

Ticagrelor inverse agonist activity at the P2Y₁₂ receptor is non-reversible versus its endogenous agonist ADP.

Short running title: Inverse agonism at the P2Y₁₂ receptor

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Total Word Count: 7284

Acknowledgements: The following grants supported work performed in this study: British Heart Foundation Project Grants PG/17/62/33190 (supporting J.K.) and PG/20/1/34617 (supporting J.L.H.) and the British Pharmacological Society AJ Clark Studentship (supporting M.K.).

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.

Competing Interests' Statement: None

Abstract

Background: Ticagrelor is labelled as a reversible, direct-acting platelet P2Y₁₂ receptor (P2Y₁₂R) antagonist that is indicated clinically for the prevention of thrombotic events in patients with acute coronary syndrome (ACS). As with many antiplatelet drugs, ticagrelor therapy increases bleeding risk in patients which in emergency situations requires platelet transfusion although there is ongoing debate on its effectiveness following ticagrelor therapy. The aim of this study was to further examine the reversibility of ticagrelor at the P2Y₁₂R.

Methods: Studies were performed in human platelets with both P2Y₁₂R-stimulated GTPase activity and platelet aggregation assessed. Cell-based bioluminescence resonance energy transfer (BRET) assays were also undertaken to assess G protein subunit activation downstream of P2Y₁₂R activation.

Results: Initial studies revealed a range of P2Y₁₂R ligands including ticagrelor displayed inverse agonist activity at the P2Y₁₂R. Of these only ticagrelor was resistant to wash-out. In both human platelets and cell-based assays, washing failed to reverse ticagrelor-dependent inhibition of ADP-stimulated P2Y₁₂R function in contrast to other P2Y₁₂R antagonists. The P2Y₁₂R agonist 2MeSADP, which was also resistant to wash-out, was able to effectively compete with ticagrelor. In silico docking revealed that ticagrelor and 2MeSADP penetrated more deeply into the orthosteric binding pocket of the P2Y₁₂R than other P2Y₁₂R ligands.

Conclusion: Ticagrelor binding to the P2Y₁₂R is prolonged and more akin to that of an irreversible antagonist especially versus the endogenous P2Y₁₂R agonist ADP. This study highlights the potential clinical need for novel ticagrelor reversal strategies in patients with spontaneous major bleeding and bleeding associated with urgent invasive procedures.

Keywords: P2Y₁₂ receptor, acute coronary syndrome, blood platelets, ticagrelor, irreversibility,

Introduction

Since its introduction, in 2011, the anti-platelet agent ticagrelor has established itself as a standard of care in the management of patients with acute coronary syndrome (ACS)(Collet et al., 2021). The drug selectively binds the P2Y₁₂R on the platelet surface and provides faster, greater and more consistent platelet inhibition when compared to other anti-platelet drugs including clopidogrel (Van Giezen et al., 2009; Wallentin et al., 2009). Unlike the thienopyridine-based orally administered P2Y₁₂R antagonists (ticlopidine, clopidogrel, and prasugrel), which are all prodrugs requiring hepatic metabolism to produce active compounds that bind irreversibly to P2Y₁₂R to exert anti-aggregatory activity, ticagrelor is a non-thienopyridine (cyclopentyl-triazolopyrimidine) not requiring bioactivation to act on the P2Y₁₂R(Butler & Teng, 2010). In the Platelet Inhibition and Patient Outcomes (PLATO) trial, ticagrelor's more potent platelet inhibition provided greater clinical benefit with a decreased risk of major adverse cardiovascular events and improved survival in patients with ACS, when compared to clopidogrel(Wallentin et al., 2009). However, similar to prasugrel, ticagrelor is associated with an increased risk of major bleeding, which persists for days after drug discontinuation(Wallentin et al., 2009; Wiviott et al., 2007). The management of bleeding risk represents a major clinical challenge especially in patients who present with spontaneous life-threatening bleeding or who require urgent surgical procedures, especially since there are no standardized reversal strategies or clinically available antidotes(Buchanan et al., 2015).

In the absence of specific antidotes, platelet transfusion is often used in an emergency to reverse the effect of anti-platelet drugs(Sousa-Uva et al., 2014). In the absence of platelet transfusion the offset of ticagrelor activity effects are approximately 5 days (Gurbel et al., 2009). This approach is primarily based on the hypothesis that substituting drug-inhibited platelet populations with functional donor platelets could result in overall improved haemostatic response. Notably platelet transfusion efficiently reverses the inhibitory effect of clopidogrel and prasugrel on platelets in a dose-dependent manner (Bonhomme, Bonvini, Reny, Poncet, & Fontana, 2015; Li, Hirsh, Xie, Johnston, & Eikelboom, 2012; Schoener et al., 2017). However, the effectiveness of this approach with ticagrelor has been questioned in a number of recent studies(Godier, Taylor, & Gaussem, 2015; Trenk et al., 2019; Willeman et al., 2018; Zhang et al., 2019). Persistent inhibition of platelet aggregation is observed for several days after discontinuation of ticagrelor and can still be observed when plasma

concentrations of ticagrelor are undetectable, with platelet reactivity only returning to near normal levels about 5 days following cessation of treatment (Storey et al., 2011). In this study, we sought to further probe the reversibility of ticagrelor binding to the P2Y₁₂R and compared it against other receptor antagonists. Importantly we found that the endogenous P2Y₁₂R agonist ADP was unable to restore P2Y₁₂R activity following ticagrelor treatment even after extensive washing of antagonist in either cell lines or human platelets.

Methods

Plasmids and reagents

FLAG-tagged human wild-type P2Y₁₂ receptor construct using pcDNA3.1 were generated as previously described (Hardy et al., 2005), and their validity confirmed with sequencing (Eurofin Genomics). Xanthine amine congener (XAC), AR-C 66096 tetrasodium salt, AR-C66931MX (Cangrelor), ticagrelor, elinogrel, AZD1283, SAR246471 and forskolin were procured from Tocris Bioscience (Bristol, UK). R-138727 was received from Eli Lilly Research Laboratories (Indianapolis, IN). Luciferase substrate (Coelenterazine 400a) was acquired from Insight Biotechnology Limited (Wembley, UK). Cell culture reagents included Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and Lipofectamine 2000 were from Invitrogen (Paisley, UK). All other reagents were purchased from Sigma-Aldrich.

Isolation of Human platelets

Samples were obtained from healthy consented male and female volunteers, who confirmed that they were not receiving any medication that affects platelet activity. Whole blood was collected in 4 % sodium citrate and acid citrate dextrose (29.9 mM Na₃C₆H₅O₇, 113.8 mM glucose, 72.6 mM NaCl, and 2.9 mM citric acid [pH 6.4]). Platelet-rich plasma was obtained by centrifugation at 180g for 17 min, treated with 0.02 U/ml apyrase and 10 µM indomethacin and was then centrifuged at 550g for 10 min. The platelet pellet was then resuspended in wash buffer (36mM citric acid, 10 mM EDTA, 5 mM glucose, 5 mM KCl, 9 mM NaCl) containing 0.02 U/ml apyrase and 10 µM indomethacin and centrifuged at 550g for 10 min. Platelets (1x10⁹/ml) were resuspended in modified Tyrode's buffer (150 mM NaCl, 5 mM N-2-

hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES), 0.55 mM NaH₂PO₄, 7 mM NaHCO₃, 2.7 mM KCl, 0.5 mM MgCl₂, 5.6 mM glucose [pH 7.4]) supplemented with 0.02 U/ml apyrase and 10 µM indomethacin and rested at 30 °C for at least 30 min before experimentation. All platelet preparations were processed room temperature.

Platelet aggregation

Platelet rich plasma (PRP) was pre-treated with either ARC66096, ticagrelor or vehicle (0.1% DMSO) for 30 minutes. Platelets were then washed by centrifugation and resuspension of the platelet pellet in fresh 300 µl PPP (platelet poor plasma) with 500 µl PPP as control. Platelets were incubated for 10 mins between wash-steps, except in those experiments assessing long time periods of wash-out where platelets were incubated after the second wash step for 1, 4 or 24 hrs. Aggregation was initiated by 10 µM ADP under constant stirring conditions (1000 rpm) for 5 min at 37 °C using light transmission aggregometry with a CHRONO-LOG 700 aggregometer (Labmedics, Manchester, United Kingdom).

GTPase activity assay

Washed platelet suspension (1x10⁹/ml) were either treated or untreated with different agonist/antagonists at indicated time points and reactions were stopped with an equal volume of fractionation buffer (320 mM sucrose, 4 mM HEPES, 0.5 mM Na₃PO₄, Ph 7.4) supplemented with protease inhibitor cocktail. Samples were subjected to 5 freeze-thaw cycles in liquid nitrogen. Unbroken platelets were removed by centrifugation at 5000g for 5 min at 4 °C before ultracentrifugation at 180,000g for 90 min at 4 °C. The supernatant was removed, and the pellet fraction was washed 2 times with 1 ml of fractionation buffer and resuspended in 50 µl of GTPase assay buffer. The intrinsic GTPase activity of Gαi in human platelets was measured using the GTPase Glo-assay (Promega). Briefly, the membrane fraction of human washed platelets were resuspended in assay buffer consisting of 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mg/mL BSA and treated in the presence or absence of different agonists/antagonists at indicated time points before incubation with recombinant GTP (2 µM) and DTT (1mM) for 1 hr at 24°C. Then GTPase Glo reagent, including 5 µM ADP, were added, briefly mixed, and incubated for 30 min with shaking at 24°C. Finally, detection reagent was added for 5 min in the dark and GTP hydrolysis

(luminescence) was measured using a Tecan Infinite M200 Pro microplate reader (Männedorf, Switzerland).

Cell culture and transfection

Human Embryonic Kidney (HEK293T) cells were maintained in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were grown in 100 mm dishes to 70-90% confluence and transiently cotransfected with 1-1.5 µg DNA using Lipofectamine 2000 according to manufacturer's protocol. Briefly cells were incubated with DNA/Lipofectamine complexes for 5 hrs, the media was replaced, and cells were analysed for BRET2 assay after 48 hrs transfection.

Bioluminescence Resonance Energy Transfer measurement

To investigate the effect of a drug on heterotrimeric G-protein activation, vectors encoding N-terminal FLAG-P2Y₁₂ receptor, RlucII-Gai1 (a mutated brighter version of the Rluc), Gβ1 and GFP2-Gγ2 were transiently cotransfected into HEK293T cells as previously described (Zhao et al., 2021). In Brief, 48 hrs post-transfection, cells were detached using phenol free Trypsin-EDTA, washed 2x and resuspended in phenol free DMEM at room temperature. Then 80 µl of 100,000 cells per well were seeded in a 96 well white flat-bottom microplate (Griener Bio-One, Austria) and rested for at least 30 min at 37 °C before experimentation. Cells were then treated with or without different concentrations of ligands at indicated time points. BRET2 signal between RlucII and GFP was immediately measured following addition of the Coelenterazine 400a (luciferase substrate) at a final concentration of 2 µM using a FLUOstar® Omega plate reader (BMG Labtech, UK). The BRET signal was calculated as the ratio of the light emitted by acceptor (GFP2) (510-540 nm) to donor (RlucII) (410-480 nm). To determine the delta BRET (δBRET), the value obtained in the vehicle condition was subtracted from the one measured with ligand.

In Silico Ligand Docking and Molecular Dynamic Simulations

ADP, 2MeSADP, ticagrelor and cangrelor were docked as outlined below. ADP and 2MeSADP were docked within the agonist-bound crystal model of the receptor (PDB ID: 4PXZ; (J. Zhang et al., 2014)). The antagonists, ticagrelor and Cangrelor, were docked within the antagonist-bound crystal structure of the P2Y₁₂R (PDB ID: 4NTJ;

(K. Zhang et al., 2014)). Before docking, to take account for ligand flexibility, 10,000 conformations of each ligand were generated. To generate these ligand conformations, molecular dynamic (MD) simulations of each ligand were produced. Solvated in a box of TIP3P H₂O and 0.15M NaCl, each 'free in solution' ligand was simulated for 1us, employing AMBER gaff forcefields. The 10,000 ligand conformations generated were docked using the Bristol University Docking Engine (BUDE) within the appropriate crystal model of the P2Y₁₂R, and the free energy of binding, predicted. Based on the free energy and 125 ns MD simulations of several ligand-receptor complexes, a final ligand-receptor complex was selected for each ligand. For MD simulation of the ligand-receptor complex, the complex was embedded in a membrane of POPC and 20% cholesterol and solvated in TIP3P H₂O with 0.15M NaCl. The system was minimised over 10,000 steps and then heated in two steps, first to 100K, then to 310K. The heated system was then equilibrated over ten rounds of 500 ps simulations, under anisotropic pressure scaling. Subsequent simulation was conducted in the presence of ff14SB, gaff and Lipid14 forcefields, using the Langevin thermostat and anisotropic Berendson barostat. With a timestep of 0.002 ps, coordinates were written to the trajectory file every 100 ps. Based on root-mean square deviation (RMSD) analysis of the stability of the ligand-receptor complex during the 125 ns MD simulation, a final binding pose for each ligand was selected. The plane of the extracellular membrane of the ligand-receptor simulation system was used to calculate the depth of each ligand within the P2Y₁₂R. The distance of the deepest point of each ligand and the geometric centre of each ligand, from the extracellular membrane plane, was calculated. The initial docked poses were used for the distance calculations.

Data and statistical analysis

Results are presented as average \pm standard error of the mean (SEM) of at least five independent experiments. Data analyses were performed using GraphPad Prism 8 (San Diego, CA). One-way analysis of variance (ANOVA), unless otherwise specified, was used to detect statistically significant differences with Bonferroni post hoc analysis applied for multiple comparisons. A *P* value less than 0.05 was considered significant.

Results

Our initial experiments aimed to recapitulate our previous findings demonstrating that ticagrelor has inverse agonist activity at the P2Y₁₂R (Aungraheeta et al., 2016). In these studies we used a standard BRET based approach which measures agonist-stimulated changes in Gα/βγ disassociation using the functionally validated BRET pair RlucII-Gai1 and GFP10-Gγ2 (Gales et al., 2005; Zhao et al., 2021). As expected, receptor activation with the P2Y₁₂R agonists ADP or 2MesADP promoted rapid RlucII-Gai1 and GFP10-Gγ2 disassociation leading to a decrease in ΔBRET signal in a concentration-dependent manner (Figure 1A). In agreement with our previous studies (Aungraheeta et al., 2016) and those of Garcia et al., (Garcia et al., 2019) ticagrelor decreased RlucII-Gai1 and GFP10-Gγ2 disassociation leading to an increase in ΔBRET signal whilst the neutral P2Y₁₂R antagonist ARC66096 had no effect. We subsequently assessed if a range of P2Y₁₂R antagonists, including both clinically used drugs such as cangrelor or experimental compounds including elinogrel and AZD1283 had inverse agonist activity at the P2Y₁₂R (Figure 1B). Interestingly all of these compounds did indeed display a degree of inverse agonism although with a range of potencies and Emax values (see Table 1).

As outlined above there is still considerable controversy regarding the reversibility of ticagrelor at the P2Y₁₂R versus ADP, the endogenous agonist at this receptor. We therefore sought to examine this using our BRET based approach (Figure 2A). Transfected cells were treated with ticagrelor (10 μM; 10 mins) or vehicle control. Ticagrelor reversibility was assessed by washing cells with either 3 x 10 minute washes (30 minutes total) or 3 x 30 minutes washes (90 minutes total). Ticagrelor activity, either inverse agonism in the absence of ADP, or antagonism of ADP (10 μM)-stimulated P2Y₁₂R activity was subsequently assessed. In the absence of washes, ticagrelor inverse agonism was evident whilst ADP-stimulated P2Y₁₂R activity was completely attenuated by ticagrelor pre-treatment. Notably, neither the shorter or more extended wash protocols were able to effectively reverse either ticagrelor inverse agonism or antagonism of ADP-stimulated P2Y₁₂R activity. Further study was undertaken comparing ticagrelor with the potent P2Y₁₂R agonist 2MeSADP (10 μM; Figure 2B). Notably 2MeSADP was more effective than ADP in reversing ticagrelor inverse agonism in unwashed cells. However as in Figure 2A, ticagrelor antagonism of 2MeSADP-stimulated P2Y₁₂R activity was unchanged by washing cells.

We next tested the reversibility of a range of P2Y₁₂R antagonists and inverse agonists to ensure that our wash protocol was effective (Figure 3). As expected, pre-treatment with the reversible P2Y₁₂R antagonist ARC66096 (10 µM; 10 mins) was able to effectively attenuate ADP-stimulated P2Y₁₂R activity (Figure 3A) an effect that was reversed by washing cells. Inverse agonism and antagonism of ADP-stimulated P2Y₁₂R activity by cangrelor pretreatment (10 µM; 10 mins) was also effectively reversed by washing cells (Figure 3B). Pre-treatment with the active metabolite of prasugrel (R-138727; 10 µM; 10 mins), an established irreversible P2Y₁₂R antagonist effectively blocked ADP-stimulated P2Y₁₂R activity an effect that was not reversed by washing cells (Figure 3C). Further study focussing on the reversibility of the inverse agonist or agonist activity of the range of P2Y₁₂R agonists and inverse agonists identified in Figure 1 revealed that the activity of ADP, AZD6140, cangrelor and elinogrel (all at 10 µM; 10 mins) was lost following cell washing. Intriguingly the activity of ticagrelor (10 and 0.4 µM) and the agonist 2MeSADP (10 µM) was resistant to cell washing (Figure 4).

One residue in the P2Y₁₂R identified as critical for ticagrelor activity is cysteine 194(Hoffmann et al., 2014). We therefore investigated if this residue, in part, was also responsible for the irreversibility of ticagrelor. As expected, inverse agonism of ticagrelor at the P2Y₁₂R was significantly attenuated in a P2Y₁₂R in which we mutated cysteine 194 to an alanine (C194A; Figure 5). In addition, ticagrelor's ability to effectively antagonize ADP-stimulated P2Y₁₂R activity was significantly compromised in the C194A P2Y₁₂R. Notably, the residual inverse agonism still present in C194A P2Y₁₂R or antagonism of ADP-stimulated P2Y₁₂R activity still appeared resistant to wash-out.

Following on from our cell line studies we next focussed on endogenous P2Y₁₂R activity in human platelets. Our initial studies used a GTPase Glo-assay to measure G-protein activity following P2Y₁₂R activation. As expected, stimulation of platelet cell membranes with ADP (10 µM) produced a pronounced increase in GTP hydrolysis indicative of increased P2Y receptor stimulation (Figure 6A and B). Pre-treatment of platelets with either ticagrelor (10 µM; 30 mins; Figure 6A) or AR-C66096 (10 µM; 30 mins; Figure 6B) effectively antagonized ADP-stimulated rises in GTP hydrolysis. As in our cell line studies, and previously reported in human platelets(Aungraheeta et al., 2016) ticagrelor but not AR-C66096 pre-treatment effectively attenuated basal levels of platelet GTP hydrolysis indicative of reduced G protein activity and inverse agonist

activity of ticagrelor at the P2Y₁₂R. Neither antagonism of ADP-stimulated or basal GTP-hydrolysis by ticagrelor pre-treatment was reversed by platelet washing (3 x 10 minutes washes) prior to platelet membrane preparation (Figure 6A). AR-C66096-dependent antagonism of ADP-stimulated GTP-hydrolysis was readily reversed by platelet washing (Figure 6B).

We next examined the reversibility of inhibition of ADP-stimulated platelet aggregation by ticagrelor and AR-C66096 (Figure 7). As shown in Figure 5A, pre-treatment of human platelet rich plasma (PRP) with ticagrelor (1 μ M; 30 min) effectively antagonized ADP (10 μ M)-stimulated platelet aggregation. This effect of ticagrelor was not reversed by extensive platelet washing (either one 10 minute wash; washout 1 or two 10 minute washes; washout 2) prior to ADP-stimulated platelet aggregation (Figure 5A and B). AR-C66096 block of ADP-stimulated platelet aggregation was effectively reversed by washing (Figure 7B). To further probe our inability to washout ticagrelor we further extended our wash-times to 1, 4 and 24 hrs (Figure 7C). As expected at the longer periods of wash (4 and 24 hrs) ADP-stimulated platelet aggregation began to significantly reduce as the viability of our platelet preparation began to reduce. Notably however ticagrelor antagonism of ADP-stimulated platelet aggregation remained stubbornly resistant to reversal even following 24 hrs of wash, whilst that of AR-C66096 was rapidly reversed within an hour. These studies confirmed our cell line studies which indicated that ticagrelor appeared to show an irreversible mode of action at the P2Y₁₂R versus the endogenous receptor agonist ADP.

In order to further understand why both ticagrelor and 2MeSADP appeared resistant to wash-out we performed in-silico docking and all-atom molecular dynamic simulations of the P2Y₁₂R, and compared the binding profile of the agonists ADP, 2MeSADP and inverse agonists ticagrelor and cangrelor. Employing both the agonist- and antagonist-bound crystal structures of the P2Y₁₂R, agonists were docked within the agonist model, antagonists in the antagonist model (Figure 8). Based on the suggested binding poses ticagrelor, 2MeSADP and cangrelor occupy a binding pocket within the orthosteric site which is distinct to that of ADP (Figure 8A). This is in accordance with previously published data (J. Zhang et al., 2014; K. Zhang et al., 2014). The geometric centre (centroid) of each ligand was estimated and interestingly, cangrelor was found to sit higher in the receptor orthosteric cavity when compared to the other ligands (Figure 8B). The deepest penetration point for each ligand was

identified and distance to extracellular membrane plane calculated (Figure 8C-E). Intriguingly we predict that the wash-resistant compounds ticagrelor and 2MeSADP penetrate more deeply into the binding pocket than cangrelor.

Discussion

The mechanism of action of ticagrelor is distinct from that of previously described antiplatelet agents targeting the P2Y₁₂R (Aungraheeta et al., 2016; Hoffmann et al., 2014; Van Giezen et al., 2009). Ticagrelor has been demonstrated to be an inverse agonist at the platelet P2Y₁₂R (Aungraheeta et al., 2016; Garcia et al., 2019). In addition ticagrelor has been shown to inhibit the platelet adenosine ENT1 transporter; resulting in the accumulation of extracellular adenosine that further dampens down platelet reactivity (Aungraheeta et al., 2016). The central aim of this current study was to further elucidate the mode of action of ticagrelor antiplatelet therapy focussing predominantly on drug reversibility which is crucially important to consider when assessing the safety and efficacy of pharmacological therapeutics.

Given a number of recent clinical studies (Godier et al., 2015; Trenk et al., 2019; Willeman et al., 2018; Zhang et al., 2019) we sought to probe the reversibility of ticagrelor binding to the P2Y₁₂R in comparison with other receptor antagonists. Ticagrelor has demonstrated clinical superiority over many antiplatelet agents (Bonaca et al., 2015; Wallentin et al., 2009). However, the significant side effect of spontaneous major bleeding and bleeding during invasive procedures as with other P2Y₁₂R antagonists remains with ticagrelor. In respect to the thienopyridines, clopidogrel and prasugrel, the cause for this unwanted bleeding is thought in part to be due to their mode of action and irreversible blockade of the P2Y₁₂R. These drugs effectively attenuate platelet function for the duration of their lifespan. There is a clear need therefore for reversible P2Y₁₂R antagonists especially in the context of patients undergoing emergency invasive procedures or requiring abrupt cessation secondary to significant bleeding. Our study demonstrates that the endogenous agonist ADP was unable to restore P2Y₁₂R activity following ticagrelor treatment even after extensive washing in either cell lines or human platelets.

Although ticagrelor has been licensed as the first perorally active and reversible P2Y₁₂R antagonist evidence contests ticagrelor's reversibility at the P2Y₁₂R (Gerrits, Jakubowski, Sugidachi, Michelson, & Frelinger, 2017). For example, ticagrelor displayed a similar bleeding profile to clopidogrel during invasive procedures (James

et al., 2009) where ticagrelor was withheld for 24-72 hours and clopidogrel for 5 days. More recent studies indicate that platelet supplementation via transfusion does not rescue platelet inhibition resulting from ticagrelor action (Godier et al., 2015; Trenk et al., 2019; Willeman et al., 2018; Zhang et al., 2019). Potentially this is because ticagrelor and its active metabolite (AR-C124910XX) have much longer half-lives (9 and 12 hrs, respectively) than the thienopyridine derivatives prasugrel and ticagrelor with their ongoing presence able to inhibit fresh platelets at the time of the transfusion (Butler & Teng, 2010; Cave et al., 2019; Zhu et al., 2019). Intriguingly, ticagrelor-targeted monoclonal antibody fragments have been engineered, appearing efficacious in the rapid reversal of the drug (Bhatt et al., 2019). The data from these studies ultimately underscores the inconsistencies in the reported reversibility of ticagrelor.

Our study is the first, to our knowledge, to demonstrate that ticagrelors' inverse agonism and antagonism of ADP-stimulated P2Y₁₂R activity are not readily reversed in cell lines or more importantly human platelets. Previous seminal studies by Van Giezen et al., have demonstrated that [³H]-ticagrelor binds to the P2Y₁₂R in a reversible manner with a $t_{1/2}(\text{on})$ and $t_{1/2}(\text{off})$ of 3.8 ± 0.9 and 13.5 ± 1.9 min respectively (Van Giezen et al., 2009). These studies were undertaken in P2Y₁₂R-transfected CHO-K1 membranes and, as is standard for such studies, [³H]-ticagrelor (40 nM) was displaced with unlabelled ligand (10 μ M). Clearly, therefore, ticagrelor is not a classical irreversible antagonist at the P2Y₁₂R like the thienopyridines, clopidogrel and prasugrel. Intriguingly, our studies have demonstrated that ticagrelor activity was maintained following extensive washing, both in our whole cell studies (BRET and platelet aggregation) and in cell membrane (GTPase Glo Assays). Studies in CHO cells expressing the P2Y₁₂R also show that ticagrelor shows little demonstrable reversibility versus ADP (data not shown).

In agreement with van Giezen et al (Van Giezen et al., 2009) and consistent with our previous studies (Aungraheeta et al., 2016) we showed that 2MeSADP, a P2Y₁₂R agonist, with a 100 fold higher potency than ADP, was more readily able to compete with ticagrelor. Again, as with ADP, wash steps did not reverse residual 2MeSADP antagonism by ticagrelor. We (Aungraheeta et al., 2016) and others (Van Giezen et al., 2009) have demonstrated that ticagrelor shows non-competitive antagonism versus ADP at therapeutic antagonist concentrations. Ticagrelor is suggested to bind to a distinct site to that of ADP on the P2Y₁₂R and act through a non-competitive, allosteric

mechanism to prevent ADP-stimulated receptor activation. Previous mutagenesis analysis has shown that cysteine 194 of the P2Y₁₂R plays a key role in coordinating ticagrelor binding (Hoffmann et al., 2014). Interestingly we found that the resistance of ticagrelor to removal by washing was maintained in a P2Y₁₂R construct (C194A) with demonstrably reduced ticagrelor activity.

Notably Gerrits et al., reported that prolonged incubation of ex-vivo platelets with ticagrelor (24 h) resulted in incomplete reversibility of platelet reactivity, a phenomenon not observed after shorter periods of exposure of ticagrelor (Gerrits et al., 2017). This study suggested that the process of irreversible inhibition was time-dependent. Our study in cell lines and human platelets indicate a more rapid emergence of irreversible inhibition. The possibility of ticagrelor acting at a different off-target site to cause irreversible platelet inhibition cannot be excluded although would seem less plausible in our HEK293 cell line system. Given the short time periods of ticagrelor treatment (30 minutes or less) we would also suggest that changes in protein expression are unlikely to explain the apparent irreversibility of ticagrelor reactivity. Importantly we show that ticagrelor's resistance to washing is retained at therapeutically relevant plasma concentrations of drug (0.4 µM). Potentially ticagrelor binding may alter the P2Y₁₂R conformation or more likely some ticagrelor may remain bound to the P2Y₁₂R. Intriguingly the SWAP-2 study demonstrated a failure of prasugrel to significantly block P2Y₁₂R function when administered 36 and 60 hours after the last ticagrelor dose (Angiolillo et al., 2014). This would support the theory that residual ticagrelor binding or ticagrelor-dependent changes in P2Y₁₂R conformation prevent interaction with prasugrel's active metabolite.

Our studies suggest that ticagrelor is not the only P2Y₁₂R ligand to show significant resistance to reversal following washing. Notably 2MeSADP, a potent agonist at the P2Y₁₂R appeared resistant to wash out. Unfortunately, comparison of the structure of 2MeSADP with that of ticagrelor or the more reversible P2Y₁₂R ligands fails to give any obvious structure / function or chemical differences, for example, ligand lipophilicity, to indicate why these two compounds appear resistant to wash-out.

Although the resolution of the P2Y₁₂R by x-ray crystallography provided significant insights into its structure (J. Zhang et al., 2014; K. Zhang et al., 2014), including the presence of two binding pockets, there is still not a definitive binding pose for ticagrelor at the P2Y₁₂R. An extensive molecular docking study of the major classes of substances, previously reported as P2Y₁₂R ligands was unable to dock ticagrelor to

the agonist-bound P2Y₁₂R structure. A “hybrid” receptor for successful ticagrelor docking was required which resembled the agonist-bound P2Y₁₂R except for the top portion of TM6, which was taken from the antagonist-bound P2Y₁₂R structure(Paoletta et al., 2015). We also performed in-silico docking and all-atom molecular dynamic simulations of the P2Y₁₂R, and compared the binding profile of the agonists ADP, 2MeSADP and inverse agonists ticagrelor and cangrelor. Employing both, the agonist- and antagonist-bound crystal structures of the P2Y₁₂R, agonists were docked within the agonist model, antagonists in the antagonist model. We found that such a protocol did not require the design of a ‘hybrid’ receptor. Interestingly we found that cangrelor sits higher and penetrates less deeply into the receptor orthosteric cavity when compared to the wash-resistant ticagrelor and 2MeSADP. Further extensive mutagenesis studies, beyond the scope of this work, may help define how the potential deeper penetration of these ligands into the binding pocket may relate to their wash-resistance.

In conclusion, our study highlights incomplete reversibility of platelet and P2Y₁₂R inhibition following exposure to ticagrelor. This has obvious and clear clinical implications for patients requiring surgical intervention following ticagrelor therapy and underscores current guidelines, which state that in patients on P2Y₁₂R antagonists who need to undergo non-emergency major non-cardiac surgery, postponing surgery for at least 5 days after cessation of ticagrelor should be considered if clinically feasible and unless the patient is at high risk of ischemic events.

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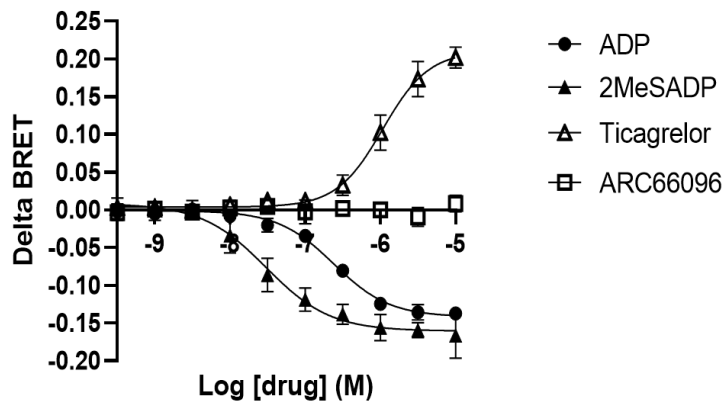
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Table 1

Drug	EC50	E _{max} (A.U)
Ticagrelor	744.3nM (551.0-1180.0nM)	0.2076 (0.1864 to 0.2410)
Cangrelor	41.5nM (32.8-53.1nM)	0.1634 (0.1541 to 0.1730)
Elinogrel	21.4nM (12.1-38.6nM)	0.1081 (0.09770 to 0.1220)
AZD1283	12.7nM (8.3-19.1nM)	0.1197 (0.1110 to 0.1302)

Potency (nM) and E_{max} inverse agonist activity values of range of P2Y₁₂R ligands as assessed by BRET based assay data taken from Figure 1A. Data represent the means of at least 5 independent experiments, each performed in triplicate with numbers in brackets representing 95% confidence intervals.

A



B

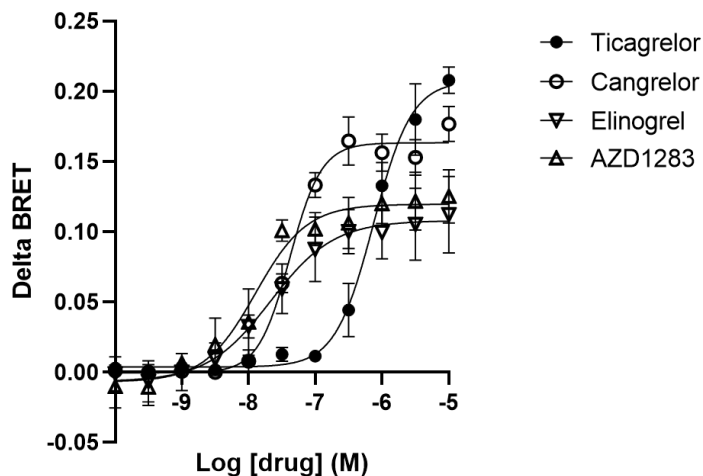


Figure 1: Ligand-dependent regulation of P2Y₁₂R responsiveness as assessed by a BRET based assay. HEK 293T cells were co-transfected with human FLAG-P2Y₁₂R and heterotrimeric G-proteins, RlucII-Gαi, untagged Gβ and GFP-Gγ. Forty-eight hours post transfection cells were treated with ligand for 5 min at 37 °C and subsequent changes in BRET signal measured with reduction in BRET signal a consequence of G protein-subunit disassociation. Results are expressed as delta BRET. (A) Ligand-induced changes in receptor activation as assessed BRET following treatment with ticagrelor, 2MeSADP, ADP, AR-C66096 versus vehicle (0.1% DMSO) control (B) Ligand-induced changes in receptor activation as assessed BRET following treatment with ticagrelor, cangrelor, elinogrel and AZD1283 versus vehicle (0.1% DMSO) control. Data shown are the means ± SEM of at least 5 independent experiments, each performed in triplicate.

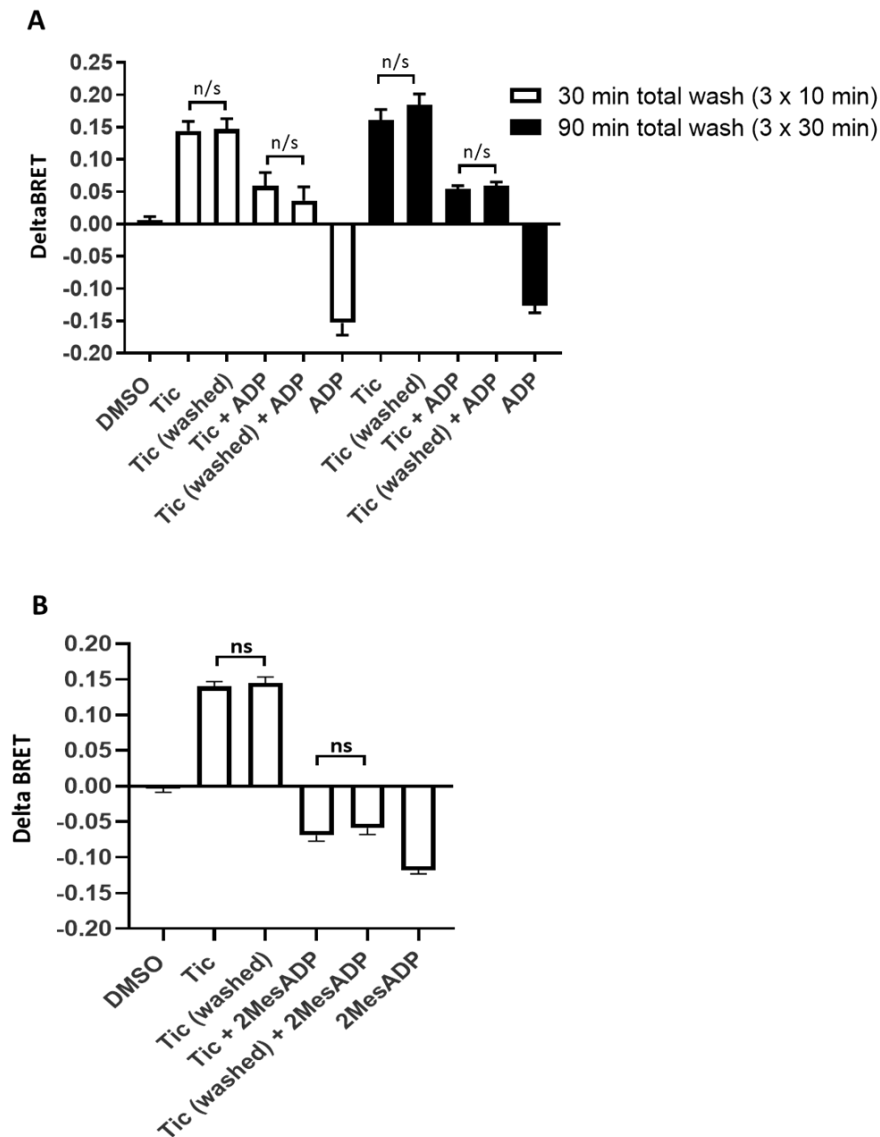


Figure 2: Ticagrelor-dependent activity at the P2Y₁₂R is resistant to wash-out.

HEK 293T cells were co-transfected with human FLAG-P2Y₁₂R and heterotrimeric G-proteins, RlucII-Gαi, untagged Gβ and GFP-Gγ. Forty-eight hours post transfection cells were treated with ticagrelor (10 μM) or vehicle control for 30 min at 37 °C. Following ticagrelor treatment cells were washed (Tic washed) for either 3 x 10 min (**A** and **B**) or 3 x30 min washes (**A**) and receptor activity compared with that following acute ticagrelor treatment (10 μM; 5 min; Tic). In (**A**) ADP-stimulated (10 μM; 5 min) activity was assessed in non-ticagrelor treated cells (ADP), in cells treated acutely with ticagrelor (Tic + ADP) or in cells following more prolonged ticagrelor-treatment and subsequent washing (Tic washed + ADP). In (**B**) 2MeSADP-stimulated (10 μM; 5 min) activity was assessed in non-ticagrelor treated cells (2MeSADP), in cells treated acutely with ticagrelor (Tic + 2MeSADP) or in cells following more prolonged ticagrelor-

treatment and subsequent washing (Tic washed + 2MeSADP). Data shown are the means \pm SEM of at least 5 independent experiments, each performed in triplicate. Statistical analysis was performed using one-way ANOVA and followed by Bonferroni's multiple comparison test.

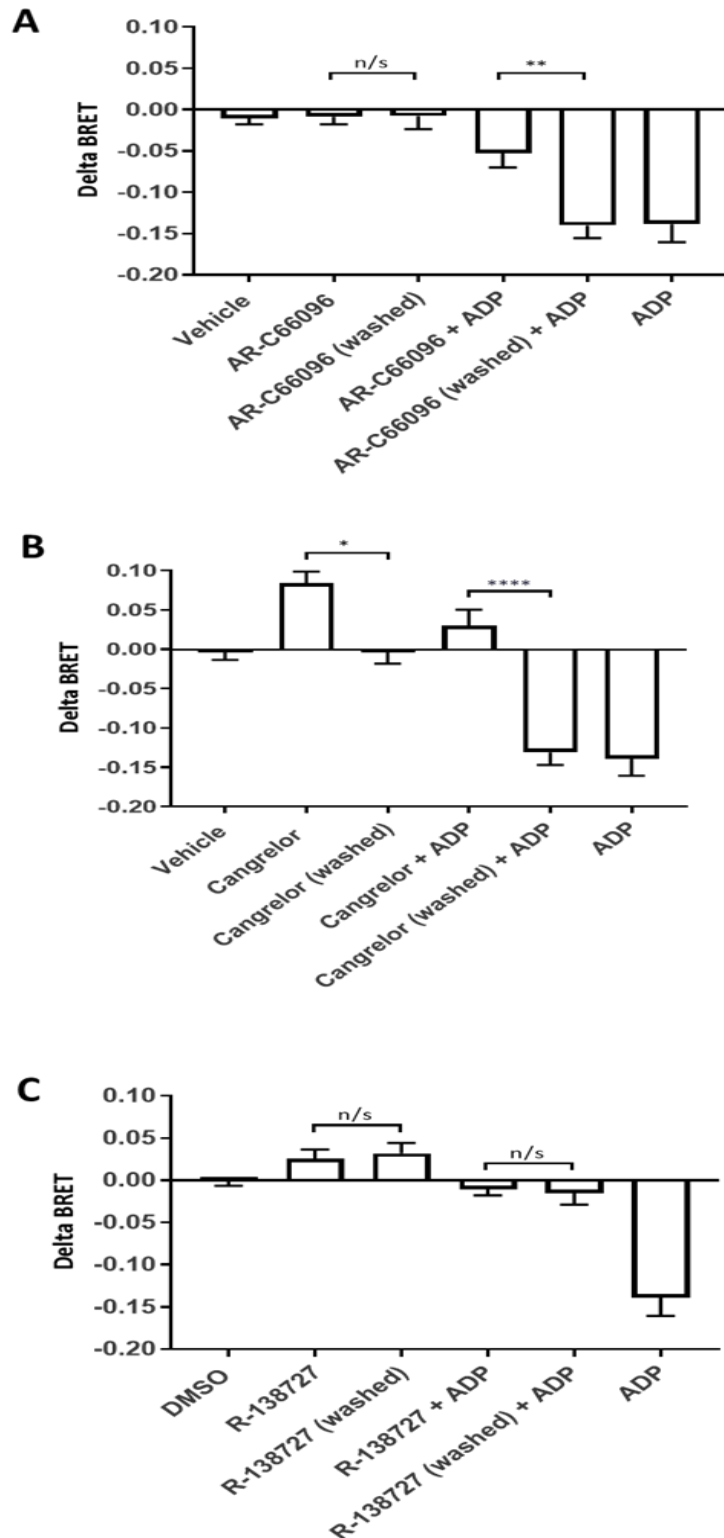


Figure 3: Prasugrel active metabolite but not cangrelor or AR-C66096 activity at the P2Y₁₂R is resistant to wash-out. HEK 293T cells were co-transfected with human FLAG-P2Y₁₂R and heterotrimeric G-proteins, RlucII-G α i, untagged G β and GFP-G γ . Forty-eight hours post transfection cells were treated with P2Y₁₂R antagonist (AR-C66096 (10 μ M; **A**), cangrelor (10 μ M; **B**) or the active metabolite of prasugrel (1

μM R138-727; **C**) for 30 min at 37 °C. Following antagonist treatment cells were washed for 3 x 10 min and receptor activity compared with that following either acute antagonist treatment alone (5 min) ADP-stimulated (10 μM ; 5 min) activity was assessed in non-ticagrelor treated cells (ADP), in cells treated acutely with ticagrelor (Tic + ADP) or in cells following more prolonged ticagrelor-treatment and subsequent washing (Tic washed + ADP) . In (B) 2MeSADP-stimulated (10 μM ; 5 min) activity was assessed in non-ticagrelor treated cells (2MeSADP), in cells treated acutely with ticagrelor (Tic + 2MeSADP) or in cells following more prolonged ticagrelor-treatment and subsequent washing (Tic washed + 2MeSADP). Data shown are the means \pm SEM of at least 5 independent experiments, each performed in triplicate. Statistical analysis was performed using one-way ANOVA and followed by Bonferroni's multiple comparison test ((**A**) ** $p > 0.01$ AR-C66096 + ADP vs AR-C66096-washed + ADP; (**C**) * $p > 0.05$ cangrelor vs cangrelor-washed and **** $p > 0.001$ cangrelor + ADP vs cangrelor-washed + ADP).

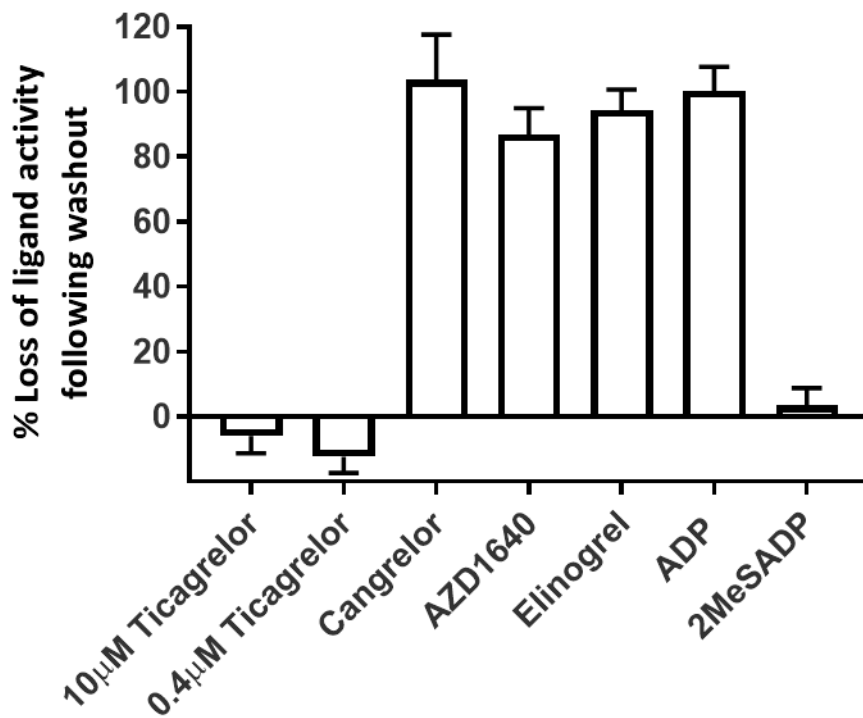


Figure 4: Ticagrelor and 2MeSADP activity at the P2Y₁₂R is resistant to wash-out. HEK 293T cells were co-transfected with human FLAG-P2Y₁₂R and heterotrimeric G-proteins, RlucII-G α i, untagged G β and GFP-G γ . Forty-eight hours post transfection receptor activity (either inverse agonist: ticagrelor, 0.4/10 μ M; AZD1640, 10 μ M; cangrelor, 10 μ M; elinogrel 10 μ M or agonist: ADP, 10 μ M 2MeSADP, 10 μ M) was compared in cells treated with P2Y₁₂R ligand (30 min) versus that in cells treated with ligand for 30 min followed by wash out for 3 x 10 min. Data are expressed as % loss of P2Y₁₂R-ligand induced activity following washing and represent means \pm SEM of at least 5 independent experiments, each performed in triplicate.

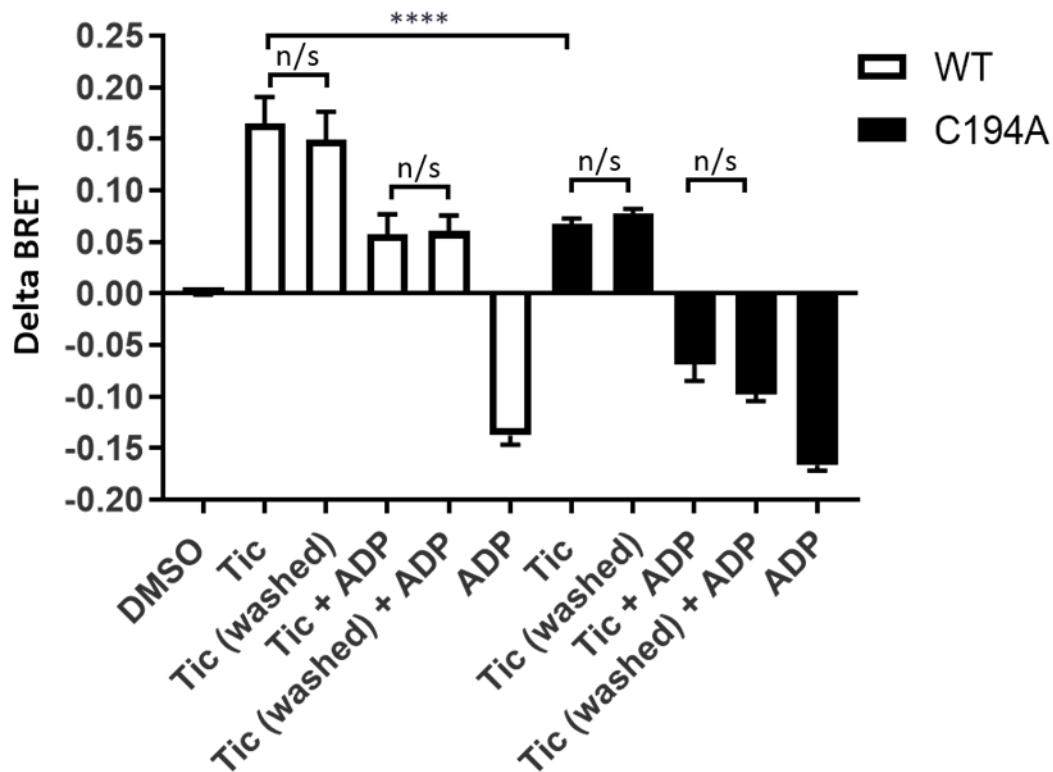


Figure 5: Resistance to wash-out of ticagrelor-dependent activity is maintained in a P2Y₁₂R mutant (C194A) displaying reduced ticagrelor activity. HEK 293T cells were transfected with either the human FLAG-P2Y₁₂R or FLAG-C194A-P2Y₁₂R and heterotrimeric G-proteins, RlucII-Gai, untagged G β and GFP-G γ . Forty-eight hours post transfection cells were treated with ticagrelor (10 μ M) or vehicle control for 30 min at 37 °C. Following ticagrelor treatment cells were washed (Tic washed) for either 3 x 10 min and receptor activity compared with that following acute ticagrelor treatment (10 μ M; 5 min; Tic). ADP-stimulated (10 μ M; 5 min) activity was assessed in non-ticagrelor treated cells (ADP), in cells treated acutely with ticagrelor (Tic + ADP) or in cells following more prolonged ticagrelor-treatment and subsequent washing (Tic washed + ADP). Data shown are the means \pm SEM of at least 5 independent experiments, each performed in triplicate. Statistical analysis was performed using one-way ANOVA and followed by Bonferroni's multiple comparison test. (****p>0.001 Ticagrelor response in FLAG-P2Y₁₂R versus FLAG-C194A-P2Y₁₂R).

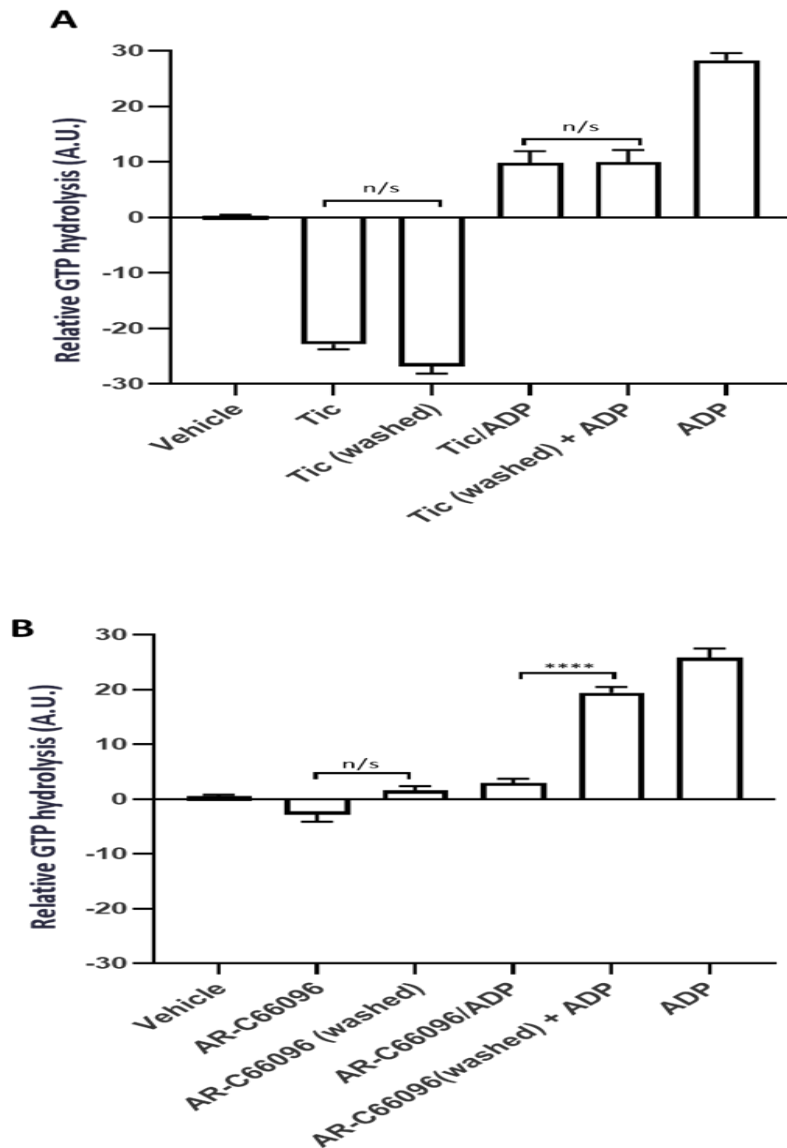


Figure 6: Ticagrelor-dependent effects on P2Y₁₂R activity are unaffected by washout in human platelets. Human washed platelets ($1 \times 10^9/\text{ml}$) were untreated or treated with (A) ticagrelor ($10 \mu\text{M}$) (B) AR-C66096 ($10 \mu\text{M}$) or vehicle (0.1% DMSO) for 30 min at 37°C . Platelets were then washed 3 times with intervals of 10 min before snap frozen in equal volume of fractionation buffer in liquid nitrogen. Membrane fractions were collected by ultracentrifugation, treated with ADP ($10 \mu\text{M}$) or vehicle (0.1% DMSO) before incubation with recombinant GTP ($2 \mu\text{M}$) and DTT (1 mM) for 1 hr at room temperature. GTPase Glo-reagents were added to measure GTP hydrolysis. Data shown are the means \pm SEM of at least 5 independent experiments. Statistical analysis was performed using one-way ANOVA and followed by Bonferroni's multiple comparison test ((B) **** $p < 0.0001$ AR-C66096 + ADP vs AR-C66096-washed + ADP).

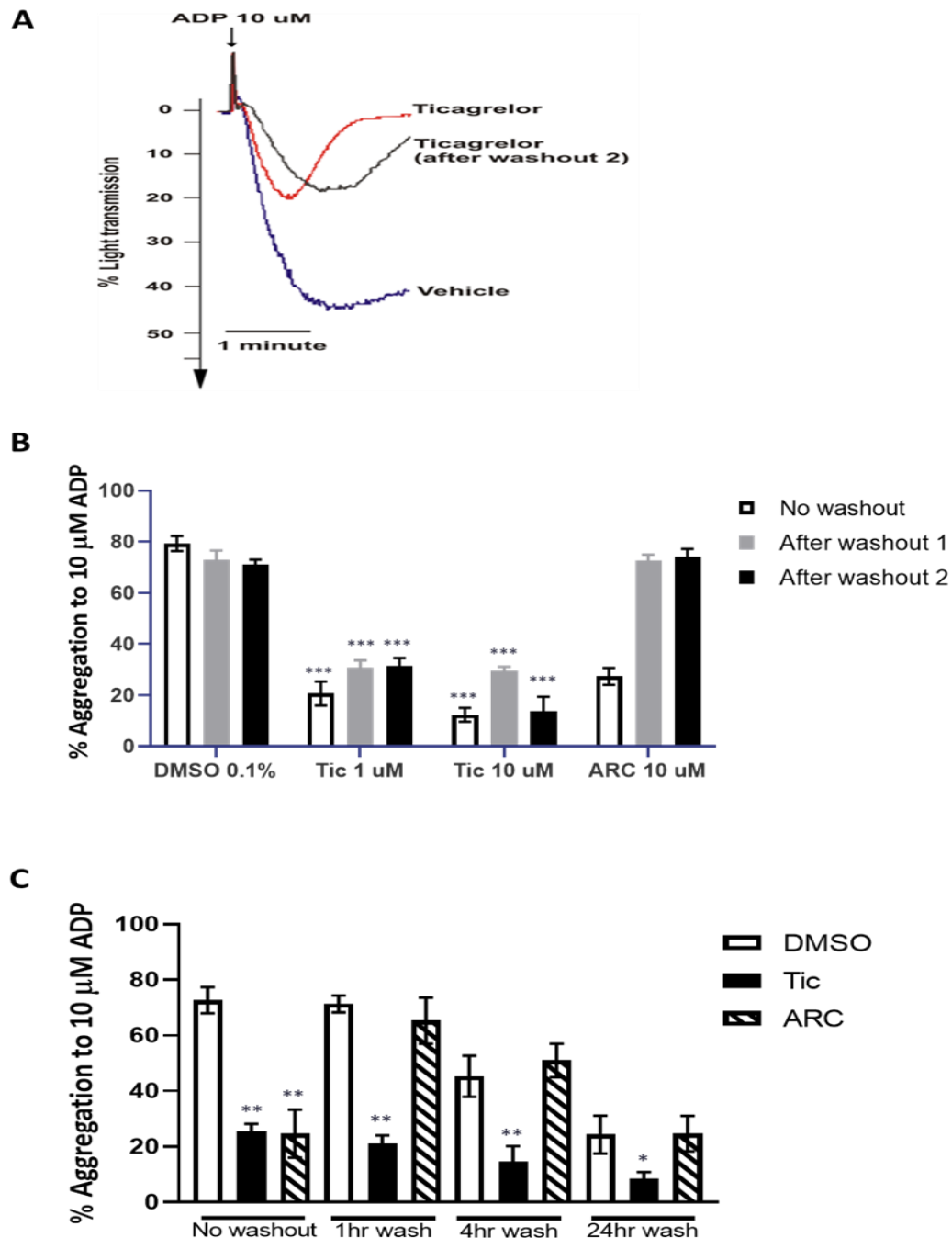


Figure 7: Inhibition of ADP-stimulated platelet aggregation by ticagrelor is not reversed by extensive washout. (A and B) PRP were treated with vehicle (0.1% DMSO), ticagrelor (1 or 10 μ M) or AR-C66096 (10 μ M) for 30 min at 37 $^{\circ}$ C. Treated samples were either unwashed (before washout), washed once (after washout 1) or twice (after washout 2) with intervals of 10 min between wash steps. As outlined in the methods following centrifugation steps platelets were resuspended in PPP. In all cases aggregation responses were recorded following the addition of ADP (10 μ M) or vehicle (0.1% DMSO). **(A)** shows a representative aggregatory traces **(B)** Data shown

are the means \pm SEM of at least 5 independent experiments. Statistical analysis was performed using one-way ANOVA and followed by Bonferroni's multiple comparison test (** $p > 0.05$ DMSO vs Tic (1 μ M) or DMSO vs Tic (1 μ M) comparing before washout, after washout 1 or after washout 2). **(C)** As above PRP was treated with either vehicle (0.1% DMSO), AR-C66096 (10 μ M) or ticagrelor (10 μ M) for 30 min at 37 °C. Samples were washed 2 times and resuspended in PPP for 1, 4 or 24 hrs and aggregation responses were recorded following the addition of ADP (10 μ M) or vehicle (0.1% DMSO). Data shown are the means \pm SEM of at least 5 independent experiments.

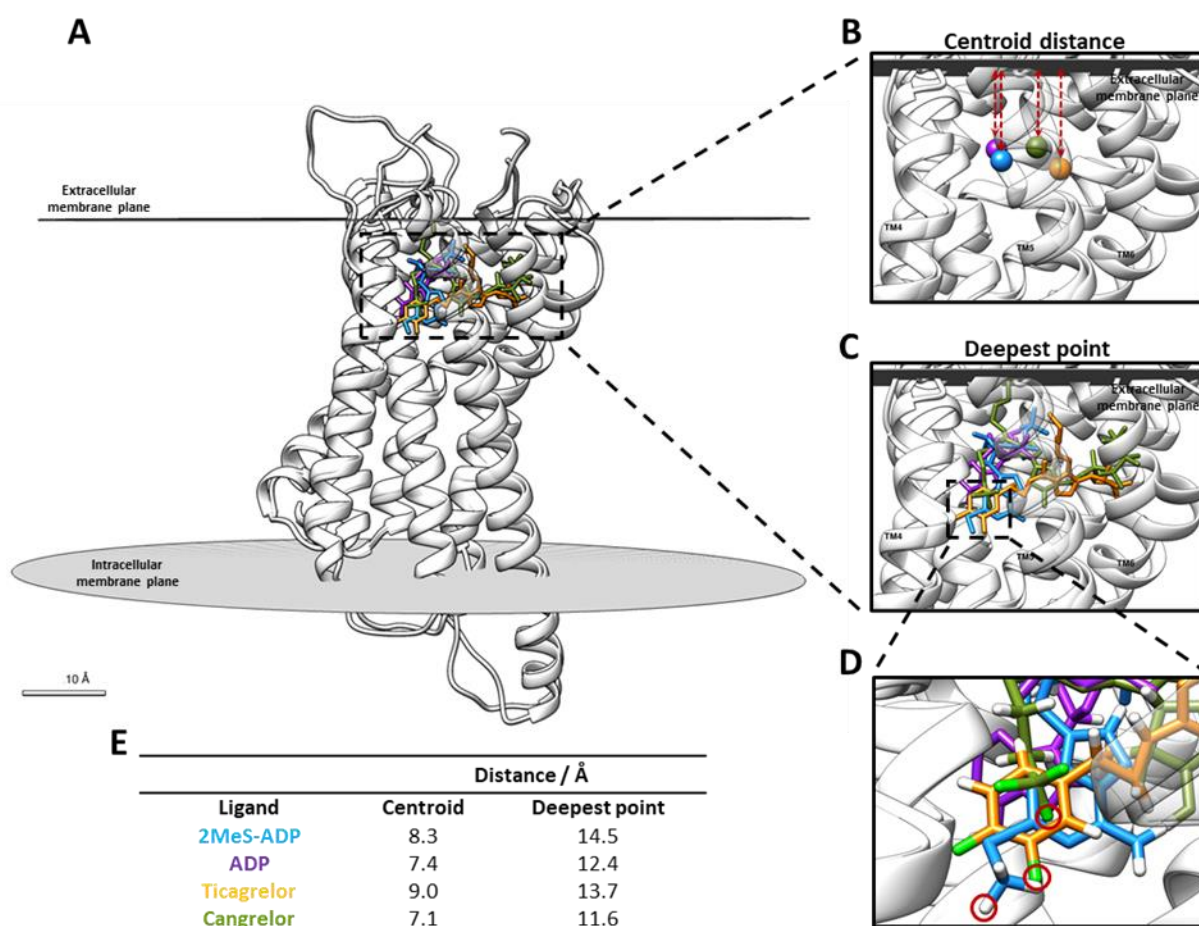


Figure 8: In silico docking reveals that ticagrelor and 2MeSADP penetrate more deeply into the orthosteric binding pocket of the P2Y₁₂R than cangrelor. **A)** Overlay of the agonist and antagonist models of the P2Y₁₂R with 2MeS-ADP (blue), ADP (purple), ticagrelor (orange), and cangrelor (green) docked within the receptor orthosteric site. Model also shows the outmost plane of both the extracellular and intracellular membrane. The 2MeSADP binding pose based on reported crystal structure whilst that for ADP, ticagrelor and cangrelor is based on simulated docking results. **B)** Geometric centre (centroid) of each ligand shown. Centroid distance to the extracellular membrane for each ligand calculated and summarised **E**. **C)** Deepest point for each ligand identified and distance to extracellular membrane plane calculated and displayed in **E**. **D)** Focus zoom in of residues of 2MeS-ADP, ticagrelor and cangrelor identified to show deepest penetration into orthosteric binding pocket. **E)** Summary of distances for the deepest penetration points and ligand centroid from the extracellular membrane plane for each ligand.