.

31 Stasch JP, Hobbs AJ. NO-independent, haem-dependent soluble guanylate cyclase stimulators. *Handb Exp Pharmacol*. 2009: 277-308.

32 Holt AW, Martin DN, Shaver PR, Adderley SP, Stone JD, Joshi CN, Francisco JT, Lust RM, Weidner DA, Shewchuk BM, Tulis DA. Soluble guanylyl cyclase-activated cyclic GMP-dependent protein kinase inhibits arterial smooth muscle cell migration independent of VASP-serine 239 phosphorylation. *Cell Signal*. 2016; **28**: 1364-79.

33 Mendes-Silverio CB, Leiria LO, Morganti RP, Anhe GF, Marcondes S, Monica FZ, De Nucci G, Antunes E. Activation of haem-oxidized soluble guanylyl cyclase with BAY 60-2770 in human platelets lead to overstimulation of the cyclic GMP signaling pathway. *PLoS One*. 2012; **7**: e47223.

34 Sitbon O, Channick R, Chin KM, Frey A, Gaine S, Galie N, Ghofrani HA, Hoeper MM, Lang IM, Preiss R, Rubin LJ, Di Scala L, Tapson V, Adzerikho I, Liu J,

Moiseeva O, Zeng X, Simonneau G, McLaughlin VV, Investigators G. Selexipag for the Treatment of Pulmonary Arterial Hypertension. *N Engl J Med*. 2015; **373**: 2522-33.

35 Kuwano K, Hashino A, Asaki T, Hamamoto T, Yamada T, Okubo K, Kuwabara K. 2-[4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy]-N-(methylsulfonyl)acetamide (NS-304), an orally available and long-acting prostacyclin receptor agonist prodrug. *J Pharmacol Exp Ther*. 2007; **322**: 1181-8.

FIGURE LEGENDS

Figure 1: GC-1 activator BAY-70 potentiates anti-platelet actions of the $P2Y_{12}$ 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 TxA_2 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 $P2Y_{12}$ receptor antagonists as anti-platelet therapy. (A) In the healthy circulation endothelial-derived mediators NO and PGI_2 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 PGI_2, lowering intra-platelet cyclic nucleotide tone and decreasing the threshold for activation. (C) A synergic relationship exists between intra-platelet cyclic nucleotides and $P2Y_{12}$ receptor blockade such that pharmacological modulators of cyclic nucleotides, to compensate for reduced endothelial cell function, combined with $P2Y_{12}$ receptor antagonist produces a focused anti-platelet effects at low-doses of each associated with reduced drug side effects.