Platelet inhibition by $\text{P2Y}_{12}$ antagonists is potentiated by adenosine signalling activators

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Background and Purpose: $\text{P2Y}_{12}$ receptor antagonists reduce platelet aggregation and the incidence of arterial thrombosis. Adenosine signalling in platelets directly affects cyclic nucleotide tone, which we have shown to have a synergistic relationship with $\text{P2Y}_{12}$ inhibition. Several studies suggest that ticagrelor inhibits erythrocyte uptake of adenosine and that this could also contribute to its antiplatelet effects. We therefore examined the effects on platelet activation of adenosine signalling activators in combination with the $\text{P2Y}_{12}$ receptor antagonists ticagrelor and prasugrel.

Experimental Approach: Human washed platelets, platelet-rich plasma and whole blood were used to test the interactions between ticagrelor or prasugrel and adenosine or $5'-N$-ethylcarboxamidoadenosine (NECA). Platelet reactivity to thrombin, protease-activated receptor 1 (PAR-1) activation or collagen was assessed by a combination of 96-well plate aggregometry, light transmission aggregometry, whole blood aggregometry, ATP release assay and levels of cAMP.

Key Results: The inhibitory effects of ticagrelor and prasugrel on platelet aggregation and ATP release were enhanced in the presence of adenosine or NECA. Iso-bolographic analysis indicated a powerful synergy between $\text{P2Y}_{12}$ receptor inhibition and adenosine signalling activators. Increased levels of cAMP in platelets were also observed. In all cases, ticagrelor showed similar synergistic effects on platelet inhibition as prasugrel in the presence of adenosine or NECA.

Conclusion and Implications: These results indicate that $\text{P2Y}_{12}$ antagonists have a synergistic relationship with adenosine signalling and that their efficacy may depend partly upon the presence of endogenous adenosine. This effect was common for prasugrel and ticagrelor despite reports of differences in their effects upon adenosine reuptake.

KEYWORDS
adenosine signalling activators, $\text{P2Y}_{12}$ receptor, platelet, prasugrel, synergism, ticagrelor
INTRODUCTION

Platelets, responsible for the prevention of blood loss at sites of vascular injury, are also central to the formation of arterial thrombi and subsequent ischaemic events including myocardial infarctions (Ruiz-Nodor et al., 2020). Antiplatelet drug therapy impairs the formation and progression of thrombi and thus constitutes the cornerstone of cardioprotective medications. P2Y12 receptors, activated by ADP released from platelets, are central to the haemostatic response. The advent of P2Y12 receptor antagonists, particularly clopidogrel, led to a significantly reduced incidence of arterial thrombosis in at-risk individuals (Committee, 1996; Diener et al., 2004; Dorsam & Kunapuli, 2004). However, almost 30% of patients show inadequate platelet inhibition with clopidogrel due to its complicated pharmacokinetic and pharmacogenomic factors (Ellis et al., 2009). As a result, newer P2Y12 receptor antagonists have been developed and largely superseded clopidogrel.

Prasugrel, like clopidogrel, is a member of the thienopyridine class of antagonists. It is a prodrug that is converted by cytochrome P450 enzymes (CYP) to an active compound (prasugrel active metabolite [PAM]) that irreversibly blocks receptor activation (Ding et al., 2003). Crucially however, its conversion has less dependence upon CYP enzymes than clopidogrel (Farid et al., 2007; Wallentin et al., 2007) resulting in more rapid and less variable platelet inhibition at lower doses. In contrast, ticagrelor from the cyclopentyltriazolopyrimidine chemical class is a direct-acting P2Y12 receptor antagonist that does not require metabolic activation. Another key difference is that it binds reversibly to the P2Y12 receptor at a site different from the ADP-binding site, rendering the P2Y12 receptor unfunctional in a non-competitive manner (Teng & Butler, 2010; van Giezen & Humphries, 2005). Interestingly, there is evidence that ticagrelor inhibits erythrocyte uptake of adenosine via equilibrative nucleoside transporter 1 (ENT1; SLC29A1) and with the potential to increase plasma levels of adenosine (Armstrong et al., 2014).

Adenosine is an endogenous nucleoside that mediates several physiological processes, including vasodilation, anti-inflammation and platelet inhibition (Layland et al., 2014). Human platelets are rich in A2A and A2B adenosine receptors, and A2A and A2B receptor knockout mice have higher platelet aggregation activity and lower antplatelet aggregation properties than wild-type mice (Ledent et al., 1997; Yang et al., 2010). Both A2A and A2B are Gs-protein-coupled receptors that stimulate AC activity and increase cAMP production (Amisten et al., 2008).

Intraplatelet levels of the cyclic nucleotides cAMP and cGMP increase in response to endothelial-derived prostacyclin (PGI2) and NO and are critical to maintaining platelet quiescence. We have previously demonstrated a synergistic relationship between the actions of PGI2 and NO and anti-thrombotic blockade of platelet P2Y12 receptors (Chan et al., 2016; Kirkby et al., 2013). Following on from this work, we also reported that coupling prasugrel with soluble GC (sGC) stimulators can produce powerful antiplatelet effects (Armstrong et al., 2020). Considering the potential downstream action of adenosine on intraplatelet cAMP levels, we hypothesized that adenosine may also amplify P2Y12 receptor antagonism. In this study, we investigate this hypothesis and through comparison of prasugrel and ticagrelor, we also examine any additional contribution from ticagrelor’s ability to inhibit adenosine reuptake.

METHODS

2.1 Blood collection and platelet preparation

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 samples were collected into trisodium citrate (0.32% final; Sigma Aldrich, UK) by venepuncture from healthy volunteers who had abstained from antiplatelet drugs consumption for 2 weeks previously. Platelet-rich plasma was obtained by centrifugation of whole blood (175 × g, 15 min, 25°C) and platelet-poor plasma was obtained by centrifugation of red blood cell fraction (15,000 × g, 2 min, 25°C). All platelet-rich plasma experiments were completed within 2 h of blood taken.

Platelets were pelleted by further centrifugation of platelet-rich plasma in the presence of apyrase (0.02 U/ml; Sigma Aldrich) and PGI2 (1 μg/ml; Tocris, UK) at 1100 × g for 10 min at room temperature. The resulting pellets were resuspended twice in modified Tyrode’s buffer (134-mM NaCl, 2.9-mM KCl, 0.34-mM Na2HPO4, 12-mM NaHCO3, 20-mM HEPES, 1-mM MgCl2, 0.1% glucose,
0.35% BSA and 0.02 U·ml⁻¹ apyrase). Washed platelets were then adjusted to 3 × 10⁶ platelets·ml⁻¹ and allowed to rest for at least 30 min.

2.2 | Light transmission aggregometry

Platelet-rich plasma was preincubated for 30 min at 37°C with ticagrelor (3 μM; kindly supplied by AstraZeneca, Sweden), PAM (3 μM; kindly supplied by AstraZeneca) or vehicle (0.5% DMSO). Platelet aggregation stimulated by thrombin receptor activator peptide SFLRN (TRAP-6 amide, 10 μM; Bachem, Switzerland) or Horm collagen (10 μg·ml⁻¹; Nycomed, Austria) was measured by a Bio/Data PAP-8E aggregometer (Alpha Laboratories, Eastleigh, UK) after incubation (15 min, 37°C) with adenosine (1 or 10 μM; Sigma Aldrich) or 5′-N-ethylcarboxamidoadenosine (NECA, 1 μM; Sigma Aldrich). The percentage of final aggregation after 7 min was recorded.

2.3 | 96-well plate aggregometry

To facilitate a broader and more high-throughput assessment of platelet function, a 96-well plate aggregometry method was used (Armstrong et al., 2009). Briefly, washed platelets were preincubated with vehicle (0.5% DMSO) or different concentrations of ticagrelor (0.033, 0.11, 0.36, 1.2, 4 μM) or PAM (0.033, 0.11, 0.36, 1.2, 4 μM) for 30 min at room temperature before placing 100 μl into individual wells of 96-well plate (Nunc, UK). Different concentrations of adenosine (0.03, 0.1, 0.3, 1, 3, 10 μM) or NECA (0.03, 0.1, 0.3, 1, 3, 10 μM) were then added to the wells in the presence of ticagrelor or PAM. After 15-min incubation, washed platelets were activated by the addition of thrombin (0.5 U·ml⁻¹; Sigma Aldrich) or Horm collagen (3 μg·ml⁻¹), in the presence of 1 mg·ml⁻¹ human fibrinogen (Sigma Aldrich). The plate was shaken vigorously on a microplate shaker (BioShake IQ, Quantifoil Instruments, Germany) at 1200 rpm for 5 min at 37°C and then we measured the absorbance at 595 nm on a 96-well plate reader (Tecan Sunrise; Tecan, Weymouth, UK). Percentage of platelet aggregation was calculated with reference to the absorbance of modified Tyrode’s buffer as a surrogate for 100% aggregation.

2.4 | Whole blood aggregometry

Whole blood was preincubated with ticagrelor (3 μM), PAM (3 μM) or vehicle (0.033% DMSO) for 30 min at room temperature. Adenosine (10 μM) or NECA (1 μM) was then added to the whole blood in the presence of ticagrelor or PAM for 15-min incubation. Platelet aggregation to TRAP-6 amide (30 μM) or Horm collagen (5 μg·ml⁻¹) was measured by whole blood lumi-aggregometer (Chronolog, Havertown, PA) in blood diluted 1:1 with PBS.

2.5 | ATP release

Platelet-rich plasma was preincubated with ticagrelor (3 μM), PAM (3 μM) or vehicle (0.5% DMSO) for 30 min at 37°C and then incubated with adenosine (10 μM) or NECA (1 μM) for 15 min at 37°C. After adding CHRONO-LUME reagent (1:10 v/v; Chronolog), platelet ATP release was triggered by TRAP-6 amide (10 μM) or Horm collagen (4 μg·ml⁻¹) and measured in whole blood lumi-aggregometer (Chronolog). The luminescence of an ATP standard (LabMedics, UK) was used to calculate the levels of ATP release.

2.6 | cAMP measurement

Platelet-rich plasma was preincubated with ticagrelor (3 μM), PAM (3 μM) or vehicle (0.5% DMSO) for 30 min at 37°C and then incubated with adenosine (10 μM) or NECA (1 μM) for 15 min at 37°C. TRAP-6 amide (10 μM) or Horm collagen (4 μg·ml⁻¹) was used to activate platelets. After 3-min incubation, platelets were lysed with the solution containing isobutylmethylxanthine (250 μM), potassium fluoride (0.5 M) and Triton-X-100 (0.625%). We used homogenous time-resolved fluorescence-based competitive immunoassays (Cisbio) to detect the concentrations of cAMP.

2.7 | Isobolographic analysis

Isobolographic analysis was used to present the interaction between two drugs (Kirkby et al., 2013; Tallarida, 2006). Firstly, adenosine or NECA concentration–response curves were generated for the inhibition of thrombin- or collagen-induced aggregation by ticagrelor or PAM. These data were incorporated to a logistic equation by using the method of least squares. Then, the concentration required to inhibit 25% aggregation was calculated from each curve and isobolograms were constructed with the concentrations of ticagrelor or PAM to inhibit aggregation on the X-axis and the concentrations of adenosine or NECA to inhibit aggregation on the Y-axis. The combinations of adenosine/NECA and ticagrelor/PAM that caused 25% inhibition were used to produce a scatter plot. For comparison, the theoretical additive line was plotted by connecting the inhibitory concentrations of ticagrelor (0.033–4 μM) or PAM (0.033–4 μM) applied alone and the inhibitory concentrations of adenosine (0.03–10 μM) or NECA (0.03–10 μM) applied alone. Synergy or antagonism was indicated when the line was located below or above the theoretical additive line, respectively.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis (Curtis et al., 2018). All experiments were designed to generate
groups of equal size in the randomized and blinded manner where appropriate. The data are showed as mean ± SEM; n represents the number of healthy volunteers studied. The sample number subjected to analysis achieves 80% power for each experiment and is of at least five independent values from different donors (not technical replicates). Statistical evaluation was performed by one-way ANOVA followed by a multiple comparisons test (Bonferroni test). Two-way ANOVA and Bonferroni post-tests were used to determine concentration–response curves. P < 0.05 was considered to be statistically significant. Post hoc tests were conducted only when F-value in ANOVA was achieved P < 0.05 and there was no significant variance in homogeneity. All statistical tests were performed by using GraphPad Prism 6 (La Jolla, CA, USA; RRID:SCR_002798).

2.9 | Materials

Ticagrelor and prasugrel active metabolite were kindly supplied by AstraZeneca, Sweden. Prostaglandin I2 was obtained from Tocris, TRAP-6 amide from Bachem and Horm collagen from Nycomed. CHRONO-LUME reagent was from Chronolog and ATP standards from LabMedics (UK). cAMP immunoassays were made by Cisbio and 96-well plates by Nunc. All other chemicals and reagents including trisodium citrate, apyrase, adenosine, NECA, thrombin and human fibrinogen were obtained from Sigma Aldrich, UK.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Combined effects of ticagrelor or prasugrel with exogenous adenosine or NECA on platelet aggregation in washed platelets

Initial studies were undertaken in washed platelets to observe the direct effects of adenosine. This approach also excluded any effect of adenosine reuptake effects by red blood cells. The inhibitory effects of PAM on thrombin- and collagen-induced aggregation were strongly increased by the addition of adenosine (Figure 1a,b). Ticagrelor similarly produced much stronger inhibition of platelet aggregation in the presence of adenosine (Figure 1c,d). Similarly, in the presence of the adenosine receptor agonist NECA, both PAM (Figure 1e,f) and ticagrelor (Figure 1g,h) exhibited much stronger inhibitory effects against platelet aggregation.

3.2 | Determination of synergism between P2Y12 receptor antagonists and exogenous adenosine or NECA on platelet aggregation by isobolographic analysis

Isobolographic analysis was used to analyse whether the observed interactions between P2Y12 receptor antagonists and exogenous adenosine or NECA were additive or synergistic with regard to inhibition of platelet aggregation. Isobolograms were constructed showing the individual and combined concentrations of P2Y12 receptor antagonists and adenosine required to produce 25% inhibition of platelet aggregation. The isoboles of both PAM and ticagrelor combined with adenosine curved strongly towards the axis, revealing PAM and adenosine had synergistic antiplatelet effects (Figure 2a–d). Similarly, the IC50 isobolograms of PAM and ticagrelor combined with NECA revealed synergistic antiplatelet effects (Figure 2e–h).

3.3 | Combined effects of ticagrelor or prasugrel with exogenous adenosine or NECA on platelet aggregation in platelet-rich plasma

Adenosine (10 μM) or NECA (1 μM) alone concentrations chosen did not decrease platelet aggregation in platelet-rich plasma induced by TRAP-6 or collagen, nor was it reduced by PAM or ticagrelor alone. In the presence of adenosine, PAM significantly inhibited aggregation induced by both TRAP-6 (Figure 3a; inhibition of 50% ± 11%) and collagen (Figure 3b; inhibition of 49% ± 7%) as did ticagrelor (Figure 3c; TRAP-6, inhibition of 33% ± 6%; Figure 3d; collagen, inhibition of 32% ± 3%). In the presence of NECA, PAM produced significant inhibition of platelet aggregation induced by TRAP-6 (Figure 3e; inhibition of 84% ± 4%) and collagen (Figure 3f; inhibition of 78% ± 3%) as did ticagrelor (Figure 3g; TRAP-6, inhibition of 74% ± 10%; Figure 3h, collagen; inhibition of 69% ± 7%).

3.4 | Combined effects of ticagrelor or prasugrel with exogenous adenosine or NECA on the release of ATP from platelets

ATP release from platelets induced by TRAP-6 or collagen was not reduced by PAM, ticagrelor or adenosine applied alone but was inhibited by NECA applied alone (Figure 4; TRAP-6, inhibition of 51% ± 7%; collagen, inhibition of 35% ± 6%). PAM combined with NECA (inhibition of 88% ± 5%) or adenosine (inhibition of 71% ± 4%) significantly decreased ATP release from platelets activated by TRAP-6 (Figure 4a). The same effect was observed when platelets were activated by collagen (Figure 4b; inhibition of 78% ± 3% PAM and NECA; inhibition of 65% ± 5% PAM and adenosine). Similarly, the inhibitory effects of ticagrelor were
FIGURE 1  Combined effects of prasugrel active metabolite (PAM) or ticagrelor with exogenous adenosine or 5′-N-ethylcarboxamidoadenosine (NECA) on platelet aggregation in washed platelets. Effects on aggregation of washed platelets of (a) PAM in combination with adenosine against thrombin (0.5 U ml⁻¹); (b) PAM in combination with adenosine against collagen (3 μg ml⁻¹); (c) ticagrelor in combination with adenosine against thrombin; (d) ticagrelor in combination with adenosine against collagen; (e) PAM in combination with NECA against thrombin; (f) PAM in combination with NECA against collagen; (g) ticagrelor in combination with NECA against thrombin and (h) ticagrelor in combination with NECA against collagen (n = 6 for all). Data expressed as mean ± SEM. *P < 0.05 for difference from DMSO by two-way ANOVA plus Bonferroni post-tests.
FIGURE 2  IC\textsubscript{25} isobolograms for the inhibition of thrombin- or collagen-induced platelet aggregation in the presence of prasugrel active metabolite (PAM) or ticagrelor and/or exogenous adenosine or S'-N-ethylcarboxamidoadenosine (NECA). IC\textsubscript{25} isobolograms of PAM and/or adenosine against (a) thrombin (0.5 U ml\textsuperscript{-1}) or (b) collagen (3 μg ml\textsuperscript{-1}); ticagrelor and/or adenosine against (c) thrombin or (d) collagen; PAM and/or NECA against (e) thrombin or (f) collagen and ticagrelor and/or NECA against (g) thrombin or (h) collagen (n = 6 for all)
FIGURE 3  Combined effects of prasugrel active metabolite (PAM) or ticagrelor with exogenous adenosine or 5'-(N-ethylcarboxamido)adenosine (NECA) on platelet aggregation in platelet-rich plasma (PRP). Effects on aggregation in PRP of PAM (3 μM) in combination with adenosine (10 μM) against (a) TRAP-6 (10 μM) and (b) collagen (10 μg/ml); ticagrelor (3 μM) in combination with adenosine against (c) TRAP-6 and (d) collagen; PAM in combination with NECA (1 μM) against (e) TRAP-6 and (f) collagen; and ticagrelor in combination with NECA against (g) TRAP-6 and (h) collagen (n = 6 for all). Representative aggregation traces (i) for PAM or ticagrelor in combination with adenosine or NECA against TRAP-6. Data expressed as mean ± SEM. *P < 0.05 for difference from DMSO and #P < 0.05 for difference from PAM or ticagrelor applied alone by one-way ANOVA plus Bonferroni post-tests.
increased greatly in the presence of NECA or adenosine against ATP release stimulated by TRAP-6 (Figure 4c; inhibition of 85% ± 5% ticagrelor and NECA; inhibition of 76% ± 5% ticagrelor and adenosine) or collagen (Figure 4d; inhibition of 81% ± 2% ticagrelor and NECA; inhibition of 70% ± 2% ticagrelor and adenosine).

**3.5 Combined effects of P2Y₁₂ antagonist with exogenous adenosine or NECA on the levels of cAMP in platelets**

Levels of cAMP in platelets following activation by TRAP-6 were not affected by ticagrelor, PAM, adenosine or NECA (Figure 5) but were
increased in the presence of ticagrelor and adenosine (Figure 5a) and in the presence of ticagrelor and NECA (Figure 5b). However, levels of cAMP in platelets were not significantly affected by PAM in the presence of adenosine (Figure 5c) or NECA (Figure 5d) after TRAP-6 stimulation. In addition, adenosine did not augment the effects of ticagrelor or PAM on the levels of cAMP in platelets after collagen stimulation (Figure S1).

3.6 Combined effects of P2Y₁₂ antagonist with exogenous adenosine or NECA on platelet aggregation in whole blood

PAM, ticagrelor, adenosine or NECA alone did not inhibit TRAP-6- or collagen-stimulated platelet aggregation in whole blood. Adenosine increased the inhibitory effect of PAM or ticagrelor on TRAP-6-induced platelet aggregation (Figure 6a, inhibition of 51% ± 10%; Figure 6c, inhibition of 55% ± 4%, respectively) but had no effect on collagen-induced platelet aggregation (Figure 6b, d). PAM or ticagrelor combined with NECA significantly reduced platelet aggregation induced by both TRAP-6 (Figure 6e, inhibition of 72% ± 15%; Figure 6g, inhibition of 48% ± 17%) and collagen (Figure 6f, inhibition of 43% ± 8%; Figure 6h, inhibition of 42% ± 6%).

4 DISCUSSION

Our studies show that adenosine signalling increases the inhibitory effects of prasugrel or ticagrelor on aggregation and suggests this effect centres on the ability of the drug combinations to maintain higher intraplatelet cyclic nucleotide tone. Isobolographic analyses also demonstrate that these are strongly synergistic antiplatelet effects. Finally, the similar inhibitory patterns of ticagrelor and prasugrel in whole blood experiments provide no evidence for an additional influence of adenosine reuptake being exerted by ticagrelor.

Platelets circulate in a quiescent manner under the continual influence of endothelial derived mediators, PGI₂ and NO (Knowles & Warner, 2019). Each acts to raise cAMP and cGMP levels, both of which are regarded as critical cytosolic regulators of platelet function (Eigenthaler et al., 1992). The P2Y₁₂ receptor on platelets couples to a Gi protein that acts to rapidly decrease levels of cAMP. However, this action is countered by both ticagrelor and prasugrel. We have previously shown that P2Y₁₂ receptor antagonists in vitro only weakly inhibit thrombin- or collagen-induced platelet aggregation (Armstrong et al., 2011; Kirkby et al., 2013), however, their effect is strongly related to cyclic nucleotide levels in platelets (Knowles & Warner, 2019). In this way, when platelet cyclic nucleotide levels are elevated, as may well be the case within the circulation where
FIGURE 6  Combined effects of prasugrel active metabolite (PAM) or ticagrelor with exogenous adenosine or 5′-N-ethylcarboxamidoadenosine (NECA) on platelet aggregation in whole blood. Effects on platelet aggregation in whole blood of PAM (3 μM) in combination with adenosine (10 μM) against (a) TRAP-6 (30 μM) and (b) collagen (5 μg/ml); ticagrelor (3 μM) in combination with adenosine against (c) TRAP-6 and (d) collagen; PAM in combination with NECA (1 μM) against (e) TRAP-6 and (f) collagen; and ticagrelor in combination with NECA against (g) TRAP-6 and (h) collagen (n = 5 for all). Data expressed as mean ± SEM. *P < 0.05 for difference from DMSO and #P < 0.05 for difference from PAM or ticagrelor applied alone by one-way ANOVA plus Bonferroni post-tests.
platelets are bathed in NO and PGI2, the antiplatelet effects of P2Y12 inhibition are greatly potentiated. This synergistic relationship holds clear clinical relevance as a therapeutic target, as we have recently demonstrated by coupling the P2Y12 antagonist prasugrel with sGC stimulators (Armstrong et al., 2020).

In this study, we have explored the above relationship further by examining the effects of adenosine signalling activators in combination with prasugrel or ticagrelor. Like the PGI2 IP receptor, platelet A2 receptors couple to Gs protein which stimulates AC activity and raise levels of cAMP. However, adenosine in whole blood undergoes rapid uptake by erythrocytes and metabolism by adenosine deaminase and adenosine kinase. Therefore, in this study, we chose to test both adenosine and NECA, in combination with ticagrelor or PAM, as NECA is a stable adenosine derivative that is resistant to deamination and phosphorylation metabolism and a weak substrate for adenosine reuptake system (Dawicki et al., 1986).

For our experimental design, we used a previously established in vitro concentration of ticagrelor or PAM that is physiologically relevant and exhibits almost complete inhibition of ADP-induced platelet aggregation whilst thrombin and collagen stimulation remain largely insensitive (Kirkby et al., 2011). Similarly informed by our initial aggregation studies, we selected concentrations of adenosine and NECA that alone do not affect thrombin- and collagen-induced platelet function, thereby allowing us to assess any potentiation or synergy of effect.

We found strong synergistic antiplatelet effects on thrombin- and collagen-induced aggregation when adenosine or NECA was used in combination with ticagrelor or PAM. Moreover, a similar pattern of inhibition persisted from our initial studies in washed platelet preparations and platelet-rich plasma, where there are no red blood cells to uptake adenosine, through to our studies in whole blood, where adenosine metabolism and reuptake will be present. Significantly, in whole blood, we observed no differences in our combinations of adenosine receptor activators with either prasugrel or ticagrelor.

Despite its demonstrated clinical benefits, our understanding of the pharmacology of ticagrelor has remained uncertain. From a receptor binding site that is independent of ADP (Van Giezen et al., 2009), reported inverse agonism of the P2Y12 receptor (Aungraheeta et al., 2016) and inhibition of ENT1 on both erythrocytes (Armstrong et al., 2014) and platelets (Aungraheeta et al., 2016), there has been continued study and discussion over the relative contribution of each factor to ticagrelor’s anti-thrombotic efficacy and associated bleeding risk. In particular, adenosine plasma levels and their variation resulting from ticagrelor treatment have been of concern. Contributing to this concern was a study reporting an increase in plasma adenosine levels in percutaneous coronary intervention (PCI) patients receiving ticagrelor 6 h after loading dose administration (Bonello et al., 2014). However, more recently, the STEEL-PCI study found no impact of ticagrelor on plasma adenosine levels in patients following 1 month of ticagrelor treatment (Orme et al., 2018). Our experiments in this in vitro setting found no evidence for adenosine leading to preferential potentiation for the antiplatelet effects of ticagrelor over prasugrel, implying that any inhibition of adenosine reuptake has little impact on platelet function.

Inhibition of AC by ADP plays a crucial role in platelet aggregation in vivo because it could negatively regulate the antiplatelet effects of endogenous modulators (i.e. prostacyclin or adenosine) that increase the platelet cAMP levels under physiological conditions. We did observe increases in levels of cAMP in platelets suggesting that the synergistic antiplatelet effects, irrespective of chemical class, are

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**Synergistic effects on platelet activation of adenosine signalling activators in combination with P2Y12 antagonists**

**Compensating for variations in endogenous adenosine levels could enhance therapeutic responses to P2Y12 antagonists in specific at-risk patients**

(a) Treatment with P2Y12 antagonists in HTPR patients

(b) Combination adenosine signalling activators with P2Y12 antagonists in HTPR patients

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**FIGURE 7** Summary of the synergistic effects of P2Y12 receptor antagonists with exogenous adenosine signalling activators on platelet aggregation. The effectiveness of ticagrelor or prasugrel on thrombin- or collagen-induced platelet activation could be augmented by supplementing adenosine or 5’-N-ethylcarboxamidoadenosine (NECA). HTPR, high on-treatment platelet reactivity.
linked directly to intraplatelet cyclic nucleotide levels. However, the signalling event downstream from Gi, independent of platelet AC inhibition, also contributes to platelet activation by ADP. The PI3K pathway and potassium channels are implicated in P2Y12 receptor-mediated platelet activation (Dangelmaier et al., 2001; Shankar et al., 2004). Thus, more studies will be required to fully elucidate the precise mechanisms of the interplay between P2Y12 antagonists and adenosine receptor agonists.

In addition to building upon our previous observations of a synergistic relationship between P2Y12 receptor inhibition and platelet cyclic nucleotide levels, our findings have potential clinical implications. In particular, it adds to the continuing discussion of how it may be possible to potentiate the efficacy of P2Y12 inhibitors in specific patient groups. For example, it is known that lower plasma adenosine levels are associated with platelet over-activation in multiple sclerosis (MS) patients (Mayne et al., 1999) and in atrial fibrillation (AF) patients (Minamino et al., 1999), both of whom are at increased risk of thromboembolic events (Björck et al., 2013; Christensen et al., 2012). Moreover, poor responsiveness to prasugrel and ticagrelor are still present in certain patient populations with high on-treatment platelet reactivity (HTPR) and ‘resistance’ to antiplatelet agents is associated with poor outcomes (Bonello et al., 2010; Michelson et al., 2005). Thus, further optimization of antiplatelet therapy could provide a viable alternative to prevent thromboembolic events in patients with high on-treatment platelet reactivity (Figure 7).

5 | CONCLUSION

In conclusion, our study indicates that P2Y12 receptor antagonists strongly synergize with adenosine receptor agonists to inhibit platelet aggregation, an interplay related to changes in cyclic nucleotide levels in platelets. We found no evidence for a greater interaction with ticagrelor than with prasugrel associated to inhibition of adenosine uptake. These results imply that the in vivo benefits of P2Y12 antagonists depend partly on the presence of endogenous adenosine. Variations in endogenous adenosine levels could therefore explain interindividual and interdisease differences in response to P2Y12 antagonists. Thus developing novel combinations of antiplatelet drugs that focus upon compensating for low adenosine levels could provide a viable therapeutic approach.

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AUTHOR CONTRIBUTIONS

All authors participated in the design of this study. CCS, MVC, NSK and IV performed experiments. CCS, MVC, NSK, JAM, PCA and TDW analysed the data. CCS, PCA and TDW wrote the manuscript. All authors edited and approved the final manuscript.

CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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