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ORIGINAL ARTICLE

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

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Abstract

Background: Endothelium-derived prostacyclin and nitric oxide elevate platelet cyclic nucleotide levels and maintain quiescence. We previously demonstrated that 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 synergize 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 while minimizing vasodilator side effects.

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KEYWORDS
blood platelets, cyclic, nucleotides, pharmacology, purinergic P2Y receptor antagonists, thrombosis

1 | 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] While 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 phosphodiesterase (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 synergizes with PGI_2 and NO[17,18] to produce profound platelet inhibition. We therefore hypothesized 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 produce systemic vasodilator side effects. Here we report in vitro, ex vivo, and in vivo studies that support this hypothesis.

Essentials

- A synergistic relationship exists between cyclic nucleotides and $P2Y_{12}$ receptor inhibition.
- Approved drugs that modulate cyclic nucleotide tone in platelets produce numerous side effects including headache.
- Low dose guanylyl cyclase activators synergize with $P2Y_{12}$ inhibition to produce a powerful anti-platelet effect without altering blood flow.
- This novel combination can provide a strong and focused anti-thrombotic regimen.

2 | METHODS

2.1 | 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 venipuncture 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 × *g* for 15 minutes to obtain platelet-rich-plasma (PRP). Where appropriate, washed platelets (WP) were isolated from PRP by further centrifugation (1000 × *g*, 10 minutes) in the presence of PGI_2 (1 μg/mL; Tocris) and apyrase (0.02 U/mL; Sigma). The resulting pellet was washed in modified Tyrode's (MTH) buffer (containing 134 mmol/L NaCl, 2.9 mmol/L KCl, 0.34 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$, and 1 mmol/L $MgCl_2$; pH 7.4) containing HEPES (20 mmol/L; Sigma) and 0.02 U/mL apyrase (Sigma) and resuspended in MTH buffer to a concentration of 3×10^8 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), ARC66096 tetrasodium (Tocris), and/or GC-1 activator BAY 60-2770 (BAY-70; kind gift from Dr Johannes-Peter Stasch, Bayer AG) for 30 minutes at 37°C.

2.2 | Mouse strains

C57Bl/6 wild-type (WT) mice were purchased from Charles River UK. All mice were 8 to 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 license 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.

2.3 | Mouse dosing and obtaining blood

To maximize clinical relevance in vivo we replaced PAM with prasugrel and BAY-70 with cinaciguat (BAY 58-2667, kind gift from Bayer AG), as both are approved for human administration. However, given the short half-life of intra-platelet cyclic nucleotides we coadministered the clinically used PDE inhibitor dipyridamole so as to maximize the detection of anti-platelet effects ex vivo. In total four test groups were conducted: (a) vehicle alone; (b) prasugrel alone; (c) cinaciguat and dipyridamole; or (d) prasugrel, cinaciguat, and dipyridamole together (combined therapy).

Prasugrel (0.3 mg/kg, Sigma) or vehicle (0.6% DMSO) intravenous plus dipyridamole (2 mg/kg; Sigma) or vehicle (0.03% v/v HCl) intraperitoneal was administered 2 hours prior to blood collection or arterial injury. Mice were subsequently anesthetized with ketamine (Narketan, 100 mg/kg; Vetoquinol) and xylazine (Rompun, 10 mg/kg; Bayer, Germany) intraperitoneal cinaciguat (0.3 mg/kg) or vehicle (2% DMSO) intravenous was administered 10 minutes prior to blood collection or arterial injury. Blood was collected from the inferior vena cava into trisodium citrate (0.32%).

2.4 | Light transmission aggregometry

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

2.5 | Platelet adhesion under physiological flow

Flow chamber slides (VI0.1 µ-slide, Ibidi) were coated with Horm collagen (100 µg/mL) followed by blocking with bovine serum albumin (BSA, 4%, Sigma). Whole blood, treated with mepacrine (10 µmol/L, Sigma) to label platelets, was perfused across the coated surface at 1000 s^{-1} for 5 minutes. Post-flow images were taken (four per experiment) at x40 magnification using a TE-2000S, Nikon Eclipse inverted microscope connected to a RT slider CCD camera (Diagnostic Instruments Inc). Images were analyzed using Image J (NIH).

2.6 | 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 µmol/L), the PAR-4 activating peptide AYPGKF amide (PAR4-amide, 30 µmol/L; Bachem), or the TxA_2 mimetic U46619 (3 µmol/L; Cayman Chemical Company) 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) at 37°C, mixing at 1200 rpm, for 5 minutes. 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) for 30 minutes. Alternatively, mouse platelets were labelled using APC-conjugated anti-CD41 monoclonal antibody (clone MWReg30, Life Technologies) for 30 minutes. 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 analyzed using a FACSCalibur flow cytometer (BD Biosciences).

2.7 | Mouse ferric chloride arterial injury

In mice anesthetized 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 minutes. The carotid artery was then flooded with saline and the filter paper removed. A Doppler flow probe (Transonic) was then placed around the artery and flow monitored for up to 30 minutes. The time to stable occlusion (defined as flow 0.0 ± 0.2 mL/min for 1 minute) was recorded.

2.8 | Mouse tail bleeding assay

Anesthetized 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 seconds.

2.9 | 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). 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 $P < .05$. 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 analyzed using FlowJo v7.4 (Tree Star). For analysis, the "single platelet" population was gated based on side scatter and anti-platelet immunoreactivity (fluorescence intensity).

3 | RESULTS

3.1 | 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,B). A threshold concentration of PAM (3 µmol/L) that had little inhibitory effect when used alone was determined (64 ± 1% vehicle versus 59 ± 1% PAM, $P > .05$). Increasing concentrations of the GC-1 activator BAY-70 produced concentration-dependent potentiation of the inhibitory effect of PAM. At the maximum tested concentration of BAY-70 (10 µmol/L) in presence of PAM, aggregation was reduced by 54 ± 4% ($P < .05$). The same concentration of BAY-70 in the absence of PAM had little inhibitory action, with aggregation being reduced by 7 ± 3% ($P > .05$). 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 10 nmol/L in the presence of PAM, compared to a 17 ± 5% decrease without PAM ($P < .05$).

In experiments using PRP, thrombin was replaced by the activator peptide TRAP-6. As in WP, PAM 3 µmol/L had little effect on TRAP-6 induced aggregation (77 ± 10% vehicle; 70 ± 8% PAM; Figure 1C,D). Similarly, BAY-70 potentiated the effect of PAM against both TRAP-6 and collagen. Aggregation (Figure 1D) conducted in the presence of 10 µmol/L 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 ($P < .05$ versus BAY-70 plus PAM). In a separate mechanistic study (Figure S1 in supporting information) we found that PAR4, as compared to PAR1, agonism was more sensitive to $P2Y_{12}$ antagonism in the presence of BAY-70.

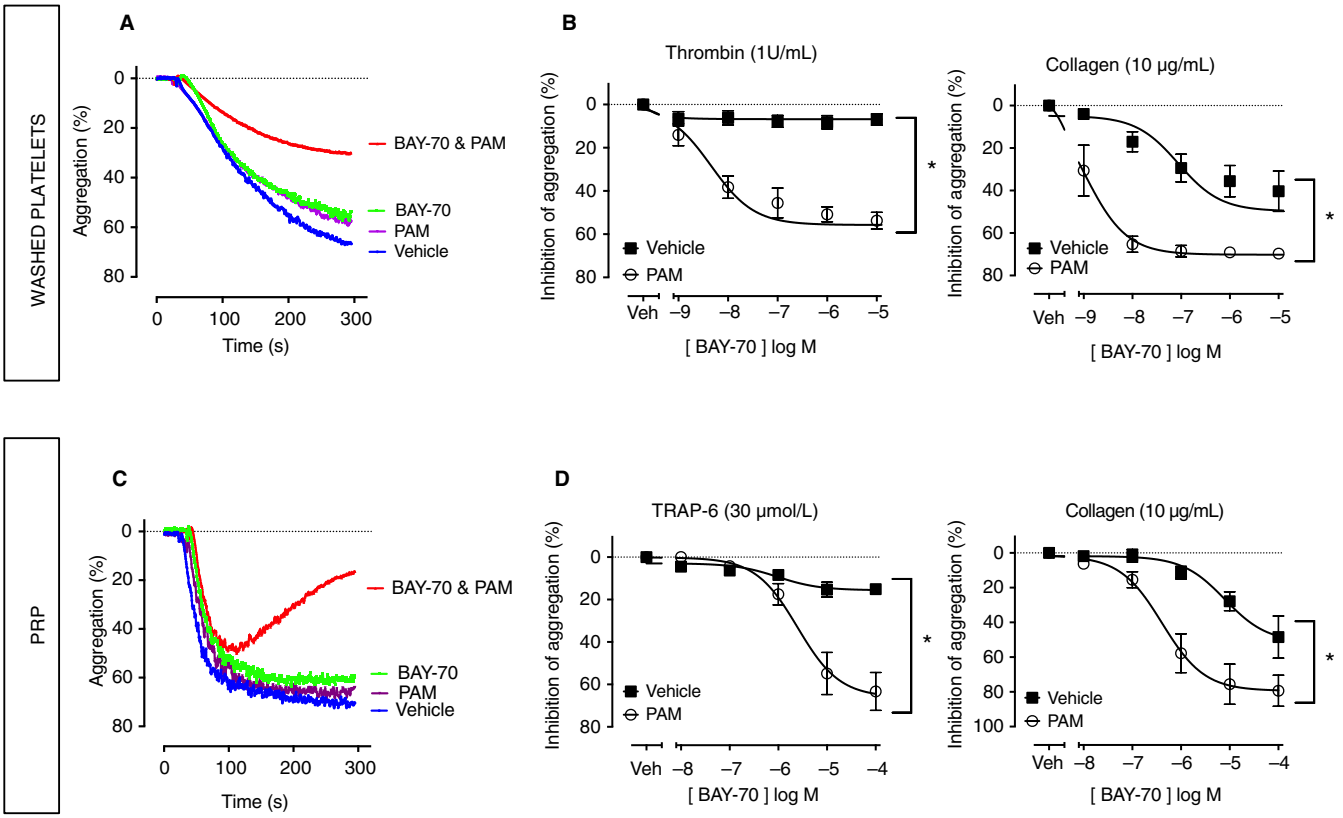

FIGURE 1 Guanylyl cyclase (GC-1) activator BAY-70 potentiates anti-platelet actions of the $P2Y_{12}$ inhibitor prasugrel active metabolite (PAM) in vitro. A, Representative light transmission aggregometry (LTA) trace of aggregation in response to thrombin (1 U/mL) of washed platelets treated with BAY-70 (1 µmol/L) and/or PAM (3 µmol/L). 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 µmol/L) of platelet-rich plasma (PRP) treated with BAY-70 (10 µmol/L) and/or PAM (3 µmol/L). 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 ± standard error of the mean. *$P < .05$ by two-way analysis of variance, n = 4 for all

3.2 | 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% ($P < .05$ versus vehicle; Figure 2A,B).

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, $P > .05$). In contrast BAY-70 and PAM strongly combined to reduce aggregation by 71 ± 7% ($P < .05$ versus vehicle; Figure 2C).

3.3 | 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% versus 53 ± 9%; PAR-4 peptide, 7 ± 4% versus 37 ± 9%; U46619, 13 ± 6% versus 48 ± 11%; $P < .05$ for all).

In the FeCl3-induced arterial thrombosis injury model prasugrel alone (time to occlusion, 438 ± 87 seconds) or cinaciguat plus dipyridamole (482 ± 110 seconds) had no effect relative to vehicle (432 ± 50 seconds). In contrast, the combination of all three caused a significant increase in the time to occlusion ($P < .01$) with only two of six mice fully occluding within 30 minutes. Consistent with these results we also observed a significantly extended bleeding time ($P < .01$) in a tail transection model. No differences in blood flow in the carotid artery immediately prior to injury were noted (vehicle, 0.7 ± 0.1 mL/min; prasugrel alone, 0.8 ± 0.1 mL/min; cinaciguat plus dipyridamole, 0.8 ± 0.1 mL/min; combination therapy, 0.9 ± 0.1 mL/min) demonstrating that at the doses used the drugs did not cause systemic vasodilation alone or in combination.

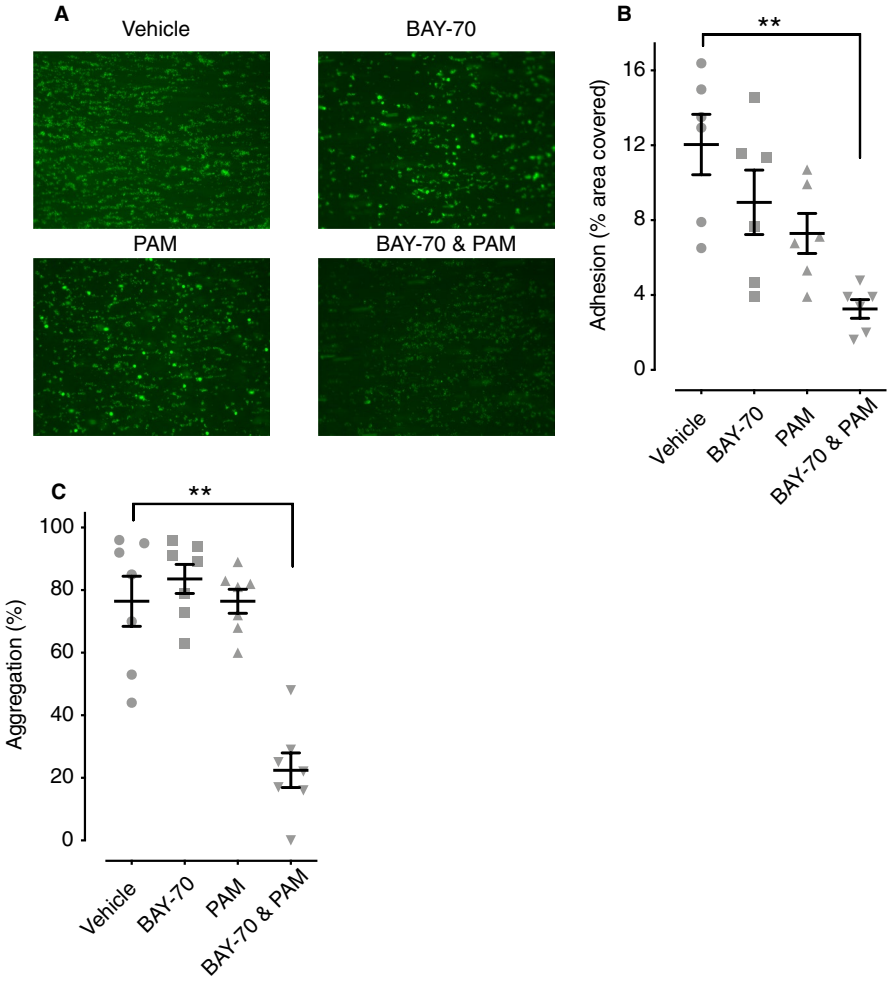

FIGURE 2 Guanylyl cyclase (GC-1) activator BAY-70 potentiates inhibition by prasugrel active metabolite (PAM) of platelet adhesion and aggregation in whole blood. A, Representative images of platelet (green) adhesion to collagen (100 µg/mL) following perfusion (1000 s^{-1} for 5 minutes). Images acquired at ×40 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 µmol/L) using flow cytometry. Data presented as mean ± standard error of the mean. **$P < .01$ versus vehicle by paired analysis of variance

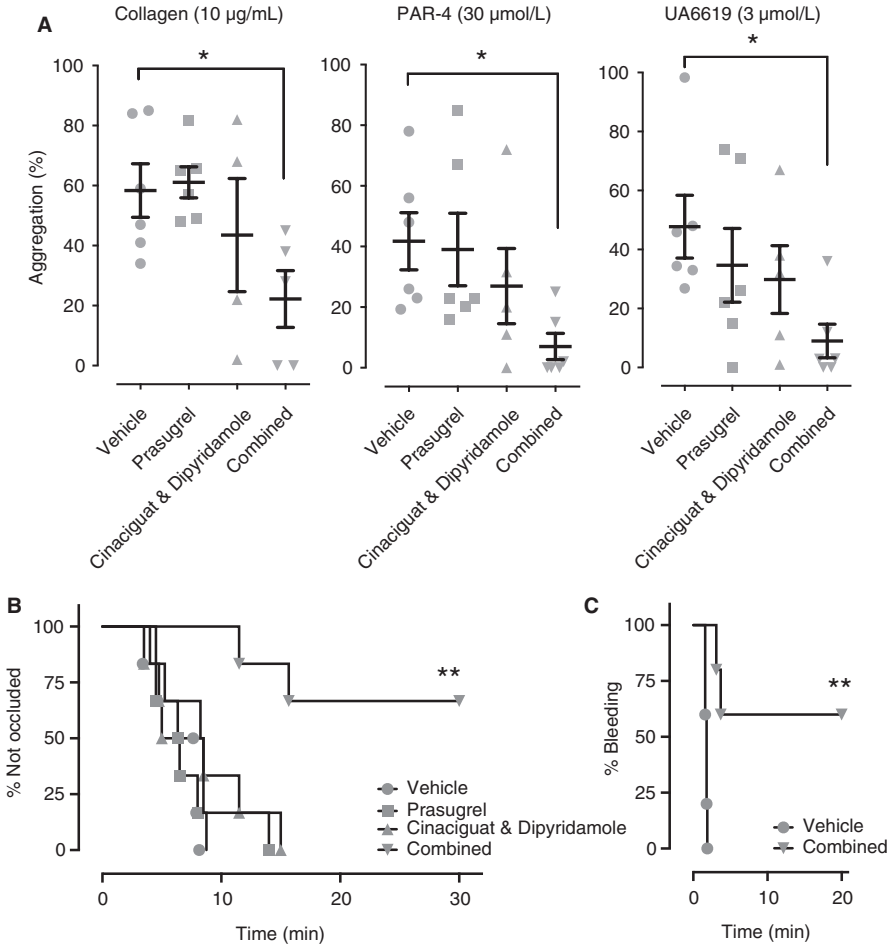

FIGURE 3 Guanylyl cyclase (GC-1) activator cinaciguat, in combination with dipyridamole, potentiates the inhibitory effects of prasugrel against ex vivo aggregation and in vivo thrombosis in mice. A, Aggregation in whole blood in response to collagen (10 µg/mL), PAR-4 amide (30 µmol/L), and TxA_2 mimetic U46619 (3 µmol/L). *P < .05 versus vehicle, n = 5-7 per group. B, Kaplan Meier plot for occlusion against time (minutes) following FeCl3-induced arterial injury in mice. **P < .01 versus 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 < .01 versus vehicle by Mantel-Cox test, n = 6 per group

4 | 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 toward 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. Because 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 exist 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 heme 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 ischemic stroke. Its use, however, like that of other drugs targeting cyclic nucleotides modulation, is associated with localized 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 hypothesized 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 4A-C). Indeed, we did not observe any significant change to arterial blood flow in our in vivo studies in any of our treatment groups while observing clear anti-thrombotic effects only in our combined treatment group.

While our study primarily centered 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-dependent cAMP production.[35] Equally, selective PDE isoform inhibitors, such as cilostazol, which 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. While more work and optimization will be required to clinically translate this in human

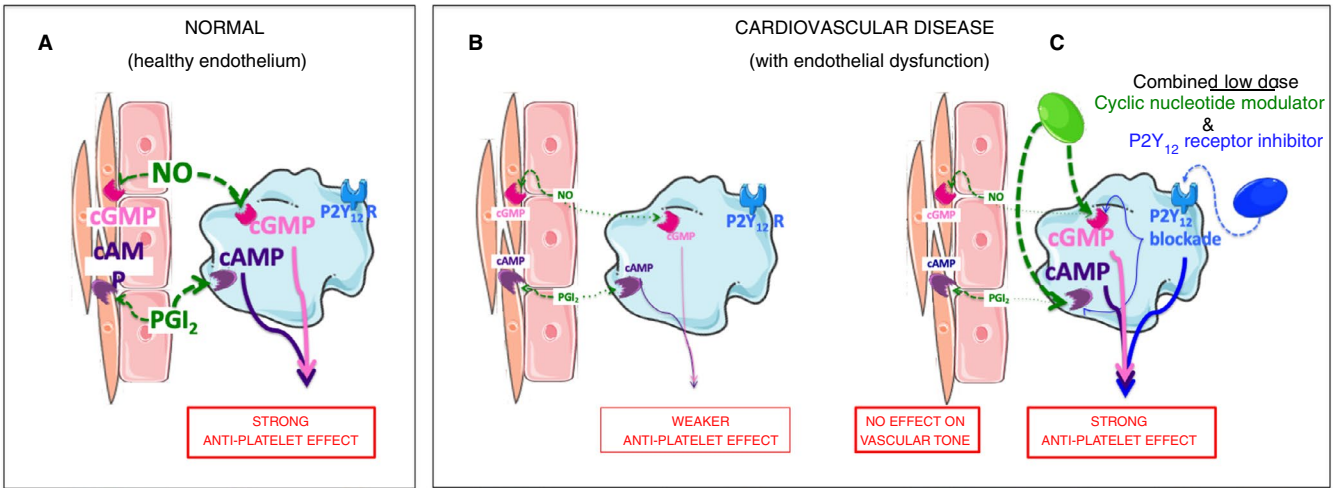

FIGURE 4 Summary of the pathophysiological rationale for the efficacy of combined cyclic nucleotide modulators with $P2Y_{12}$ receptor antagonists as anti-platelet therapy. A, In the healthy circulation endothelial-derived mediators nitric oxide (NO) and prostaglandin I_2 (PGI_2) act upon platelets 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

studies, such combined pharmacological approaches represent a focused anti-platelet regimen while potentially sparing associated off-target side effects.

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CONFLICTS 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.

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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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