Mode of action of P2Y12 antagonists as inhibitors of platelet function

David Iyú1; Jackie R. Glenn1; Ann E. White1; Sue C. Fox1; Hans van Giezzen2; Sven Nylander2; Stan Heptinstall1

1Cardiovascular Medicine, University of Nottingham, Nottingham, UK; 2Department of Bioscience, Astra Zeneca R&D Mölndal, Mölndal, Sweden

Summary
P2Y12 receptor antagonists are anti-thrombotic agents that inhibit platelet function by blocking the effects of adenosine diphosphate (ADP) at P2Y12 receptors. However, some P2Y12 receptor antagonists may affect platelet function through additional mechanisms. It was the objective of this study to investigate the possibility that P2Y12 antagonists inhibit platelet function through interaction with G-protein-coupled receptors other than P2Y12 receptors. We compared the effects of cangrelor, ticagrelor and the prasugrel active metabolite on platelet aggregation and on phosphorylation of vasodilator-stimulated phosphoprotein (VASP). We compared their effects with those of selective IP, EP4 and A2A agonists, which act at Gi-coupled receptors. All three P2Y12 antagonists were strong inhibitors of ADP-induced platelet aggregation but only partial inhibitors of aggregation induced by thrombin receptor activating peptide (TRAP) or the thromboxane A2 mimetic U46619. Further, after removing ADP and its metabolites using apyrase and adenosine deaminase, the P2Y12 antagonists produced only minor additional inhibition of TRAP or U46619-induced aggregation. Conversely, the Gi-coupled receptor agonists always produced strong inhibition of aggregation irrespective of whether ADP was removed. Other experiments using selective receptor agonists and antagonists provided no evidence of any of the P2Y12 antagonists acting through PAR1, TP, IP, EP4, A2A or EP3 receptors. All three P2Y12 antagonists enhanced VASP phosphorylation to a small and equal extent but the effects were much smaller than those of the IP, EP4 and A2A agonists. The effects of cangrelor, ticagrelor and prasugrel on platelet function are mediated mainly through P2Y12 receptors and not through another G-protein-coupled receptor.

Keywords
ADP, Gi-coupled receptors, platelet aggregation, P2Y12 antagonists, VASP phosphorylation

Introduction
Platelet activation contributes to thrombus formation in coronary and cerebral arteries leading to acute coronary syndromes and stroke. Platelet activation mediated by exposed collagen and newly formed thrombin leads to platelet aggregation, TXA2 generation, dense body and α-granule secretion, platelet-leukocyte interactions and enhanced procoagulant activity (1), and all of these processes contribute to the thrombotic event (2). One of the materials secreted from dense bodies is adenosine diphosphate (ADP) and this serves to amplify the platelet activation induced by other agents. ADP acts via P2Y1 and P2Y12 receptors on the platelet surface, and in particular the P2Y12 receptor (also formerly known as the P2Y1 receptor) plays a central role in platelet function (3–8). The P2Y12 receptor has become an attractive molecular target for anti-thrombotic therapy and agents that block the effect of ADP at the P2Y12 receptor are already in use and in development as anti-thrombotic agents (9–12).

The P2Y12 receptor antagonists that are already in use are the thiopyridines clopidogrel and prasugrel, and two of the newer agents in development are ticagrelor and cangrelor. Clopidogrel and prasugrel are prodrugs which are converted to active metabolites that bind covalently to cysteine residues within the P2Y12 receptor and thereby interfere with the ability of ADP to bind to the receptor (13–16). Ticagrelor (also known as AZD6140) is a non-competitive antagonist of the P2Y12 receptor which binds directly to a site distant from the ADP-binding site (17). Cangrelor (also known as AR-C69931) acts as a rapidly reversible antagonist of the P2Y12 receptor (18–20). Thus all four drugs act as ADP antagonists at the P2Y12 receptor but differ in their chemical structure and mode of action at the molecular level.

There is the possibility that some P2Y12 antagonists may modify platelet function through additional mechanisms involving interaction with other G-protein-coupled receptors on the platelet surface, of which there are many. The most important receptors associated with inhibition of platelet function are the Gi-coupled receptors that include the IP receptor, the EP4 receptor and the A2A receptor, all of which increase cAMP by stimulating adenylate cyclase (21–23). Also there is the EP3 receptor which, like the P2Y12 receptor, is a Gi-coupled receptor that reduces CAMP by inhibiting adenylate cyclase and thereby potentiates platelet function (24–26). All G-protein-coupled receptors have cysteine residues at a similar position to those in the P2Y12 receptor (27, 28), and since these cysteine residues are the means through which the active
metabolites of clopidogrel and prasugrel exert their effects at the P2Y12 receptor, it is possible that clopidogrel and prasugrel (but not other P2Y12 antagonists) could modify platelet function through interaction with other G-protein-coupled receptors as well.

It has also been reported (29) that the adenosine-based P2Y12 antagonists cangrelor (AR-C69931) and 2-methylthioadenosine 5’-monophosphate (2MeSAMP) are able to inhibit platelet aggregation through interaction with an unidentified G1-coupled receptor leading to increased cAMP levels. The authors argued that it is unlikely that the active metabolites of prasugrel and clopidogrel would act in the same way because of their different chemical structure and mode of action.

The possibility that different P2Y12 antagonists may affect platelet function through modes of action that are additional to their direct effects at the P2Y12 receptor may also be relevant to differences in clinical efficacy and/or side effects such as enhanced bleeding, as seen in clinical trials. For example, the PLATO study (30) revealed an enhanced efficacy for ticagrelor compared with clopidogrel in its ability to reduce death in patients with acute coronary syndromes, with no apparent enhancement of bleeding.

Against this background we have performed a series of investigations to look further for any indications that different P2Y12 antagonists may differ in their effects on platelet function, and that some may act via mechanisms additional to P2Y12 blockade. The P2Y12 antagonists that we investigated were cangrelor, ticagrelor and the prasugrel active metabolite (PAM).

phosphorylation measurements the Functional Bead Conjugation Buffer Set and Cell Signaling Master Buffer Kit were obtained from Becton Dickinson (Oxford, UK). IE273 antiVASP was from Alexis Biochemicals (San Diego, CA, USA). FITC-conjugated antibody 5C6 antiVASP pSer 157 was from Acris Antibodies (Insight Biotechnology, Wembley, UK). Lysis buffer consisted of Tris buffered saline, pH 7.6 from the Sigma Chemical Co containing Triton X-100 (1%), the ionic detergent sodium deoxycholate (0.25%) and phenylmethanesulfonyl fluoride (1 mM). A protease inhibitor tablet (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany), and phosphatase inhibitor (Cocktail Set II, Calbiochem, Darmstadt, Germany) were also added to the lysis buffer according to the manufacturers’ instructions. For cAMP measurements, [2,8-3H]-adenine was supplied by New England Nuclear (Buckinghamshire, UK). [Adenine U-14C]-adenosine 3’,5’-cyclic phosphate, ammonium salt was obtained from Amersham International (Buckinghamshire, UK).

Blood collection

Venous blood was obtained following informed consent from healthy volunteers who denied taking any medication in the preceding 14 days. Blood was taken into polystyrene tubes containing hirudin (fc 50 μg/ml).

Preparation of platelet-rich plasma (PRP)

Freshly collected blood was centrifuged (180 g, 10 minutes [min]) and the supernatant PRP removed. The remainder of the blood was re-centrifuged (1,500 g, 10 min) to obtain platelet-poor plasma (PPP) and the PRP diluted using autologous PPP to a standard platelet count of 350 x 10^9/l. When the P2Y12 receptor antagonists were used, the PRP was split into four different aliquots containing either vehicle (saline and DMSO 0.1%), cangrelor (1 μM), ticagrelor (10 μM) or PAM (10 μM) and incubated at room temperature for 30 min to allow maximum binding of PAM to the P2Y12 receptor.

Platelet aggregation

Aliquots of PRP (final volume 495 μl) were dispensed into pre-warmed polystyrene tubes (64 x 11 mm) containing the agent(s) under investigation and a stirrer bar. The tubes were placed in the stirring wells of a Multi-Sample Agitator (MSA, University of Nottingham, UK) operating at 1,000 rpm, 37°C. After 1 min, an aliquot (15 μl) of the sample was removed and mixed with fixative solution (30 μl) to provide a ‘starting’ platelet count. After a further minute, 20 μl of a solution of ADP (10 μM), TRAP (20 μM) or U46619 (1 μM) was added. Aliquots (15 μl) of the sample were removed at 1,
2 and 4 min and mixed with fixative solution (30 μl). The platelet count of the fixed samples was determined using the Sysmex® KX-21 analyser, an automated multi-parameter blood cell counter. Platelet aggregation was calculated as the percentage loss of single platelets compared to the ‘starting’ platelet count of PRP.

**Measurement of phosphorylation of vasodilator-stimulated phosphoprotein (VASP)**

The method used was very similar to the one used previously to determine the roles of EP receptors on modulating levels of cAMP in platelets (22). Aliquots of PRP (final volume 250 μl) were dispensed into pre-warmed (37°C) polystyrene tubes (64 x 11 mm) containing the appropriate G-protein-coupled receptor agonist/antagonist or vehicle (10 μl). After 2 min, the reaction was stopped by the addition of ice-cold phosphate-buffered saline (1 ml) and the samples were placed on ice and transferred immediately to cold Eppendorf tubes. The samples were then centrifuged (11,600 g, 10 seconds), the supernatant discarded and the resulting platelet pellet lysed by vigorous resuspension in 100 μl of lysis buffer. Samples were then frozen and stored at −20°C prior to assay. The amounts of the phosphorylated form of VASP present in the lysates were measured by flow cytometry using a cytometric bead assay which was developed using Becton Dickinson Cytometric Bead Array products as described below.

A specific APC-fluorescent bead was chosen in order to prepare a stock of capture beads by conjugation of the beads to an appropriate capture antibody. Conjugation was performed according to instructions and reagents (Functional Bead Conjugation Buffer Set) with a monoclonal antibody to VASP (mAb IE273 anti-VASP). The assay works on the principle that soluble VASP in the platelet lysate is captured by the bead-bound antibody. Addition of a secondary fluorescent antibody (FITC-conjugated antibody 5C6 anti-VASP pSer 157) enables detection of the phosphorylated form of VASP on the capture bead by flow cytometry. Stock capture beads were diluted 1 in 50 using capture bead diluent (Cell Signaling Master Buffer Kit) before use. Lysed platelet samples were thawed and vortexed immediately prior to assay. A 25 μl sample was added to 25 μl bead suspension in a FACS tube followed by the addition (7.5 μl) of FITC-conjugated antibody 5C6 anti-VASP pSer 157 (10 μg/ml) (Acris Antibodies, Düsseldorf, Germany). Samples were incubated in the dark at room temperature for 2 hours. Immediately prior to analysis samples were resuspended in 150 μl wash buffer (Cell Signaling Master Buffer Kit). Flow cytometry was performed on a Becton Dickinson LSRII flow cytometer using FACS DIVA acquisition software using both the blue (488 nm) and red laser (17 mW power, excitation 633 nm) to perform the bead analyses. Capture beads were identified by their associated APC fluorescence and 300 bead events were collected. Phosphorylated VASP was detected as FITC fluorescence expressed as median fluorescence (mf).

**Figure 1:** Effects of P2Y₁₂ receptor antagonists and Gₛ-coupled receptor agonists on ADP-induced platelet aggregation.

Platelet aggregation was measured in PRP in response to ADP (10 μM) in the presence of (A) the P2Y₁₂ antagonists cangrelor (1 μM), ticagrelor (10 μM) and PAM (10 μM), and (B) the Gₛ-coupled receptor agonists iloprost (3 nM), ONO-AE1–329 (0.1 μM) and adenosine (10 μM). Aggregation was determined by single platelet counting. The results shown are the mean ± SEM of four experiments. *p<0.05 cf vehicle.
14C-cAMP as a recovery tracer. Tubes were centrifuged (1,500 g, 30 min, 4°C) and the supernatant decanted and stored at −20°C prior to assay. The extraction of cAMP from samples of PRP using column chromatography has been described previously (35). Briefly, samples were loaded onto an alumina column primed with 10% TCA (w/v). The column was washed with 10% TCA (10 ml), water (10 ml) and ammonium formate (0.2 M, 1 ml). A further 4 ml of ammonium formate was added and the fraction collected onto an acidified Dowex column. One mM potassium phosphate buffer (pH 7.3, 3 ml) was added to the Dowex column, followed by a further 3 ml. The 3 ml fraction was collected into a glass vial and dissolved in liquid scintillation cocktail (Emulsifier Safe, approx. 18 ml) prior to scintillation counting. The percentage conversion of 3H-adenine to 3H-cAMP was calculated for each sample. Results were corrected for 3H-uptake and for cAMP recovery.

Data presentation

Data are presented as mean ± SEM and compared by either repeated measures (aggregation data) or paired t-tests (VASP and cAMP data) using SPSS 15 statistical software. Actual numbers of the different experiments performed are provided in the figure legends.

Results

Effects of P2Y12 receptor antagonists and Gs-coupled receptor agonists on ADP-induced platelet aggregation

We determined the effects of the P2Y12 receptor antagonists cangrelor, ticagrelor and PAM on platelet aggregation in PRP induced by ADP. At the same time we also looked at the ability of the Gs-coupled receptor agonists iloprost (an IP receptor agonist), ONO-AE1–329 (0.1 μM) or adenosine (10 μM). The experiments were performed in the absence (A, B) or presence (C, D) of apyrase (3 U/ml) and adenosine deaminase (1.2 U/ml). Aggregation was determined by single platelet counting. The results shown are the mean ± SEM of 3–6 experiments. *p<0.05 cf vehicle.
AE1–329 (an EP4 receptor agonist) and adenosine (an A2A receptor agonist) to inhibit the ADP-induced aggregation response. All three P2Y12 receptor antagonists caused almost complete inhibition of ADP-induced platelet aggregation (Fig. 1A). All three Gs-coupled receptor agonists also markedly inhibited ADP-induced platelet aggregation (Fig. 1B).

Effects of P2Y12 receptor antagonists and Gs-coupled receptor agonists on TRAP-induced platelet aggregation

We determined the effects of the same P2Y12 antagonists and Gs-coupled receptor agonists on TRAP-induced platelet aggregation. Here the degree of inhibition brought about by cangrelor, ticagrelor and PAM was much less than when ADP was used as the agonist; their effect was to convert the irreversible aggregation seen with TRAP to a reversible aggregation response (Fig. 2A). Also, there was no difference in the degree of inhibition brought about by any of the P2Y12 antagonists. In contrast, in the presence of iloprost, ONO-AE1–329 and adenosine, TRAP-induced aggregation was almost completely inhibited (Fig. 2B).

To ascertain the level of involvement of ADP released from activated platelets in mediating the aggregation response to TRAP, these experiments were then repeated in the presence of apyrase used at a concentration (3 U/ml) sufficient to completely prevent platelet aggregation induced by 10 μM ADP. Adenosine deaminase (ADA, 1.2 U/ml) was also added to remove any adenosine generated as a result of ADP breakdown to AMP by apyrase and subsequent conversion to adenosine via 5'-nucleotidase in plasma; the concentration of ADA used was sufficient to remove the inhibitory effects of at least 10 μM adenosine. Under these circumstances the irreversible TRAP-induced aggregation was converted into a reversible response, indicating that ADP does contribute to the extent of aggregation observed. Addition of cangrelor, ticagrelor or PAM slightly enhanced the reversibility of the response (Fig. 2C), and all three agents did so to the same extent. Once again iloprost and ONO-AE1–329 brought about marked inhibition of the aggregation response (Fig. 2D). (Note that it was not possible to...
use adenosine in these experiments as they were carried out in the presence of ADA.)

Effects of P2Y₁₂ receptor antagonists and Gₛ-coupled-receptor agonists on U46619-induced platelet aggregation

We determined the effects of the same P2Y₁₂ antagonists and Gₛ-coupled receptor agonists on the platelet aggregation that occurred in response to U46619. As with TRAP, the degree of inhibition brought about by cangrelor, ticagrelor and PAM was much less than when ADP was used as the agonist. Again their effects were to convert the irreversible aggregation to a reversible aggregation response (Fig. 3A). There was no difference in the degree of inhibition brought about by any of the three P2Y₁₂ antagonists. In the presence of iloprost, ONO-AE1–329 or adenosine, U46619-induced aggregation was completely inhibited (Fig. 3B).

In the presence of apyrase and ADA the irreversible aggregation was also converted into a more reversible response, again indicating that ADP contributes to the extent of aggregation observed. There appeared to be a small additional inhibitory effect of cangrelor, ticagrelor and PAM (Fig. 3C) under these conditions, but this was less evident than was the case using TRAP as the agonist (Fig. 2C). U46619-induced aggregation was completely inhibited by iloprost and also there was marked inhibition with the EP₄ agonist ONO-AE1–329 (Fig. 3D).

Effects of Gₛ-coupled receptor antagonists on the ability of P2Y₁₂ receptor antagonists to inhibit TRAP-induced platelet aggregation

We investigated the ability of the IP receptor antagonist CAY10441, the EP₄ receptor antagonist ONO-AE3–208 and the A₂₅ receptor antagonist SCH58261 to modify the inhibitory effect of the P2Y₁₂ receptor antagonists on TRAP-induced platelet aggregation.

![Figure 4](https://www.thrombosis-online.com/105.1/2011/101/Iyú+et+al.+Mode+of+action+of+P2Y₁₂+antagonists.png)

Figure 4: Effects of Gₛ-coupled receptor antagonists on the ability of P2Y₁₂ receptor antagonists to inhibit TRAP-induced platelet aggregation. Platelet aggregation was measured in PRP in response to TRAP (20 μM) in the presence of cangrelor (1 μM), ticagrelor (10 μM) and PAM (10 μM), and (A) vehicle, (B) the IP receptor antagonist CAY10441 (1 μM), (C) the EP₄ receptor antagonist ONO-AE3–208 (1 μM) or (D) the A₂₅ adenosine receptor antagonist SCH58261 (10 μM). The experiments were performed in the presence of apyrase (3 U/ml) and adenosine deaminase (1.2 U/ml). Aggregation was determined by single platelet counting. The results shown are the mean ± SEM of four experiments. *p<0.05 cf vehicle.
antagonists on TRAP-induced platelet aggregation. The experiments were performed in the presence of apyrase and ADA. None of these agents affected the ability of the P2Y12 receptor antagonists to inhibit the aggregation response (Fig. 4).

Effects of P2Y12 receptor antagonists on TRAP-induced platelet aggregation in the presence of Gs-coupled receptor agonists

We investigated the ability of P2Y12 antagonists to modify the inhibition of TRAP-induced platelet aggregation brought about by the IP, EP4 and A2A receptor agonists iloprost, ONO-AE1–329 and adenosine. The experiments were conducted in the presence of apyrase and ADA (except when we looked specifically at the effects of adenosine). The inhibition of aggregation brought about the Gs-coupled receptor agonists was not modified by any of the P2Y12 antagonists used (Fig. 5).

Effects of P2Y12 receptor antagonists on TRAP-induced platelet aggregation in the presence of an EP3 receptor agonist

We investigated the ability of P2Y12 antagonists to modify the potentiation of TRAP-induced platelet aggregation brought about by the EP3 receptor agonist sulprostone. Once again the experiment was conducted in the presence of apyrase and ADA. Sulprostone potentiated TRAP-induced aggregation alone and this potentiation was unaffected by any of the P2Y12 antagonists (Fig. 6).
We determined the effects of cangrelor, ticagrelor and PAM on VASP phosphorylation, and compared their effects with those of iloprost, ONO-AE1–329 and adenosine. We also looked for any kind of interaction between the P2Y12 receptor antagonists and the Gs-coupled receptor agonists. All three P2Y12 receptor antagonists brought about a small increase in VASP phosphorylation as compared to the control (Fig. 7). There was no difference between the results obtained for any of the P2Y12 receptor antagonists used. In contrast, all three Gs-coupled receptor agonists brought about a much greater increase in VASP phosphorylation. This increase in VASP phosphorylation was not modified in the presence of cangrelor, ticagrelor or PAM. Also the small increase in VASP phosphorylation brought about by cangrelor, PAM or ticagrelor was not modified by either the IP antagonist CAY10441, the EP4 antagonist ONO-AE3–208, or the A2A antagonist SCH58261 (data not shown). Neither was it modified by adding apyrase and ADA (data not shown).
Effects of P2Y12 receptor antagonists on VASP phosphorylation in the presence of an EP3 receptor agonist

The EP3 receptor agonist sulprostone effectively reduced VASP phosphorylation in platelets in which this had been raised by adding iloprost to PRP, and this was also the case when cangrelor, ticagrelor or PAM were also present (Fig. 8).

Effects of a P2Y12 receptor antagonist and a Gs-coupled receptor antagonist on cAMP production

The effects of the P2Y12 antagonist cangrelor and the Gs-coupled receptor agonist iloprost on platelet cAMP levels were determined. Cangrelor (used at a concentration of 1 μM) did not bring about any measurable change in cAMP as determined by % conversion of 3H-adenine (0.032% ± 0.001 cf 0.034% ± 0.001 for saline, n=7). In contrast, iloprost (used at a concentration of 10 nM) brought about a marked increase in cAMP (0.486% ± 0.099, n=5, p<0.01 cf saline).

Discussion

This investigation was performed to address the possibility that certain P2Y12 antagonists may inhibit platelet function by additional mechanisms distinct from P2Y12 antagonism involving interaction with G-protein-coupled receptors other than the P2Y12. The P2Y12 antagonists that we chose to investigate were cangrelor, ticagrelor and PAM. We argued that if PAM was indeed interacting with receptors additional to P2Y12 receptors via their cysteine residues, then this agent may differ from cangrelor and/or ticagrelor in its ability to inhibit platelet function. Also, if it were to interact with G-protein-coupled receptors such as the IP, EP4 or A2A receptor, PAM may interfere with the normal functioning of such receptors. We also argued that if cangrelor has a major effect at an unidentified Gs-protein-coupled receptor as well as the P2Y12 receptor, and thereby increases cAMP, the effect of this agent on platelet function may also differ from those of other P2Y12 antagonists.

As expected, all three P2Y12 antagonists strongly inhibited platelet aggregation induced by ADP in the same way as did iloprost, ONO-AE1–329 and adenosine. However, in contrast to the results obtained with iloprost, ONO-AE1–329 and adenosine, which always produced strong inhibition regardless of the aggregating agent, the P2Y12 antagonists were much less effective at inhibiting platelet aggregation induced by TRAP or U46619. Further, when steps were taken to remove any ADP secreted from the platelets following platelet activation with TRAP or U46619, there was only a small additional inhibition of aggregation brought about by cangrelor, ticagrelor or PAM. We could detect no differences in the inhibitory effects of cangrelor, ticagrelor and PAM on the measured aggregation responses in any of the experiments that were performed. Such results are not indicative of major effects of any of the investigated P2Y12 antagonists at G-protein-coupled receptors other than P2Y12 receptors.

The other experiments described in this paper also provided no positive evidence for any of several potentially plausible interactions between the P2Y12 antagonists investigated and G-protein-coupled receptors on platelets other than P2Y12. First, the small additional inhibition of TRAP-induced platelet aggregation brought about by all three antagonists was unaffected by known antagonists that act at IP, EP4 and A2A receptors. Second, none of the P2Y12 antagonists interfered with the inhibitory effects of agonists that act at these same receptors. Third, none of the P2Y12 antagonists interfered with the ability of an agonist that acts at EP3 receptors to promote platelet aggregation. Finally, there was no evidence of any differential effects of different P2Y12 antagonists at PAR1 or TP receptors given the similarity of their effects on the aggregation induced by TRAP and by U46619.

There are other potential G protein-coupled receptors on platelets with which P2Y12 antagonists may interact, particularly the purinergic receptors P2Y1 and P2Y14. However, binding studies have provided no evidence of any interaction of cangrelor (36) or PAM (37) with the P2Y1 receptor, and although the Gs-coupled P2Y14 receptor has been identified on platelets, it has not been shown to be functional (24) and so is unlikely to play a part in mediating any inhibitory effects of the P2Y12 antagonists.

Figure 8: Effects of P2Y12 receptor antagonists on VASP phosphorylation in the presence of an EP3 receptor agonist. VASP phosphorylation was measured in PRP to which iloprost (0.3 nM) had been added following which the effects of the EP3 receptor agonist sulprostone (1 μM) were determined. Experiments were also performed in the presence of the P2Y12 antagonists cangrelor (1 μM), ticagrelor (10 μM) or PAM (10 μM). VASP phosphorylation was determined by flow cytometry using a cytometric bead array and is presented as median fluorescence (mf). The results shown are the mean ± SEM of three experiments. *p<0.05 cf iloprost.
To look further for effects of the P2Y\textsubscript{12} antagonists at G\textsubscript{s}-protein-coupled receptors we determined their effects on VASP phosphorylation. Occupation of G\textsubscript{s}-protein-coupled receptors raises cAMP in platelets which is reflected in increased VASP phosphorylation (38) and this was confirmed by the large increases seen here using iloprost, ONO-AE1–329 and adenosine. In contrast, only very small effects of cangrelor, ticagrelor and PAM on VASP phosphorylation were observed, and we could find no evidence of any interference with the effects on VASP phosphorylation of agents acting via IP\textsubscript{1}, EP4, A\textsubscript{2A} or EP3 receptors. Direct measurement of cAMP revealed no effect of cangrelor, whilst iloprost, as expected, was extremely effective in raising cAMP levels.

These results provide little or no evidence for effects of P2Y\textsubscript{12} antagonists acting via G-protein-coupled receptors other than P2Y\textsubscript{12}. They contrast with the results described in a previous report which suggested a major role for an unidentified G\textsubscript{s}-protein-coupled receptor in mediating the inhibitory effects of cangrelor and 2MeSAMP on platelet function through increased levels of cAMP (29). On the other hand, they agree with a very recent contradictory report in which cangrelor and 2MeSAMP failed to increase cAMP levels in human platelets or in platelets from wild-type or P2Y\textsubscript{12}-deficient mice (39).

In our own experiments performed using PRP derived from fresh blood we found it necessary to take positive steps to exclude any contributions of ADP and adenosine before we could be confident of our conclusions. In attempting to explain why our findings differ from the previous report (29), it should be noted that in that report platelet concentrates were used, rather than fresh PRP. We think it is possible that ADP may have accumulated in those samples and this would have led to an overestimation of the ability of the P2Y\textsubscript{12} antagonists to influence platelet function induced by agonists other than ADP.

One observation made here that is unresolved is the nature of the small degree of inhibition of platelet aggregation that is still seen following addition of cangrelor, ticagrelor and PAM in the experiments in which secreted ADP was removed using apyrase and ADA, and also the small increase in VASP phosphorylation that occurred. In attempting to explain this observation, we must point out that this small degree of inhibition was the same for all three P2Y\textsubscript{12} antagonists, as was the small degree of VASP phosphorylation that was also seen using all three agents. Given that all three agents exert their effects at P2Y\textsubscript{12} receptors via different mechanisms of action, we suggest that the commonality of their effects is likely to be because these are mediated via the P2Y\textsubscript{12} receptor itself.

We consider that there are two ways in which the P2Y\textsubscript{12} antagonists could all produce these effects via P2Y\textsubscript{12} receptors. One possibility is that P2Y\textsubscript{12} receptors possess a certain constitutive activity that is continually reducing intracellular cAMP via activation of G\textsubscript{s}, and that the P2Y\textsubscript{12} antagonists, acting as inverse agonists, remove this constitutive activity. Previous observations that give credence to this are i) a report of a high level of constitutive activity in a mutated form of the human P2Y\textsubscript{12} receptor (40), and ii) an observation that transfected wild-type P2Y\textsubscript{12} receptors showed clear evidence of constitutive activation when a sensitive electrophysiological assay was used to detect this (41).

Another possibility is that, despite our attempts to remove all influence of secreted ADP and its metabolites in our experiments, a small amount of ADP, possibly inaccessible to the apyrase added, may have remained associated with P2Y\textsubscript{12} receptors and that the P2Y\textsubscript{12} antagonists were able to negate the effects of this ADP thus producing an additional small degree of inhibition of platelet function. We did experiment with the use of an even higher concentration of apyrase (10 U/ml) to remove any last traces of ADP but the results were identical to those with 3 U/ml (results not shown).

We believe that either of these possibilities would explain both the additional inhibition of aggregation seen and the small increase in VASP phosphorylation obtained with all three agents, even though we were unable to demonstrate a direct effect of cangrelor on cAMP when we looked directly for this. The latter may have been a consequence of an inferior sensitivity of the cAMP measurements compared with the measurements of VASP phosphorylation.

In this investigation we looked for evidence that one or more of cangrelor, ticagrelor or PAM may influence platelet function via effects at platelet G-protein-coupled receptors additional to P2Y\textsubscript{12}. However, we could find little or no evidence for this.

Although not within the scope of this particular investigation as such, we began the study in the belief that the results may eventually provide some insight into the differences in clinical benefit derived from the use of different P2Y\textsubscript{12} antagonists, as seen, for example, in the PLATO trial (30). Despite the finding that all three agents studied consistently yielded similar results under the experimental conditions described here, we must point out that there are other potential mechanisms through which the agents may exert different effects on platelet function that are unrelated to

What is known about this topic?

- P2Y\textsubscript{12} antagonists are widely used as anti-thrombotic agents and as experimental agents for assessing the role of P2Y\textsubscript{12} receptors in platelet function.
- However, the possibility has been raised that some P2Y\textsubscript{12} antagonists may inhibit platelet function by other modes of action, especially via effects at G-protein-coupled receptors other than P2Y\textsubscript{12}.
- Thus it was important that this possibility was further investigated and the mode of action of P2Y\textsubscript{12} antagonists further defined.

What does this paper add?

- We looked carefully for additional effects at G-protein-coupled receptors of three well-known P2Y\textsubscript{12} antagonists, cangrelor, ticagrelor and prasugrel active metabolite.
- We could find no evidence of any of the P2Y\textsubscript{12} antagonists acting through other G-protein receptors including PAR1, TP, IP, EP4, A\textsubscript{2A} or EP3 receptors.
- The selective mode of action of P2Y\textsubscript{12} antagonists at P2Y\textsubscript{12} receptors is thus confirmed.
their direct effects on platelets themselves. For example, ticagrelor is reported to inhibit adenosine uptake into red cells (42) and thus may influence platelet function indirectly through raised levels of this agent in vivo. Any antiplatelet effect of ticagrelor through a mechanism related to adenosine uptake would not be revealed by the experiments conducted in this study. We are therefore addressing this possibility in further studies that are currently being performed.

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