University of BRISTOL

School of Physiology, Pharmacology and Neuroscience

Defining the molecular pharmacology of ticagrelor

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Abstract

Platelets are the main orchestrators of processes such as wound healing and tissue regeneration post vascular injury. They not only mediate haemostasis, but also promote immune cell recruitment to the site of vascular injury. Platelets are also key promoters of angiogenesis, and they support the formation of novel 'scar' tissue. However, inappropriate platelet activation can be responsible for pathological thrombosis, most commonly in the coronary vasculature. Platelet non-reactivity is tightly regulated by several surface receptors. The $P2Y_{12}$ receptor is one such platelet receptor that is stimulated by ADP released from activated platelets to further potentiate platelet reactivity. The $P2Y_{12}$ receptor is now an established therapeutic target, with antagonism of this receptor used in the treatment of atherothrombosis. It has recently been established that this receptor exhibits a high level of agonist-independent activation (constitutive activity). Ticagrelor, a widely used therapeutic platelet inhibitor that blocks ADP-dependent $P2Y_{12}$ receptor stimulation, was recently shown to act as an inverse agonist at the $P2Y_{12}$ receptor, reducing its basal constitutive activity. Interestingly, although marketed as a reversible drug, ticagrelor was recently shown to be irreversible throughout the life span of platelets. The present study aims to characterize several molecular modulators of the $P2Y_{12}$ receptor constitutive activity and to probe why ticagrelor may be irreversible in platelets. Using a BRET-based approach, this current study discovered that Zn^{2+} acts as a non-selective positive regulator of the $P2Y_{12}$ receptor activity. Tetherin (BST-2), a molecular regulator of membrane microdomains, acts as a negative regulator at the receptor. Studies probing the mode of action of ticagrelor have found that neither cholesterol, nor $P2Y_{12}$ downstream signalling affect the ability of this drug to act in an irreversible manner. Mutant $P2Y_{12}$ receptor studies could also not decipher the irreversible nature of ticagrelor post-washout, however it discovered key residues in the receptor structure which may be important in regulation of the constitutive activity of the receptor and the inverse agonism of ticagrelor. Further structural studies are required to understand the molecular pharmacology, i.e., binding of ticagrelor at the receptor. The findings of this study will contribute to the understanding of platelet hyperreactivity and the mode of action of ticagrelor, which would ultimately improve clinical outcomes in thrombotic patients.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Chapter II: Materials & Methodology

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Chapter I: Introduction

Blood platelets are small anucleate cellular fragments originating from the cytoplasm of megakaryocytes in the bone marrow. Physiological levels of platelets range between $2x10^5$ and $5x10^5/\mu L$ of blood. The healthy human body can produce up to 10^{11} platelets in 24 hours. At rest, platelets are discoid-shaped and measure 2-3µm in diameter. Given the lack of nucleus, platelets have a relatively short lifespan of 7-10 days. Once formed, they circulate the blood in a quiescent state to promote and maintain vascular integrity.

1.1. Platelet formation

Platelet formation from bone marrow megakaryocytes is a prolonged and fascinating biological process comprising of multiple steps. Hematopoietic stem cells differentiate into megakaryocytes (MKs) in several sites in the human body, mainly in the bone marrow (BM) when exposed to thrombopoietin (TPO). Exposure to TPO promotes growth and development of platelet-specific granules in MKs, as well as endomitosis (i.e., DNA replication without cell division) to create the polyploid nucleus required to synthesize sufficient mRNA and protein for platelet formation whilst retaining the ability to function (*Zimmet and Ravid, 2000*). As they continue maturation, megakaryocytes develop a system of membranous invaginations in the cytoplasm which are continuous with the plasma membrane, referred to as the invaginated membrane system (IMS), previously known as the demarcation membrane system *(Schulze et al., 2006; Radley & Haller, 1982*). The newly formed IMS plays a key role in the formation of preplatelets, which are larger in size but similar in shape to platelets (2-10 µM), as well as proplatelets, which are barbell-like formations of platelets separated in the middle by a cytoplasmic bridge (*Thon et al., 2010; Italiano et al., 1999*). The IMS is also critical in the transfer of platelet-specific organelles from the mature MKs to the pre- and proplatelets. Megakaryocytes migrate to the BM sinusoidal capillaries and release pre- and proplatelets, which once exposed to high concentrations of S1P in the blood, initiate the process of platelet formation (*Zhang L et al., 2012*). At this stage, preplatelets can interconvert to proplatelets, and, through microtubule-mediated abscission forces, proplatelets are separated into two distinct platelets. The size of the final platelet product is determined by the microtubule coil diameter and thickness required for the separation process (*Thon et al., 2012*). The process is summarised in Figure1.1.

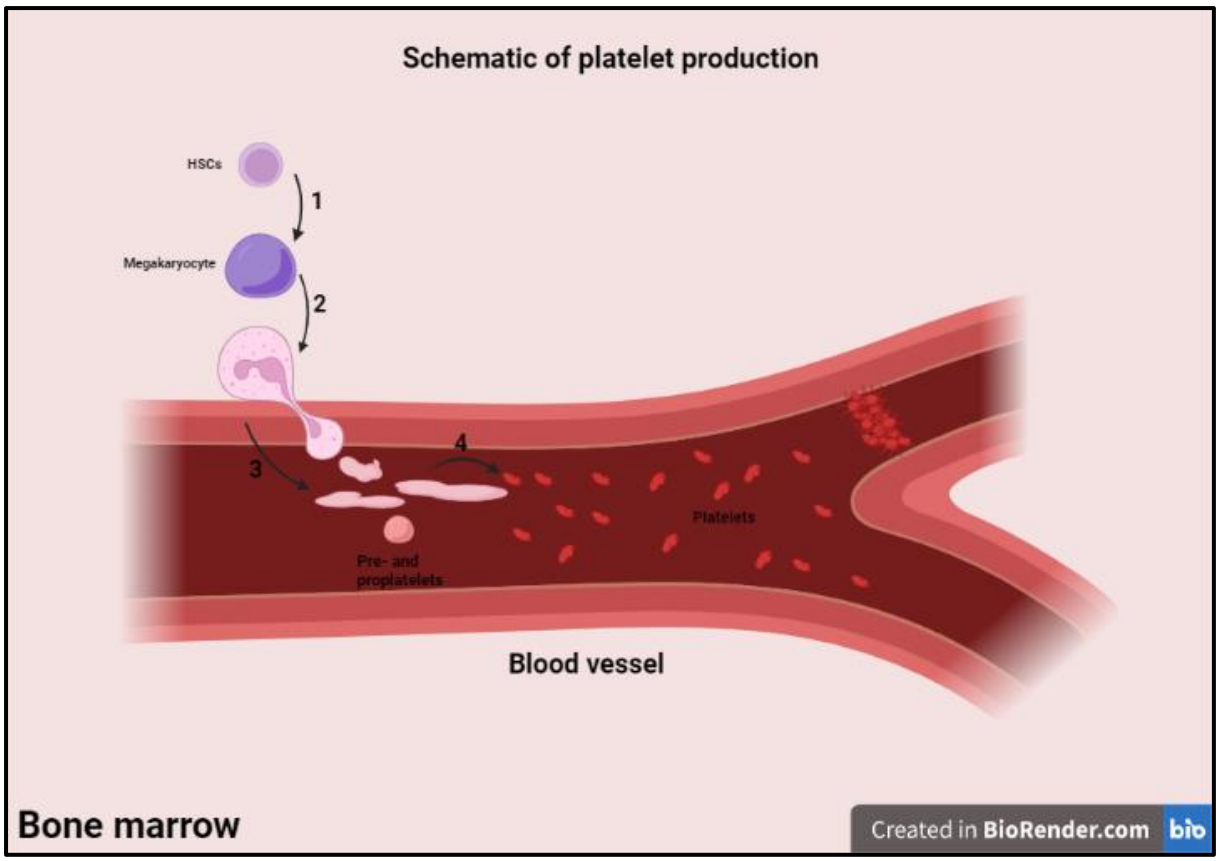


Figure1.1. Simplified schematic on platelet generation from megakaryocytes in the bone marrow. Platelet formation is a multistep process which relies on haematopoietic stem cell differentiation into megakaryocytes upon exposure to TPO (**1**). Under further TPO exposure, the newly formed megakaryocyte undergoes endomitosis, as well as cytoplasmic maturation, to prepare for the formation of new platelets (**2**). Next, the megakaryocyte migrates to the sinusoidal blood vessels in the bone marrow, where it releases newly formed proplateletes and preplatelets (**3**). Once exposed to the components of the bloodstream, preplatelets and proplatelets can interconvert and lead the final stage of platelet formation through separation into platelets (**4**).

1.2. Platelet haemostasis

In the absence of a vascular injury, platelets can be found roaming the blood circulation in a quiescent state – they do not adhere to surrounding surfaces and they do not undergo aggregation. This state is maintained by the vascular endothelium through the release of several anti-thrombogenic factors such as soluble nitric oxide or prostaglandin I_2.

The essential role of platelets is promoted by vascular injury (see Figure 1.2). Platelets form a haemostatic plug at the site of vascular injury to prevent blood loss, known as primary haemostasis. Injury to the vascular endothelium exposes the subendothelial extracellular matrix to the blood. Components of the matrix such as von Willebrand factor (vWF) and collagen bind to platelet receptors glycoprotein (GP)Ibα and GPIa/IIa, respectively, leading to the adhesion of platelets at the site of injury and initialization of platelet activation.

Under physiological conditions upon activation platelets secrete three types of granules: α-granules, which contain compounds such as vWF, fibrinogen or thrombin-activatable fibrinolysis inhibitor (TAFI), essential for platelet-platelet interaction, clot formation or clot breakdown; dense granules, which contain molecules acting on amplification of platelet activation, platelet aggregation and thrombus formation in autocrine and paracrine manners (ADP, ATP, Ca^{2+}) and lysosomal granules, which are suggested to aid the degradation of matrix components, receptor cleavage or clot disruption (*Golebiewska and Poole, 2015*). Following shape change, platelets release the contents of their granules into the neighbouring microenvironment to promote the blood clot formation through enhanced platelet activation, platelet aggregation and enhanced clotting cascade activation.

Subsequent platelet activation occurs via several intracellular signalling pathways downstream of platelet receptors such as the thromboxane (TP), protease-activate receptor (PAR) 1 and 4, GPVI, $P2Y_1$ and $P2Y_{12}$. The stimulation of receptors is mainly mediated by autocrine and paracrine release of active platelet granular contents. Once active, platelets undergo a very rapid shape change from discoid through formation of filopodia and lamellipodia to increase their surface area at the site of injury, which is facilitated by a circumferential microtubule ring, as well as an open canalicular system, which provides extra plasma membrane for platelet degranulation. The shape change is mediated by activation of the PLC pathway, alongside intracellular release of Ca^{2+} and activation of GPIIb/IIIa. Furthermore, actin cytoskeleton reorganization during shape change is dependent on PKC activation, amongst several other factors. Activation of discussed pathways has also proven highly relevant in overall platelet signalling, as GPIIb/IIIa activation by $P2Y_{12}$ stimulation occurs with Ca^{2+} levels elevation under PKC activation (*Janmey, 1994*). The simplified structure of a platelet can be observed in **Figure1.2**.

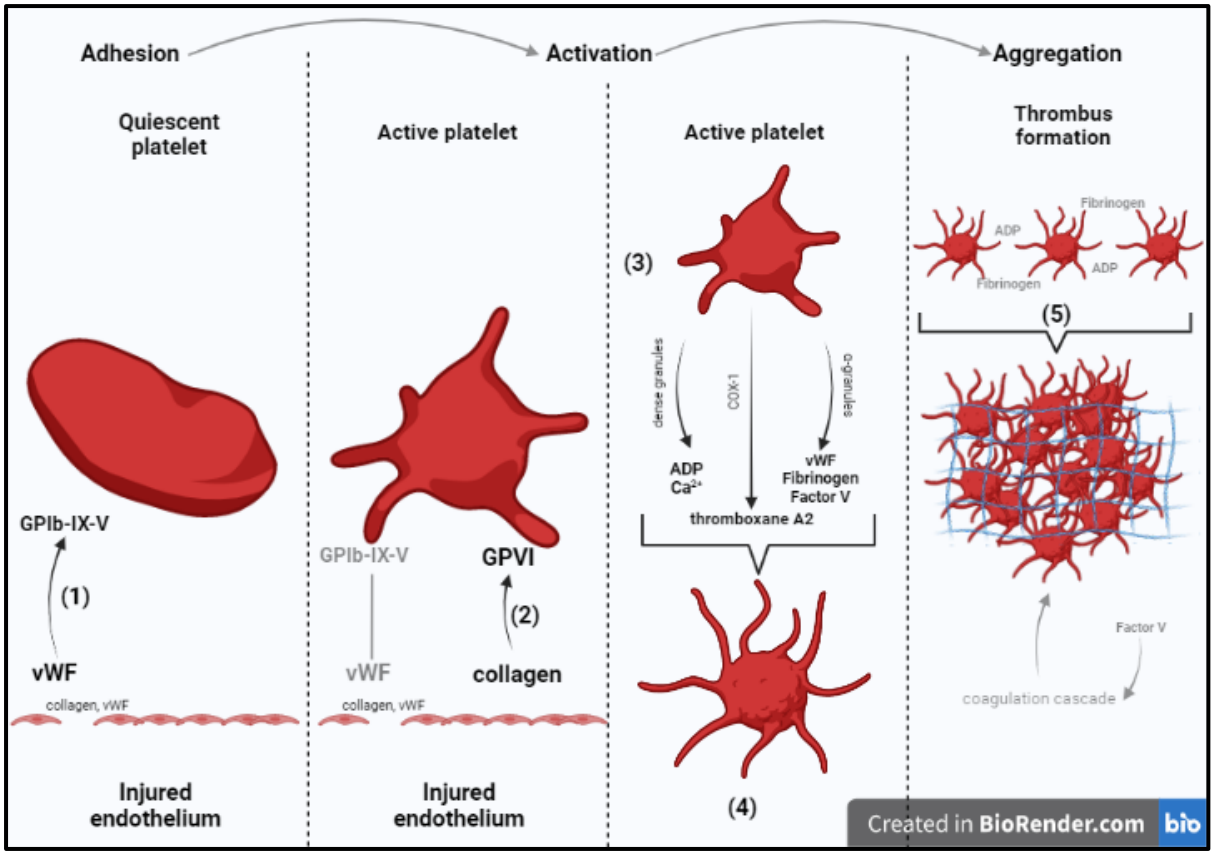


Figure1.2. Schematic of platelet structure showing surface receptors playing key roles in platelet adhesion and activation, as well as the platelet factors which are released upon platelet degranulation in the platelet microenvironment. In case of vascular injury, the endothelium releases von Willebrand factor (vWF) and exposes collagen. Upon interaction with collagen, vWF uncoils, exposing several binding sites for the platelet surface receptor GPIb-IX-V (**1**). Through GPIb-IX-V, platelets adhere to the site of injury where they collagen binds to the platelet surface receptor GPVI to initiate activation (**2**). During initial activation, platelets undergo a rapid shape change, secrete and release α- and dense granules containing platelet activation-promoting factors and coagulation-promoting factors (e.g., ADP, fibrinogen and vWF, and Factor V, respectively) and thromboxane A2 production is mediated by cyclooxygenase-1 (COX-1) (**3**). These factors act in an autocrine and paracrine manner to promote pronounced platelet activation (**4**) and subsequent aggregation (**5**).

Ultimately, platelets undergo aggregation mediated mainly by platelet-platelet interactions via autocrine release of adhesive molecules (vWF) and irreversible platelet GPIIb/IIIa activation.

Platelets also take part in the secondary haemostasis process, which relies on the stabilization of the previously formed platelet plug by insoluble fibrin, a product of the coagulation cascade. During secondary haemostasis, the platelet agonist thrombin is formed, because of prothrombin activation by Factor Xa. Thrombin, a serine protease, cleaves fibrinogen to generate insoluble fibrin; it also cleaves the PAR1 and PAR4 receptors on the cellular membrane of platelets, leading to further platelet activation. Furthermore, platelets contribute to the coagulation cascade by releasing Factor V from their α-granules.

1.3. The $P2Y_{12}$ receptor and its role in platelet activation

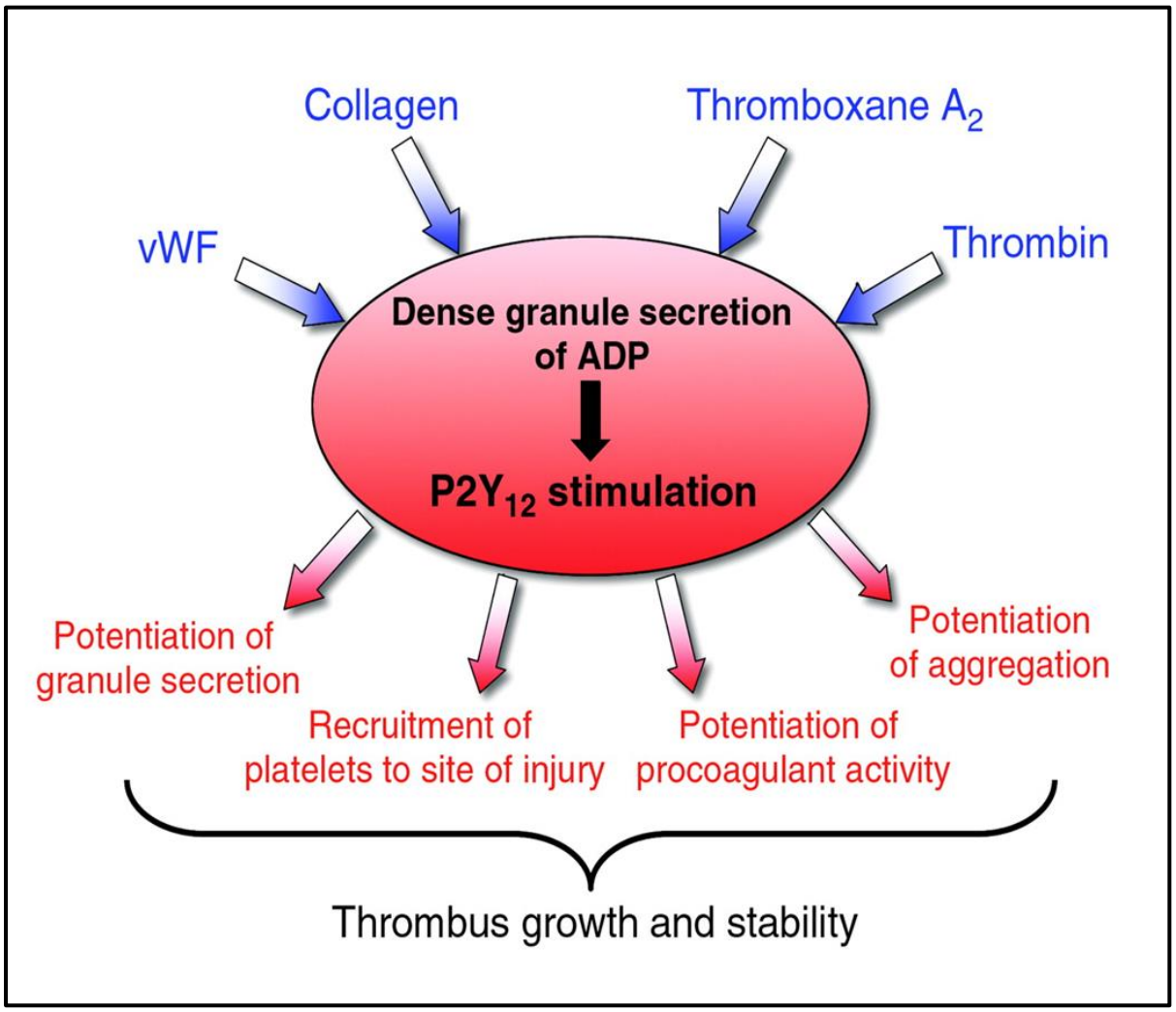


Figure1.3. The $P2Y_{12}$ receptor plays an essential role in platelet activation. Stimulation of platelet surface receptors by platelet agonists von Willebrand factor (vWF), collagen, thromboxane A2 or thrombin leads to platelet activation and subsequent platelet degranulation. The release of ADP from platelet dense granules leads to autocrine and paracrine stimulation of the $P2Y_{12}$ receptor which leads to further platelet activation and aggregation. Irreversible platelet aggregation is a pathological process and contributes significantly to atherothrombosis.

The $P2Y_{12}$ receptor has been extensively studied with its activation by ADP released from platelet dense granules required for efficient platelet aggregation (see Figure 1.3). The $P2Y_{12}$ receptor was firstly cloned in 2001 (*Hollopeter et al., 2001*). It is postulated that the $P2Y_{12}$ receptor is expressed abundantly on the surface of the resting platelet, with an estimate of around 400 receptors per platelet, with more receptor expression being induced upon initial platelet activation (*Haberstock-Debic et al., 2011*). Initial studies linked defective $P2Y_{12}$ receptor expression to increased risks of bleeding (*Cattaneo et al., 2003*) and more recent studies discovered certain mutations in residues that are potentially responsible for impaired $P2Y_{12}$ receptor function (*Patel et al., 2014; Nisar et al., 2011; Daly et al., 2009*).

The $P2Y_{12}$ receptor is a class A $G_{i/o}$ protein coupled receptor (see Figure 1.4) comprised of 342 amino acids. Through $G_{i/o}$ mobilization, the $P2Y_{12}$ receptor activation leads to inhibited Adenylyl Cyclase (AC) activity, which leads to decreased cyclic AMP production and decreased Protein Kinase A (PKA) activity. Inhibition of PKA leads to a decrease in protein phosphorylation downstream of PKA, e.g. Vasodilator-stimulated phosphoprotein (VASP), to result in increased platelet activation. Simultaneously, $P2Y_{12}$ receptor $G_{\beta\gamma}$ activity leads to an increase in Phosphoinositide 3-kinase (PI3K), which in turn leads to increased production of Akt (Protein Kinase B) to support platelet activation.

Crystal structures of the agonist and antagonist-bound $P2Y_{12}$ receptor were solved in 2014 (*Zhang J et al., 2014*; *Zhang K et al., 2014*). These and subsequent computational binding studies have looked at the $P2Y_{12}$ receptor in complex with the full agonist 2MeSADP, the partial agonist 2MeSATP and the antagonist AZD1283. These have suggested that the $P2Y_{12}$ receptor does not possess a traditional localization of its transmembrane domains perpendicularly to the plasma membrane, but rather they are angled. Other significant structural particularities consist of the apparent requirement for two cholesterol molecules bound to the structure of the $P2Y_{12}$ receptor for optimal function and the presence of two disulphide bonds, between Cys97 and Cys175, and between Cys17 and Cys270. Interestingly, the binding of the agonist produces a highly dynamic structural change in the ligand binding pocket of the $P2Y_{12}$ receptor. Agonist binding causes inward movement of the transmembrane domains and ligand binding pocket to move inwardly around the agonist molecule. Meanwhile, antagonist binding into a shared binding pocket does not produce inward movement of the transmembrane domains (*Zhang J et al., 2014; Zhang K et al., 2014*).

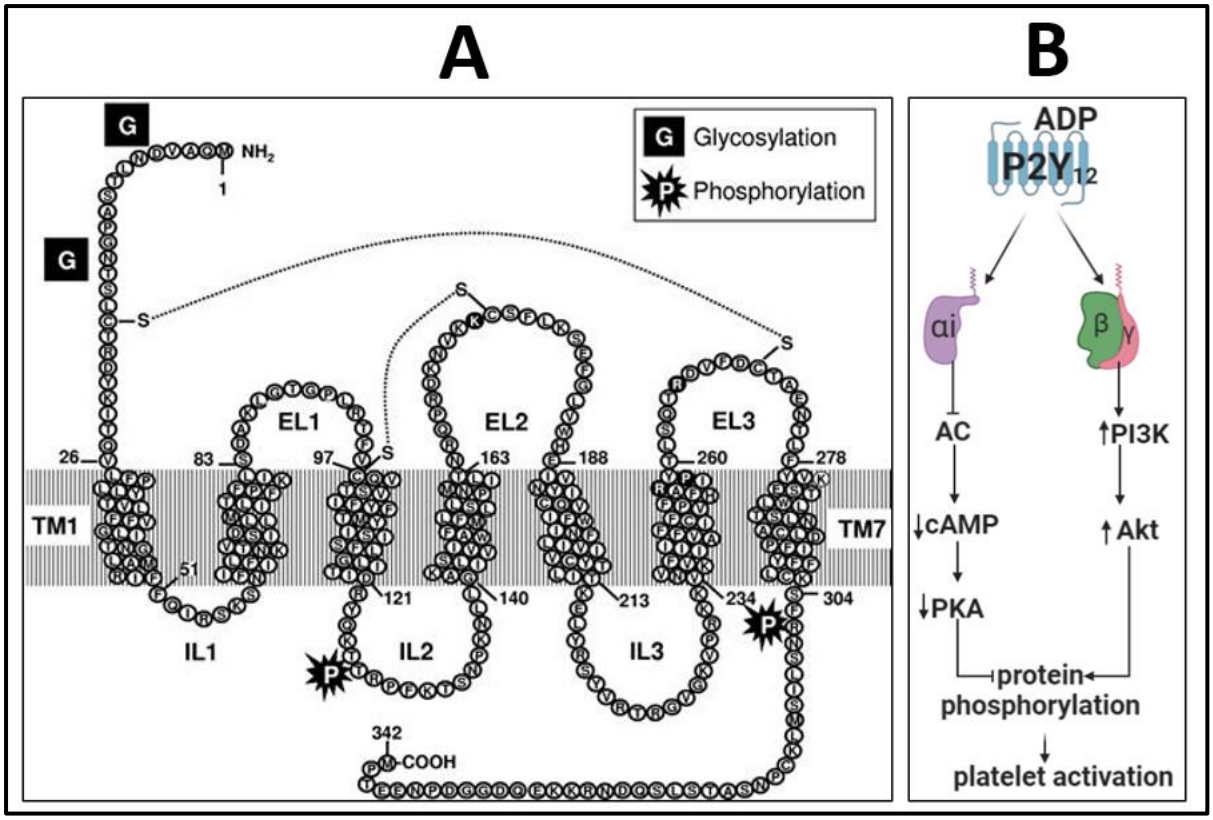


Figure1.4. The secondary protein structure of the $P2Y_{12}$ receptor with depicted disulphide bond and potential glycosylation and phosphorylation sites (*Cattaneo et al, 2011*) and the $P2Y_{12}$ receptor/G protein complex signalling cascade. Panel A: The $P2Y_{12}$ receptor is a class A GPCR composed of an extracellular N-terminus, seven transmembrane domains and an intracellular C-terminus and comprising 342 amino acid residues. There are two potential glycosylation sites in the N-terminus and two phosphorylation sites, one on intracellular loop (IL) 2 and one at the bottom of transmembrane domain (TM) 7. Studies involving molecular dynamics have extensively described the highly dynamic molecular structure of the $P2Y_{12}$ receptor and its implications in platelet pathophysiology. Panel B: Upon stimulation by ADP, the $P2Y_{12}$ receptor undergoes a conformation change to induce activation and facilitate G protein recruitment, leading to a downstream signalling cascade mediating the intracellular processes required for platelet activation. The $G_{\alpha i}$ component inhibits AC activity to reduce cAMP production and subsequently inhibit PKA activity. Reduced PKA activity leads to reduced protein phosphorylation to promote platelet activation. The $G_{\beta\gamma}$ complex component increases PI3K activity to increase PKB/Akt activity and induce protein phosphorylation required for platelet activation.

1.4. The pathophysiological role of platelets in atherothrombosis and acute coronary syndromes

As outlined above, platelet activation plays an essential role in haemostasis, reducing blood loss following vessel injury. Unfortunately, irreversible platelet haemostasis within a blood vessel can lead to pathological occlusion of the vessel known as thrombosis. Most commonly, platelet led thrombosis or atherothrombosis occurs in an artery, for example in the coronary vasculature following the rupture of an atheromatous plaque. Atherothrombosis is the main cause of acute coronary syndromes (ACS), a range of conditions associated with reduced blood flow to the heart.

Atherosclerosis is a pathological process which begins with the cross-play between chronic inflammation and dyslipidaemia leading to the formation of the atherosclerotic plaque. This process is stimulated by proinflammatory cytokines related to endothelial dysfunction under cardiovascular risk factors (i.e., hyperglycaemia and insulin resistance, hyperlipidaemia, hypertension, oxidative stress, reduced blood flow) (*Hadi et al., 2005*). During this process the endothelium presents VCAM-1, a leukocyte adhesion-promoting molecule. Through VCAM-1, blood monocytes and lymphocytes adhere to the endothelium and eventually protrude into the endothelial layer (the intima). Blood monocytes differentiate into macrophages which become foam cells following uptake of lipids.

In atherosclerotic plaque rupture, the fibrous cap that overlies the lipid core of the plaque becomes thinned by a reduction in the collagen due to extensive proinflammatory factors. This allows coagulation factors in the blood to encounter prothrombotic factors in the plaque. Exposed extracellular matrix and tissue factor from the plaque promotes platelet adhesion and activation, followed by aggregation, and formation of an acute, stable thrombus, as described in the platelet haemostasis section above. The newly formed thrombus can significantly reduce the blood flow through the vessel, leading to acute coronary syndromes. Plaque ruptures can heal by formation of fibrous tissue or can incorporate the platelet thrombus, leading to an increase in the size of the plaque and reduction in the size of the blood vessel lumen. Therefore, future plaque ruptures can lead to the formation of a thrombus that occludes blood flow and leads to cardiac ischaemia and potential acute myocardial infarction (*Kubo et al., 2010*).

Inhibition of platelet activity may prove useful in the secondary prevention of atherosclerosis, for example the formation of the stable thrombus in case of plaque rupture. Historically, platelet hyperactivation has been linked to activation of the TP receptor by thromboxane A2 and of the $P2Y_{12}$ receptor by ADP (see Figure 1.3). To counteract the activation of TP, aspirin was used to irreversibly antagonise the cyclooxygenase (COX) 1-mediated thromboxane A2 generation, whilst against $P2Y_{12}$ receptor activation, $P2Y_{12}$ antagonists clopidogrel, prasugrel, ticagrelor and cangrelor were used (*Knuuti et al., 2019*).

1.5. Anti-platelet therapy and $P2Y_{12}$ receptor antagonism

Anti-platelet therapy is the primary treatment option for patients suffering from atherothrombosis and acute coronary syndromes. There are currently four primary classes of anti-platelet drugs used clinically: the PAR1/4 receptor antagonist vorapaxar, the cyclooxygenase 1/2 inhibitor aspirin, the $P2Y_{12}$ receptor antagonists ticlopidine, clopidogrel, prasugrel, ticagrelor and cangrelor, and the GPIIb/IIIa inhibitor abciximab (see Figure 1.5). The mechanism of action behind the different treatments relies on the direct (GPIIb/IIIa inhibitors) or indirect (aspirin, $P2Y_{12}$ antagonists, PAR1/4 antagonists) inhibition of "inside-out" signalling that is required for activation of GPIIb/IIIa, leading to irreversible platelet activation and subsequent platelet aggregation. These therapies can be used alone or in combination, depending on disease severity. Vorapaxar prevents the activation of the PAR receptors by thrombin to inhibit platelet activation, whilst GPIIb/IIIa inhibitors prevent fibrinogen from binding to GPIIb/IIIa for activation. As mentioned above, aspirin acts to block COX 1-mediated thromboxane A2 generation to prevent TP receptor activation and inhibit platelet activation, whilst $P2Y_{12}$ receptor antagonists prevent the autocrine activation of the $P2Y_{12}$ receptor by ADP to inhibit platelet activation (*McFadyen et al., 2018*).

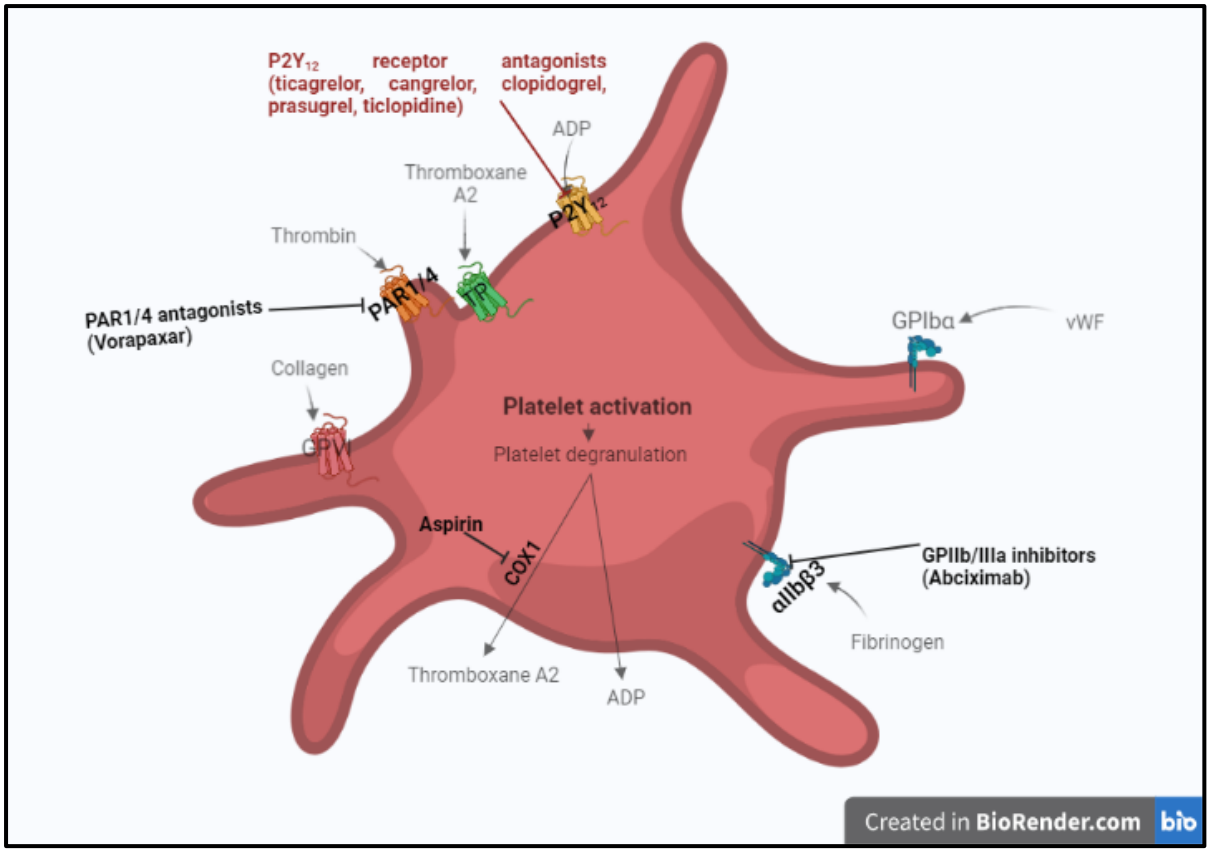


Figure1.5. Currently accepted anti-platelet targets in therapy. The primary four drug classes used in anti-platelet therapy are the $P2Y_{12}$ receptor antagonists (e.g., ticagrelor, cangrelor, clopidogrel, prasugrel, ticlopidine), the COX1 inhibitor aspirin, PAR1/4 receptor antagonists (e.g., vorapaxar) and GPIIb/IIIa inhibitors (e.g., abciximab). These treatments can be used alone or in combination to prevent platelet aggregation through activation of GPIIb/IIIa. GPIIb/IIIa activation through 'inside-out' signalling is blocked indirectly by inhibition of platelet activation (by $P2Y_{12}$ receptor antagonists, PAR1/4 antagonists, and aspirin), or directly by GPIIb/IIIa inhibitors.

As outlined above, the $P2Y_{12}$ receptor plays a pivotal role in platelet activation. This receptor is now an established target for antiplatelet therapies, with reductions in $P2Y_{12}$ receptor activation linked to reductions in platelet activation and, therefore, atherothrombus formation. There are currently a series of $P2Y_{12}$ receptor antagonists, the irreversible thienopyridines ticlopidine, clopidogrel and prasugrel, and the new generation of ATP analogues, ticagrelor and cangrelor. Initial studies indicated that aspirin showed better efficacy when paired with the only clinically available $P2Y_{12}$ inhibitor at the time, ticlopidine, than when paired with the anticoagulants heparin or warfarin for treatment of acute coronary syndrome (ACS) patients (*Cosmi et al., 2001*; *Leon et al., 1998*). This dual therapy regimen has since become the gold standard for ACS treatment. Ticlopidine was replaced by the second generation thienopyridine clopidogrel due to the very low efficacy/toxicity ratio of ticlopidine. Clopidogrel is still widely prescribed in ACS patients. However, several reports showed that clopidogrel fails to inhibit platelet aggregation to adequate levels in patients. This is due to impaired liver metabolism to its active metabolite in patients displaying certain variants of cytochrome P450 (*Gurbel et al., 2003; Järemo et al., 2002; Mega et al., 2009; Hulot et al.,*

2006). Therefore, clopidogrel proved to be a reliable anti-platelet therapy tool in only some patients. The introduction of prasugrel solved the problem of clopidogrel-related impaired metabolism (*Brandt et al., 2007*). Due to ongoing clinical concerns regarding the irreversible nature of thienopyridine binding, the ATP analogues ticagrelor (and subsequently of cangrelor) were developed. Such agents do not require hepatic metabolism into their active metabolites and showed faster, more potent platelet inhibition than the traditional thienopyridines (*Wallentin et al., 2009; Husted et al, 2006*).

1.6. Constitutive activity and the platelet $P2Y_{12}$ receptor

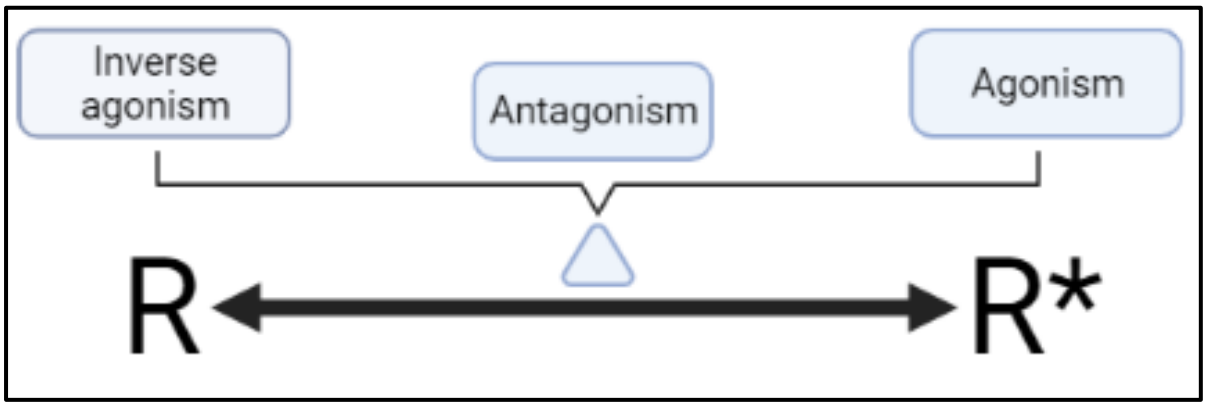


Figure1.6. Simplified schematic on the principle of constitutive activity in receptors. G protein-coupled receptors are generally found in an intermediate activation state, i.e., between an inactive state (**R**) and a fully active state (**R***). Drug-receptor interactions bring the receptor equilibrium towards a more inactive state (inverse agonists), a more active state (partial and full agonists) or do not change the equilibrium of the receptor but prevent other compounds from doing so (antagonists). Finding the balance between the inactive and the fully active state of the receptor through drug modulation represents the basis for therapeutic drug development in pharmacology.

Traditionally, receptors shift towards an active conformation upon agonist binding. To achieve this an agonist needs to possess both affinity for the receptor, which affects drug binding to the receptor and intrinsic efficacy, which determines the level of receptor activation when the ligand is bound to the receptor. However, a study by Cerione *et al.* showed that co-expression of purified β_2-adrenoreceptors from guinea pigs and purified $G_{\alpha s}$ from human red blood cells in phospholipid vesicles led to notable activity of the receptor in absence of its ligand. To enforce this, a similar study on delta opioid receptor in NG108-15 neuroblastoma cells in 1989 by *Costa and Herz* showed similar findings. These discoveries set the foundation stone to the hypothesis that all receptors elicit a certain degree of constitutive activity.

Costa and Herz, *1989* first introduced the idea of inverse agonism. They discovered that the delta opioid receptor antagonist ICI 174864 and its analogues reduced the intrinsic activity of the receptor by approximately 10%. Following this characterization of the potentially novel nature of already existing opioid receptor antagonists, several other antagonists were discovered to elicit a similar reduction in intrinsic activation of their specific receptor. Therefore, a new term, 'inverse agonism' was defined to name ligands which possess affinity for a particular receptor and negative intrinsic efficacy (the ability to reduce the activity of a receptor).

The introduction of the concept of receptor constitutive activity was a significant shift in our understanding of receptor pharmacology. Previously receptors were thought to only activate when stimulated by a ligand, until it was revealed that receptors can elicit agonist-

independent activation. Whilst agonists stabilize the receptor in an active conformation (see Figure 1.6; interchangeably between an inactive form **R** and an active form **R***) inverse agonists can shift the receptor conformation to an inactive form. Neutral antagonists, meanwhile, do not change the conformation of the receptor but prevent other ligands from binding to the receptor and doing so.

It has since been revealed that some G protein coupled receptors have a greater conformational "flexibility" that allows them to move into the **R*** form without agonist binding. Structural studies investigating mutations in key structural residues across 45 class A G protein-coupled receptors discovered that the greater conformational "flexibility" may be explained by absence of key residues in the conserved structural motifs that lock the receptor into an inactive conformation, for example the PIF, hydrophobic lock or DRY motifs (*Zhou et al., 2019*). In relation to the PIF motif, the study showed that mutation of the Isoleucine in the PIF motif to asparagine leads to formation of novel interactions between residues that stimulate outward movement of transmembrane domain (TM) 6 stabilizing the active state of the receptor. Furthermore, a mutation in the Phenylalanine residue in the PIF motif to alanine leads to a loosening of the contacts between TM3 and TM6 to make the outward movement of TM6 easier and stabilize the active state of the receptor. Mutations in residues which make up the hydrophobic lock of a receptor (e.g., L95A or I238Y) would lead to reduced stability in the lock and, conversely, weak interactions between TM3 and TM6 to facilitate the outward movement of TM6. Ultimately, the study suggests that the mutation of the aspartic acid in the DRY motif to asparagine would break the interaction with the arginine, also part of the DRY motif, to release the arginine and allow free outward movement of TM6 to ease the recruitment of G proteins. In most class A G protein-coupled receptors, the presence of the DRY motif maintains the receptor conformation in an inactive state by formation of a stabilising salt bridge between the three residues. Whereas changes in the arginine leads to loss of receptor function, as shown by the naturally occurring mutation of the arginine to a cysteine in the $P2Y_{12}$ receptor described by *Patel et al., 2014*, mutations in the aspartic acid of the DRY motif can lead to an increase in agonist-independent receptor activation, as seen for the μ-opioid receptor (*Li et al., 2001*), or a shift towards an active-like conformation, but with no changes in receptor constitutive activity, as seen for the thromboxane A2 receptor (*Capra et al., 2004*).

Therefore, the high degree of constitutive activity of the $P2Y_{12}$ receptor, as described in Aungraheeta *et al.* in 2016, may be explained by particularities in its DRY motif. In 2014, *Zhang K et al.* suggested that the structural particularities of the $P2Y_{12}$ receptor, relative to other G protein-coupled receptors, are responsible for the constitutive activity of the receptor. These particularities refer to an outward positioning of the intracellular tip of TM6 and an overall slight angle of TM6 towards the intracellular side relative to the other domains. This shift determines a different localization of the arginine residue of the DRY motif (in position 122) relative to the glutamic acid/threonine on TM6 with which the ionic lock forms in other G protein-coupled receptors to stabilise the receptor in an inactive conformation in absence of an agonist ligand. In the $P2Y_{12}$ receptor, the arginine can be found in the proximity of the hydrophobic valine in position 238, which prevents the formation of the stabilizing lock. The

absence of the lock, as described above, would permit the outward movement of TM6 to facilitate the recruitment of G proteins (*Zhang et al., 2014*). The receptor is therefore more liable to be found in an active conformation (closer to **R*** on the axis). ADP binding to the receptor promotes further receptor conformational change to the **R*** form, which interacts with downstream signalling cascades through G protein/receptor interplay. Studies evaluating platelet activation and thrombosis in mice expressing constitutively active $P2Y_{12}$ receptors linked an increase in platelet reactivity to enhanced $P2Y_{12}$ receptor signalling (*Zhang Y et al., 2012*). Furthermore, intriguing studies have also shown constitutively active $P2Y_{12}$ receptors in diabetes mellitus patients, who are known to be at increased risk of atherothrombosis due to hyperglycaemia (*Hu et al., 2017; Aronson and Rayfield, 2002*).

Importantly as discussed below, the $P2Y_{12}$ receptor blocker ticagrelor reduces agonist-independent activity shifting the conformation of the receptor towards **R**.

1.7. The mechanism of action of ticagrelor

Ticagrelor, brand name Brilinta, was introduced to the market in 2010 as a platelet inhibitor acting on the $P2Y_{12}$ receptor. Before 2016, it was thought that ticagrelor acts on the $P2Y_{12}$ receptor as a non-competitive antagonist, blocking both ADP, the physiological agonist, and 2-methylthio-ADP(2MeSADP), a synthetic agonist. The chemical structures for ticagrelor, ADP and 2MeSADP are presented in Figure 1.7.

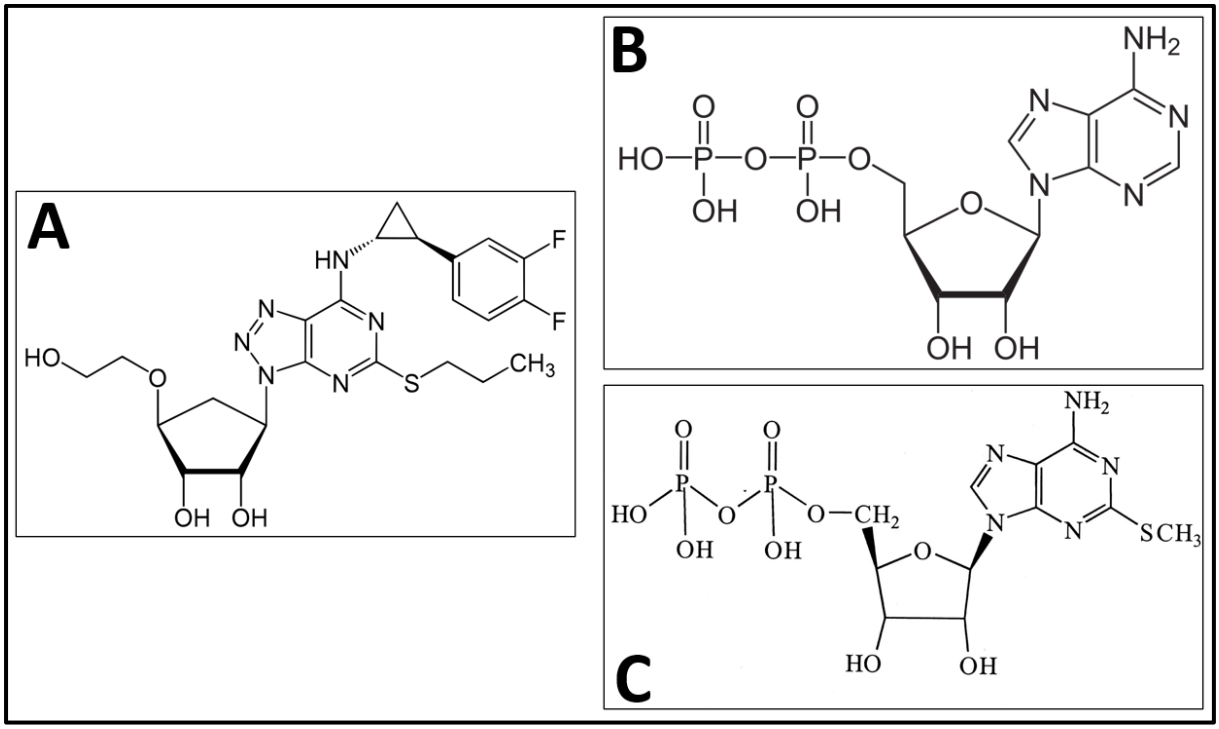


Figure1.7. The chemical structures of ticagrelor, ADP and 2MeSADP. Ticagrelor (A) is a cyclopentyl-triazolo-pyrimidine (CPTP), designed to act as a stable ATP analogue, the endogenous $P2Y_{12}$ antagonist. ADP (B) is the endogenous ligand for the $P2Y_{12}$ receptor, which is released from platelet dense granules upon platelet degranulation. 2MeSADP (C) is a synthetic ADP analogue which activates $P2Y_{12}$ with increased potency relative to ADP.

However, *Aungraheeta et al., 2016* suggested that ticagrelor exhibits its anti-platelet characteristics through a direct inverse agonistic mechanism of action at the $P2Y_{12}$ receptor and indirect platelet inhibition through inhibition of the Equilibrative nucleoside transporter 1 (ENT1) (see Figure1.8). Acting as an inverse agonist, ticagrelor reduces receptor stimulation with decreased downstream $G_{\alpha i}$ protein signalling. The loss of G proteins signalling results in increased AC activity, increased production of cAMP and PKA activity leading to a marked increase in downstream protein phosphorylation, increasing platelet inhibition. Furthermore, ticagrelor leads to an accumulation of adenosine by blockade of ENT1, the transporter responsible for uptake of residual adenosine which results from degradation of ADP to AMP by CD39 and degradation of AMP to adenosine by CD73 on surface of platelets (and potentially of red blood cells) (*Aungraheeta et al., 2016*). The resulting accumulation of adenosine leads to stimulation of adenosine A_{2A} receptor on the surface of platelets. Activation of this $G_{\alpha s}$ protein-coupled receptor leads to an increase in AC activity and

subsequent factors in the downstream signalling cascade to further halt platelet activation and aggregation.

A notable disadvantage of ticagrelor lies in the increased risk of bleeding it produces in patients (*Steg et al., 2019*). Interestingly, clinical studies have suggested the drug effects are much more long-lasting than would be expected of a reversible drug, potentially because of an irreversible effect on platelets (*Gerrits et al., 2017*). This study discovered that ticagrelor, despite its binding being hypothesized as "reversible", produced time-dependent irreversible effects in platelets. Such effects were shown by decreased levels of activated GPIIb/IIIa and P-selectin, with no difference in the platelet reactivity index, suggestive that the irreversibility is independent of VASP signalling downstream of $P2Y_{12}$ receptor activation. Therefore, it is possible that the irreversible effects of ticagrelor are through action at an off-target site. The apparent irreversibility of ticagrelor could, therefore, become clinically problematic in patients in need of emergency surgery or who are suffering from life-threating bleeding.

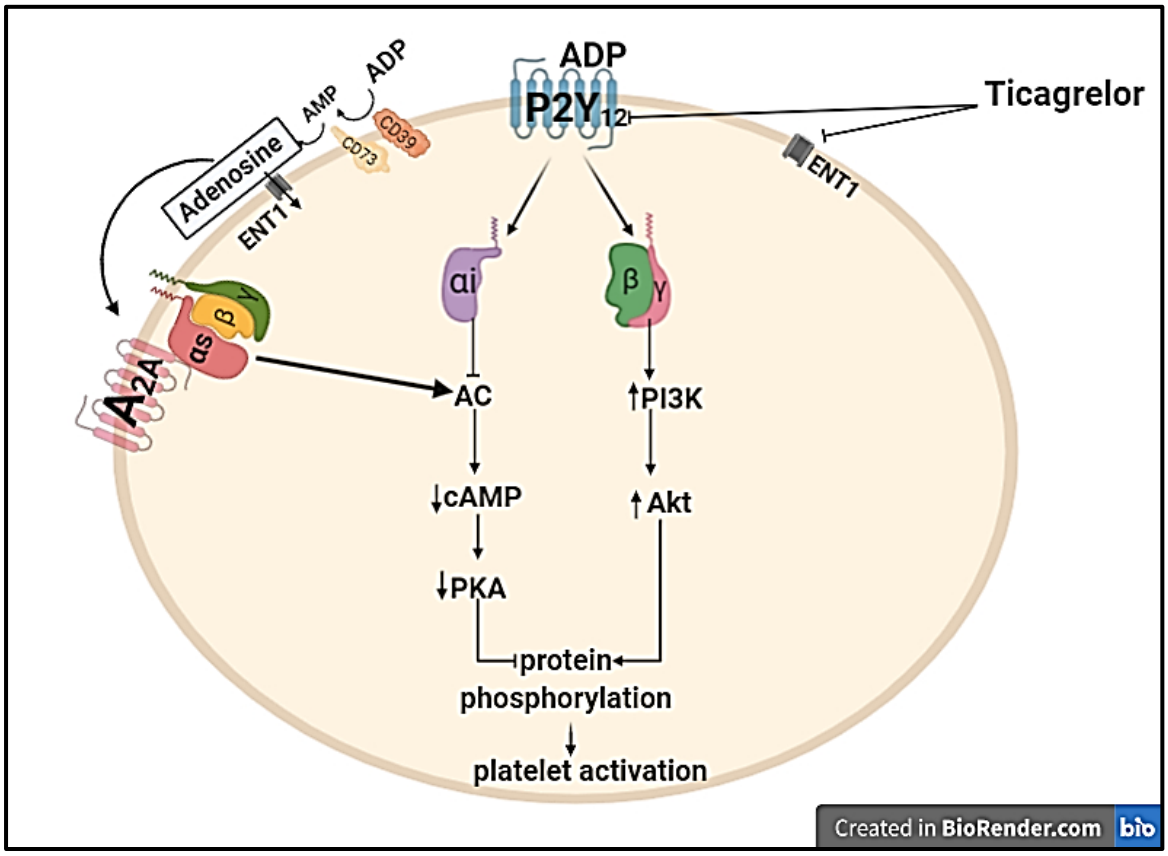


Figure1.8. The $P2Y_{12}$ receptor signalling pathway and the proposed overall mode of action of Ticagrelor on the $P2Y_{12}$ receptor in platelets. Ticagrelor is an inverse agonist at the $P2Y_{12}$ receptor. Its mechanism of action relies primarily on its inverse agonistic nature, but also on its inhibitory effect on ENT1. Through binding at the receptor, ticagrelor determines an inward movement of G proteins, shifting the receptor towards its inactive equilibrium state. This shift results in an opposite effect on the downstream signalling cascade than described in the $P2Y_{12}$-mediated platelet activation section above, resulting in inhibition of platelet activation and aggregation and reduced agonist activity upon subsequent stimulation. By blocking ENT1, ticagrelor indirectly leads to the accumulation of adenosine in the platelet

microenvironment. Adenosine then binds and activates the adenosine A_{2A} $G_{\alpha s}$ protein-coupled receptor to stimulate AC and increase activity of factors downstream of AC to suppress platelet activation.

Several studies have tried to address this issue. One research group investigated the effect of α_2-adrenoceptors agonists on ticagrelor reversibility (*Bonete et al., 2020*). Addition of the full agonist adrenaline almost fully reverses ADP and PAR1-induced platelet aggregation inhibited by ticagrelor, whilst the agonists noradrenaline, clonidine, brimonidine and dexmedetomidine only partially reduce the effect. A second group reported the development of an antigen-binding fragment (F_{ab}) antidote to cure ticagrelor irreversibility (*Buchanan et al., 2015*). The antidote showed significantly higher affinity for ticagrelor than ticagrelor shows for the receptor, whilst not binding to adenosine triphosphate (ATP), ADP, or analogues. Ultimately, the antidote decreased ticagrelor-induced bleeding in acute surgery conditions in mice (*Buchanan et al., 2015*) and produced consistent results in pigs (*Pehrsson et al., 2017*).

1.8. Aims

As described above, the discovery of the high level of constitutive activity at the $P2Y_{12}$ receptor has led to better understanding of platelet pathophysiology in patients. However, very little is still known about how the constitutive activity of the $P2Y_{12}$ receptor is regulated. Therefore, one aim of this study is to begin to characterize the molecular regulation of $P2Y_{12}$ receptor constitutive activity.

The second aim of the study focuses on the molecular pharmacology of the inverse agonist ticagrelor. Particularly, this thesis will focus on the irreversible nature of this drug at the $P2Y_{12}$ receptor. The potential importance of drug-induced post-translational modifications, the importance of membrane microdomains and drug/receptor interactions will be assessed. A greater insight into why ticagrelor appears so resistant to wash-out should provide valuable clinical resolutions in patients in immediate need of surgery who are also on treatment with ticagrelor.

Chapter II: Materials and Methodology

2.1 Materials

Name	Observations	Source
HEK293T	Human Embryonic Kidney 293 which express the SV40 T antigen	Human
CHO	Chinese Hamster Ovary cells	Chinese Hamster
Human platelets	Used in the form of Platelet-Rich Plasma in aggregation experiments and	Human

Table2.1.1. Mammalian cell lines and cell types used in the project.

Name	Description	Supplier
Penicillin	Antibiotic used in media formulation against bacterial contamination	Gibco™
Streptomycin	Antibiotic used in media formulation against bacterial contamination	Gibco™
Apyrase	Catalytic enzyme for ADP hydrolysis	Sigma-Aldrich
Indomethacin	Non-selective cyclooxygenase inhibitor	Sigma-Aldrich
Lipofectamine™ 2000	Cationic lipid transfection reagent	Invitrogen
Coelenterazine 400a	Substrate for Renilla Luciferase (Rluc)	
Phenol red-free Trypsin-EDTA (1X)	Protease-chelator complex for enhanced cell dissociation	Gibco™
Sodium citrate and Acid citrate dextrose (ACD)	Blood anti-coagulants	BioSight Ltd, Israel
Phenol red Dulbecco's Modified Eagle Medium (phenol red DMEM)	HEK293T cells culture medium	Gibco™
Phenol red Dulbecco's Modified Eagle Medium and F-12 nutrient mixture 1:1 (DMEM/F-12 1:1)	CHO cells culture medium	Gibco™
Phosphate Buffered Saline (PBS) 1X pH 7.4	Isotonic buffer solution	Gibco™
Opti-MEM® I Reduced-Serum Medium	Cationic lipid transfection medium	Gibco™
Methanol and ethanol	Solvents	Sigma-Aldrich

Table2.1.2. Table containing reagents used in the project, alongside descriptions and suppliers.

Plasmid construct	Gene name	Species	Description	Observation
pcDNA3.1	-	-	5.4 kb vector derived from pcDNA3 for stable transient transfections in mammalian host cells	Added volumes were according to final construct mass and concentration, using the formula mass(ng) = concentration (ng/uL) x Volume (uL)
FLAG-$P2Y_{12}$; FLAG-$mP2Y_{12}$	P2Y12R	*Homo sapiens*	$P2Y_{12}$ receptor (P2Y12R) tagged with FLAG epitope; point mutants of the $P2Y_{12}$ receptor tagged with FLAG epitope	
HA-MOR	OPRM1	*Homo sapiens*	μ-opioid receptor (MOR) tagged with HA epitope	
$G_{\alpha i}$-Rluc	GNAi1	*Homo sapiens*	$G_{\alpha i}$ protein tagged with Renilla luciferase	
G_{β}	GNB1	*Homo sapiens*	G_{β} protein	
G_{γ}-GFP	GNG2	*Homo sapiens*	G_{γ} protein tagged with GFP	
tetherin-WT	BST2	*Homo sapiens*	For transient transfection of tetherin	

Table2.1.3. DNA plasmid constructs and their features that were used for transient transfections in the present study.

Compound	Description	Source
Ticagrelor	$P2Y_{12}$ receptor inverse agonist	AstraZeneca
Adenosine 5'-diphosphate (ADP)	$P2Y_{1/12}$ receptor agonist	Sigma-Aldrich
2-Methylthio-adenosine-5'-diphosphate (2MeSADP)	$P2Y_{1/12}$ receptor agonist, more potent than ADP	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Aprotic solvent	Sigma-Aldrich
U46619	TP receptor agonist, TxA2 analogue	Sigma-Aldrich
DAMGO	MOR agonist	Sigma-Aldrich
$ZnCl_2$, $MgCl_2$, $CaCl_2$	Ionic salts	Sigma-Aldrich
Methyl-β-Cyclodextrin	Cholesterol chelator	Sigma-Aldrich
KT 5720, PKI	Protein Kinase A inhibitors	Sigma-Aldrich
Collagen-receptor activating peptide (CRP)	Glycoprotein VI agonist	Cambridge Bioscience
TRAP-6	Protease activated receptor 1/4 agonist	Cambridge Bioscience

Table2.1.4. Compounds used throughout the project.

Consumables	Source
Pipettes P2, P10, P20, P100, P200, P100	Gilson
Pipette tips	Corning/Rainin/STARLAB
Pipette controller: PipetBoy 2	Integra
Serological Pipets 5 mL, 10 mL, 25 mL	Thermo Fisher Scientific
15, 50 mL Falcon tubes	BD Biosciences
100 x 20 mm cell culture dishes	Corning
60 x mm cell culture dishes	Corning
T75 cell culture flasks	Corning
10 mL centrifugation vials	Thermo Fischer Scientific
0.5 mL, 1.5 mL, 2 mL Eppendorf tubes	Eppendorf
96-well plates	Greiner bio-one
Syringes/Hypodermic needles	BD biosciences
P/N 312 cuvettes	CHRONO-LOG® Corporation
Stir bars	CHRONO-LOG® Corporation
Magnetic extractor for stir bars	CHRONO-LOG® Corporation

Table2.1.5. List of consumables and the respective suppliers.

2.2. Methods

2.2.1. Platelet biology

2.2.1.1. Human platelet isolation

Human blood was donated by healthy volunteers through a written consent, according to the University of Bristol Ethics of Research Policy and Procedure and the World Medical Association Declaration of Helsinki 1964.

Blood was collected by venipuncture, via a butterfly needle connected to a 50 mL BD Plastipak™ syringe or vacutainers containing 4% sodium citrate as 10% of total blood volume. To isolate the platelet-rich plasma (PRP) from the other blood components, donated blood was divided equally into LP4 vials and centrifuged at 1000 rpm for 17 minutes. Next, PRP was carefully collected and transferred to 15 mL Falcon tubes and treated with platelet inhibitors (indomethacin and/or apyrase), according to experimental need. The PRP was then rested in a 30˚C water bath for 30 minutes before experimentation. To obtain platelet-poor plasma (PPP), 1 mL PRP was aliquoted into a 1.5 mL Eppendorf tube and centrifugated at 10000 rpm for 3 minutes. 700 µL of the resulting supernatant were carefully removed from the centrifugation Eppendorf tube and placed into a P/N 312 glass cuvette to be subsequently used as reference value.

2.2.1.2. Light-transmission aggregometry (LTA)

Platelet aggregation assays are a useful tool to assess platelet aggregation ex-vivo and evaluate the behaviour of different platelet agents (*Le Blanc et al., 2020*). Platelet aggregation was measured using 300 µL PRP were dispensed into P/N 312 glass cuvettes, which were then placed in the recording chambers of the CHRONO-LOG® Model 700 Optical Lumi-Aggregometer, and stir bars were dispensed in each cuvette. 700 µL of PPP were dispensed in a separate cuvette and placed in the reference/control chamber. Using the AggroLink software on a computer linked to the aggregometer, the baseline for each of the measurements was established according to the PPP cuvette. After the baseline was corrected, the treatments were dispensed into the PRP according to the experimental plan and ensuring that enough time was allowed between treatments to obtain clear recordings.

2.2.2. Cell biology

2.2.2.1. Sterilisation

Cell culture work was undertaken in a strict aseptic environment, in a fume hood which was prior to and subsequently disinfected with a 70% ethanol solution; lab consumables used in the fume hood (pipette tips, suction pump glass tips) were disinfected by autoclave at 120°C, whilst media bottles, Falcon tubes, pipettes, cell reservoirs were disinfected with a 70%

ethanol solution before entering the hood. The other mentioned consumables were wrapped in individual, sterile wrapping.

2.2.2.2. Mammalian cell culture

Cells used in the experiments are cultured in 100 mm x 20 mm dishes in cell growth media treated with 10% Foetal Bovine Serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. Cells were consistently being monitored for degree of confluency, so that passage was done at sub-maximal to maximal levels of confluency (90-100%) to avoid cell death through overpopulation and infection.

2.2.2.3. Subculturing of the mammalian cell culture

Subsequently, the media of the fully confluent cell plate was discarded, and cells were washed with 3 mL of PBS 1x. Next, PBS was aspirated, and cells were incubated with 1 mL trypsin-EDTA 1x solution for 2-5 minutes, to break cell to cell and cell to plate contacts. Cells were resuspended in 3 mL growth media, to allow FBS to inhibit further, undesired Trypsin activity and prevent cell breakdown. Cells are collected in a 10 mL tube and centrifuged for 2-3 minutes at 1000 rpm, a fresh 100 mm x 20 mm cell plate is prepared by addition of 9 mL fresh media and proper labelling. Post centrifugation, the resulting supernatant was aspirated, and cells were resuspended in 1 mL media, with the desired volume of new cell suspension added to the new plate, according to the next step of cell line work plan. For an efficient transient transfection, 500-600 µL are added to the new cell plate for a 1:2 split – the new cell plate would reach full confluency the next day.

2.2.2.4. Cryopreservation of mammalian cell

To prepare cell stocks to be stored in liquid nitrogen, cells are detached from the culture plate at high confluency (90-100%) and prepared as previously described. The resulting supernatant is discarded, and the cells are resuspended in a 5 mL solution containing 90% cell culture medium and 10% DMSO. The resulting cell suspension is transferred to a properly labelled cryotube and incubated in Mr Frosty™ Freezing container (containing isopropanol) in a -80˚C freezer overnight. The following day, the new cell stock is transferred to the liquid nitrogen chamber.

2.2.3. Transient transfection of mammalian cells

Cells were transfected 24 h following cell passage and at a 50-70% confluency using a standard protocol and Opti-MEM. Opti-MEM is a reduced serum medium which allows cells to 'shift focus' from cell proliferation to uptake of DNA and start expression of desired receptor.

The protocol required the use of two 1.5 mL Eppendorf tubes, the first containing 500 mL Opti-MEM and DNA plasmids and the second one containing 500 mL Opti-MEM and Lipofectamine. DNA plasmids - receptor of interest, $G_{\alpha i}$-Rluc, G_{β}, G_{γ}-GFP are added in volumes

according to a mass ratio that was established in our laboratory as most efficient for transfection. With a total mass of 5 ug DNA in the first tube, the DNA plasmids are added in a 1.5 ug: 1.5 ug: 1 ug: 1ug ratio, respectively, and incubated for five minutes at room temperature. In the second tube, Lipofectamine was added in 1:2 DNA: Lipofectamine ratio and incubated for five minutes at room temperature. Next, the Lipofectamine mix was added to the DNA mix tube and the new mix was incubated for a minimum of 20 minutes at room temperature, before it was to be added to the cell plate. Meanwhile, the cell culture medium in the transfection plate was discarded, the cells were washed with PBS and resuspended in 5 mL Opti-MEM. Finally, the DNA: Lipofectamine mix was added to the cell plate in a dropwise manner and further incubated for 4-6 h at 37˚C in the humidified incubator, to promote the uptake of the transfection mix. At the end of the incubation period, the spent transfection medium was aspirated, and 10 mL of fresh cell growth medium was added back to the cell plate to resume proliferation. Cells were further incubated for 48 hours at 37˚C in the humidified incubator before experiments were undertaken. Transfection efficiency using this protocol was never assessed during this project, however previous work performed in our laboratory suggests that the usual transfection efficiency in HEK293T cells is approximately 20%. Transfection efficiency for this project could be assessed through immunofluorescence and/or immunoprecipitation.

2.2.4. Bioluminescence Resonance Energy Transfer (BRET)

2.2.4.1. Principle

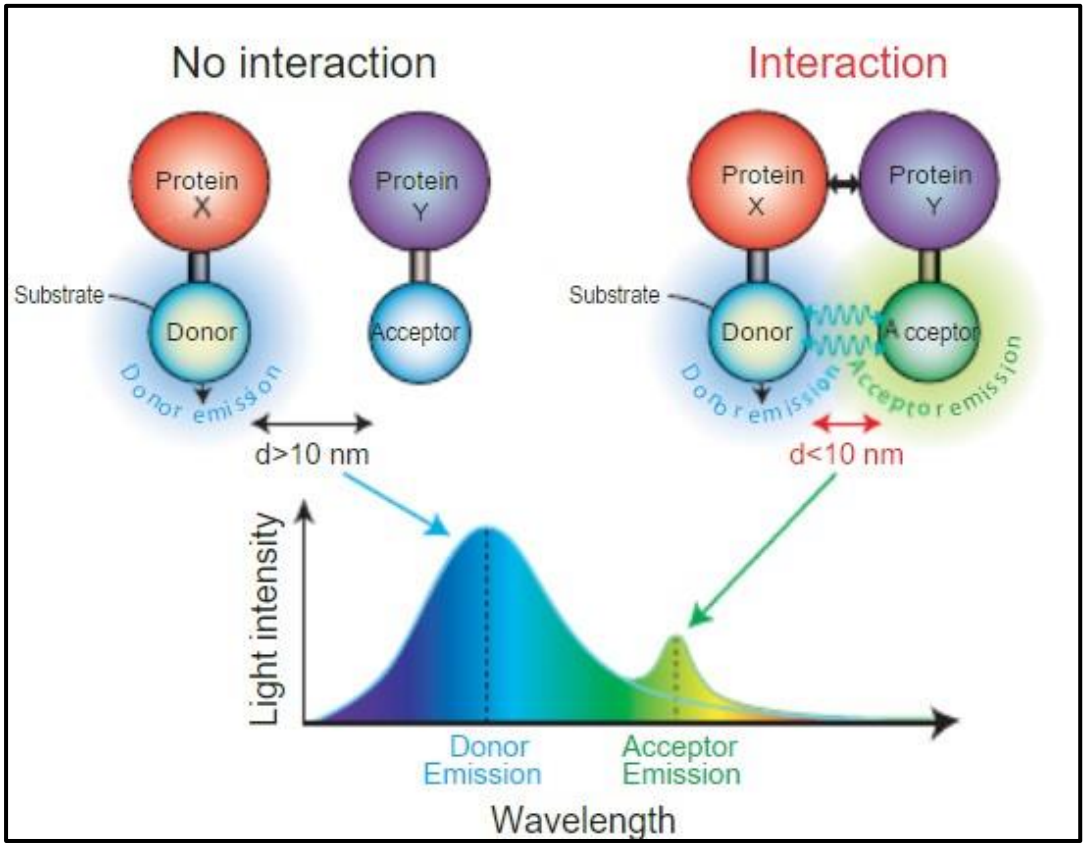


Figure1. A model describing the principle behind BRET (taken from Bacart et al., 2008)

As depicted in Figure1, the principle behind BRET analysis implies that a Donor protein, bound to Protein X, emits bioluminescence at its specific wavelength, when stimulated by its specific substrate. The bioluminescence stimulates the Acceptor protein, bound to Protein Y, to emit fluorescence at its specific wavelength. The BRET signal is equal to the ratio between Acceptor emission and Donor emission. This process occurs when the distance between the Donor and the Acceptor is less than 10 nm, otherwise, no interaction can be registered using the approach.

2.2.4.2. Assay preparation

Tailored to experiments described in this study, the Donor protein is Renilla luciferase, which is bound to $G_{\alpha i}$ protein (Protein X) and stimulated by substrate Coelenterazine 400a to emit bioluminescence at approximately 410-480 nm, which stimulates the Acceptor protein Green Fluorescent Protein (GFP), bound to G_{γ} protein (Protein Y), to emit fluorescence at approximately 515-530 nm.

Experiments were conducted 48 h after transfection day, as established in the Kelly/Mundell laboratories. Next, spent cell medium was discarded, cells were washed with room temperature PBS 1x and detached using trypsin-EDTA 1x solution. To inactivate trypsin, cells were resuspended in cell medium, centrifuged at 1000 rpm for three minutes and the supernatant discarded. Cells were washed two times using experimental medium, phenol red-free DMEM or PBS 1x supplemented with 0.1% glucose (for experiments analysing ionic effects on the $P2Y_{12}$ receptor). Finally, the cells were resuspended in 10 mL experimental medium and dispensed in a reservoir, and 80 µL cell suspension were added to each well of a 96-well white cell plate using a multichannel pipette. Cells were then incubated at 37°C 5% CO_2 for approximately 30 minutes before reading.

Meanwhile, a drug plate containing the compounds that were to be used in the experiment was prepared. Compound stock solutions and further dilutions were made using experimental medium to keep final BRET volume homogenous, unless stated otherwise.

Finally, the substrate of the experiment was prepared by a 1:25 dilution of Coelenterazine 400a stock in experimental medium. Before reading, the FLUOstar Omega Lite Microplate Reader was pre-warmed to 37°C and cleaned using 70% ethanol and dH_2O and primed using the pre-made substrate. The compounds were incubated in the wells as set in the experimental plan – ADP and ticagrelor were incubated for five minutes in each experiment, unless stated otherwise, whilst the other compounds were incubated according to experiments described in literature or for an arbitrary timeframe. The final BRET volume was set to 100 µL containing 80 µL cells, 10 µL compounds and 10 µL substrate.

2.2.4.3. Data analysis

Drug addition to the plates was randomized for each experiment (up-down or down-up) and within each experiment (random order of ticagrelor, ADP and 2MeSADP). Once recordings

are taken, data is exported into Excel, where it undergoes mathematical transformation to ΔBRET values (the difference between treatment and control-mediated signal). ΔBRET values are exported into GraphPad Prism 9 for further analysis. Where applicable, data were plotted onto graphs as mean ± S.E.M of at least three experiments (n≥3 to minimize data variability). Concentration-response curves were fitted with non-linear regression and a variable slope to generate EC_{50} values and half-life of ligands. Significant differences in mean ΔBRET obtained following ligand addition to the assays were established by one- or two-way ANOVA with multiple comparison Tukey's post-hoc test to determine the statistical differences between the means of three or more comparison groups in each experiment. In all experiments, P values smaller than 0.05 were considered significant, as follows: ns=P>0.05, *=P<0.05, **=P<0.01, ***=P<0.005, ****=P<0.001.

Chapter III: Results

Ticagrelor is considered a superior platelet activity inhibitor, reducing unnecessary ADP-mediated platelet aggregation leading to thrombosis (*Patil et al., 2010*; *Husted & van Giezen, 2009*; *Storey et al., 2009*; *van Giezen et al., 2009*). It is widely prescribed in combination with aspirin in dual antiplatelet therapy (DAPT), the gold standard for treatment in acute coronary syndrome patients. The mechanism of action of ticagrelor may underlie its superior efficacy at the $P2Y_{12}$ receptor comparative to the other available $P2Y_{12}$ receptor inhibitors (*Wallentin et al., 2009*; *van Giezen, Berntsson et al., 2009*).

In 2016, *Aungraheeta et al.* suggested that ticagrelor acts on the $P2Y_{12}$ receptor as an inverse agonist rather than a pure antagonist. This theory implies that ticagrelor decreases the constitutive activity of the receptor, i.e., shifts the equilibrium of the receptor from a natively active conformation towards an inactive state. Despite being initially marketed as a reversible agent, ticagrelor has been suggested to display irreversible effects in platelets for the entirety of a platelet cycle (*Gerrits et al., 2017*; *Buchanan et al., 2015*).

The present study aims to further understand regulation of constitutive activity of the $P2Y_{12}$ receptor and the mode of action of the inverse agonist ticagrelor. To do this, both mammalian cell lines and human platelet studies will be undertaken. As both HEK293T and CHO cell lines endogenously express the $P2Y_{12}$ receptor (*Hardy et al., 2005*), the present study aims to describe effects in both a receptor-overexpressing system and a system expressing the receptor endogenously. Bioluminescence Resonance Energy Transfer (BRET) assays will be used in mammalian cells overexpressing the $P2Y_{12}$ receptor, to directly assess drug-mediated G protein-activation downstream of the $P2Y_{12}$ receptor. Further light-transmission aggregometry assays on platelets will be used to assess ticagrelor-induced inhibition of platelet aggregation, under several experimental conditions.

3.1. Characterization of $P2Y_{12}$ receptor pharmacology in cell line systems and human platelets.

Initial studies were undertaken to recapitulate earlier unpublished studies in the Mundell laboratory characterizing the pharmacology of the $P2Y_{12}$ receptor versus the agonists ADP and 2MeSADP and the inverse agonist ticagrelor. These studies were performed on receptor overexpressed in the HEK293T or CHO cell lines or on endogenous receptor in human platelets. As outlined in the Methodology section above, a BRET based assay was used in cell lines which measures G protein subunit disassociation downstream of $P2Y_{12}$ receptor activation. Both the time-course and agonist-dependence of changes in receptor activity were assessed (see Figures 3.1.1 and 3.1.2)

As expected, the $P2Y_{12}$ receptor agonists ADP and 2MesADP caused a decrease in BRET signal because of increased G protein subunit disassociation. Ticagrelor, meanwhile, caused an increase in BRET signal indicative of increased G protein subunit association. This is likely due to ticagrelor reducing basal $P2Y_{12}$ receptor activity indicative of its action as an inverse agonist. In HEK293T cells, the calculated $t_{0.5}$ of the three compounds are approximately 1 minute [95% CI: n/a – 2.3 minutes], 2 minutes [95% CI: 0.89 – 2.82 minutes] and 6 minutes [95% CI: n/a – 19.16 minutes], respectively. The time-course studies revealed (see Figure 3.1.1, panel A) that $P2Y_{12}$ receptor stimulation is maximal after approximately 5 minutes of incubation for each of the three compounds. This timepoint was, therefore, used for the remainder of the experiments involving the use of HEK293T cells (see Table 3.1.2). The maximal response achieved by ticagrelor was approximately 0.1, whereas the maximal responses for ADP and 2MeSADP are approximately -0.3 to -0.35. The concentration-dependence of this effect (see Figure 3.1.1, panel B) was next assessed. ADP and 2MeSADP increased and ticagrelor decreased $P2Y_{12}$ receptor activity in a concentration-dependent manner (see Table 3.1.1). The EC_{50} values for ADP, 2MeSADP and ticagrelor were $3.91x10^{-7}$ M [95% CI: $1.94x10^{-7}$-$2.09x10^{-6}$ M], $4.59x\ 10^{-10}$ M [95% CI: $2.84x10^{-10}$-$7.42x10^{-10}$ M] and $5.51x10^{-7}$ M [95% CI: $2.47x10^{-7}$-$6.81x10^{-6}$ M], respectively. Notably in this experiment, ticagrelor reaches a maximal response (maximum ΔBRET) of approximately 0.1, whereas ADP and 2MeSADP reach a maximal response of approximately -0.12 and -0.14, respectively. The inherent variability in agonist and inverse agonist responses is potentially suggestive of the loss of receptor constitutive activity and may be explained by an unexpected hardware fault in the FLUOstar Omega Lite Microplate Reader.

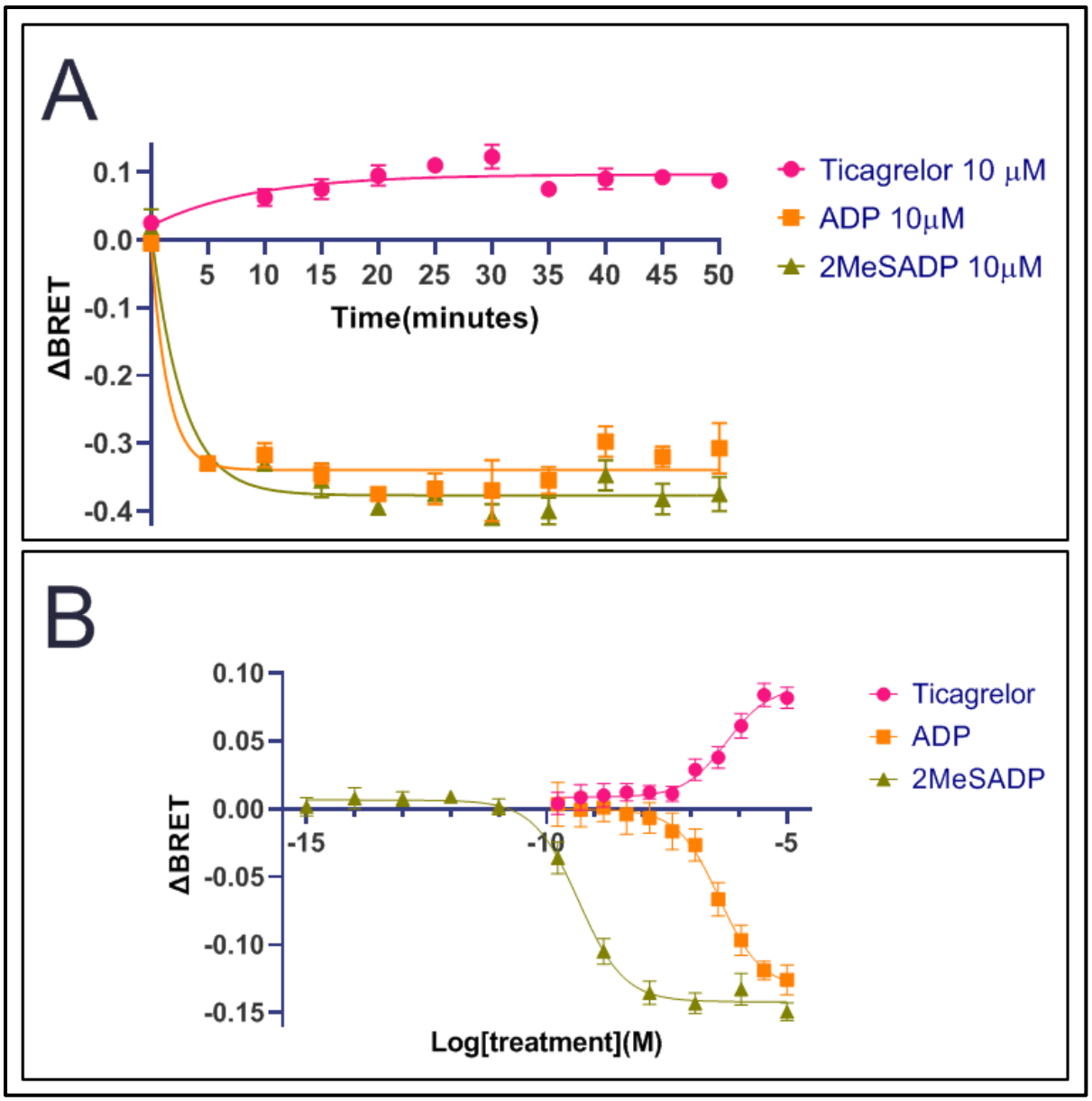


Figure3.1.1. Time (A) and concentration (B)-dependent activation of the $P2Y_{12}$ receptor by ticagrelor, ADP and 2MeSADP in HEK293T cells. HEK293T cells were transiently transfected with $P2Y_{12}$ receptor and necessary G proteins plasmids. (A) Cells were subsequently incubated with ticagrelor, ADP and 2MeSADP (all at 10 μM). Readings were taken every 5 minutes, with an initial recording at t=0 min immediately after all drug additions were performed. (B) Cells were subsequently incubated with increasing concentrations of ticagrelor, ADP and 2MeSADP for 5 minutes. In (A) and (B) data are represented ± S.E.M. of 3 independent experiments.

Similar studies were also performed in CHO cells (see Figure 3.1.2). $P2Y_{12}$ receptor stimulation is maximal after approximately 5 minutes of incubation for each compound. As with HEK293T cells, this timepoint of incubation was used for the remainder of the experiments involving the use of CHO cells (see Table 3.1.2). Ticagrelor reaches a maximal response of approximately 0.28, whereas ADP reaches a maximum of -0.04. The calculated EC_{50} values for ticagrelor and ADP are 5.98×10^{-6} M and 2.21×10^{-6} M, respectively. The calculated $t_{0.5}$ values for the two

compounds are approximately 2 minutes [95% CI: 1.15 – 2.49 minutes] and 8 minutes [95% CI: 2.45 – 98.31 minutes], respectively.

As in HEK293T cells, in CHO cells, ADP increased, whilst ticagrelor decreased, receptor activation in a concentration and time-dependent manner. Whereas ADP displays a half-life of 1 minute in HEK293T cells, in CHO cells, the half-life of ADP is longer (8 minutes). Moreover, ADP seems to be more potent in HEK293T cells than in CHO cells, as shown by the 10-fold difference in EC_{50} values. On the other hand, ticagrelor half-life in CHO cells is shorter than in HEK293T cells (2 minutes vs 6 minutes) and seems more potent in CHO cells when considering the 2.5-fold increase in magnitude of response compared to HEK293T cells which brings only a 10-fold increase in EC_{50} (Tables 3.1.1 and 3.1.2).

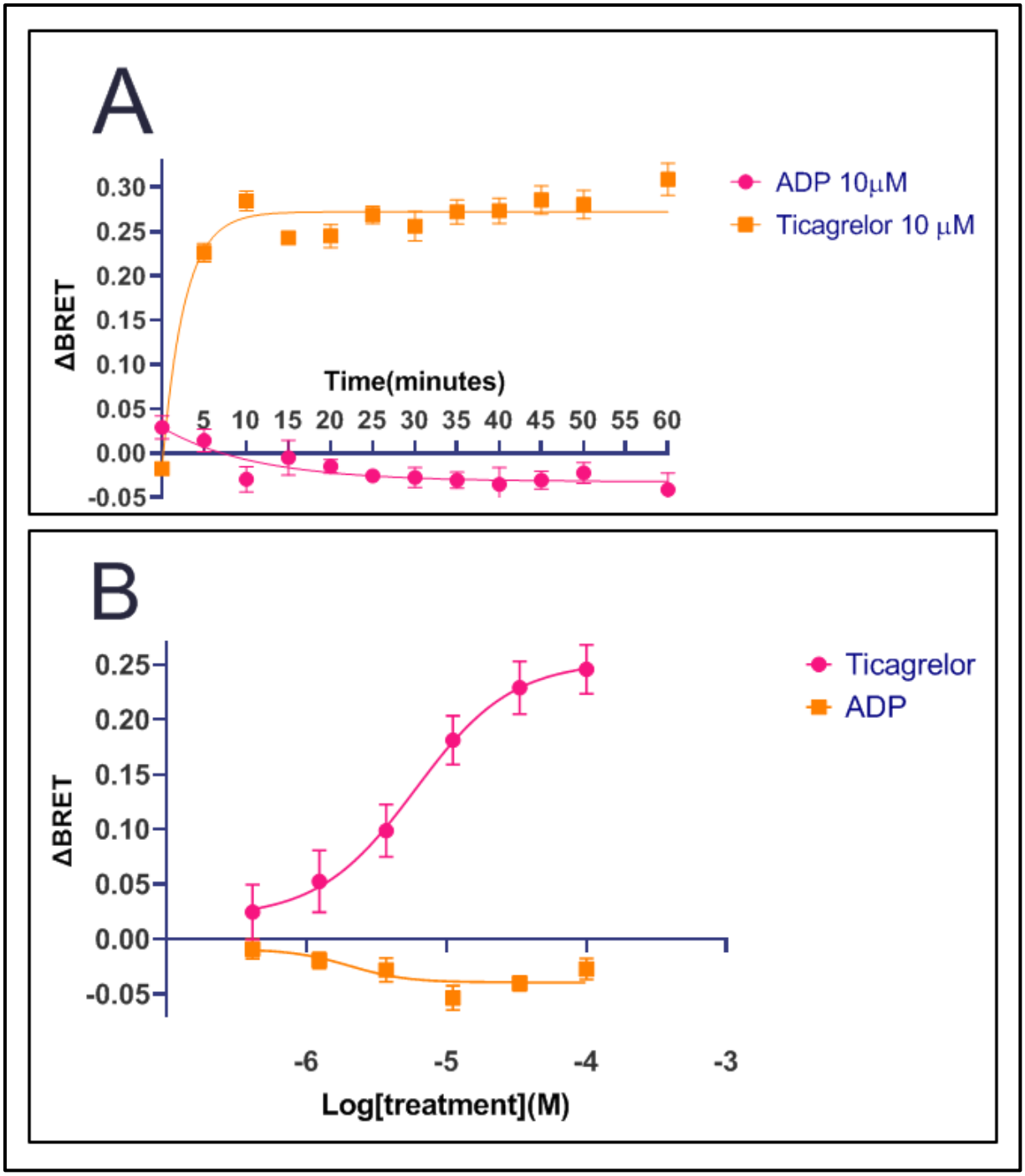


Figure3.1.2. Time (A) and concentration (B)-dependent activation of the $P2Y_{12}$ receptor by ticagrelor, ADP and 2MeSADP in CHO cells. CHO cells were transiently transfected with $P2Y_{12}$ receptor and necessary G proteins plasmids. (A) Cells were subsequently incubated with ticagrelor, ADP and 2MeSADP (all at 10 μM). Readings were taken every 5 minutes, with an initial recording at t=0 min) immediately after all drug additions were performed. (B) Cells were subsequently incubated with increasing concentrations of ticagrelor, ADP and 2MeSADP for 5 minutes. In (A) and (B) data are represented ± S.E.M. of 3 independent experiments.

Compound	EC_{50} in HEK293T	EC_{50} in CHO
ticagrelor	$5.51x10^{-7}$ M[$2.47x10^{-7}$-$6.81x10^{-6}$]	$5.97x10^{-6}$ M
ADP	$3.91x10^{-7}$ M[$1.94x10^{-7}$-$2.09x10^{-6}$]	$2.21x10^{-6}$ M
2MeSADP	$4.59x10^{-10}$ M[$2.84x10^{-10}$-$7.42x10^{-10}$]	Not undertaken

Table3.1.1. Comparison of the calculated EC_{50} values for ticagrelor, ADP and 2MeSADP at the $P2Y_{12}$ receptor in HEK293T cells and CHO cells, respectively. The 95% CI for the EC_{50} values of ticagrelor and ADP in CHO cells could not be calculated.

Compound	$t_{0.5}$ in HEK293T	$t_{0.5}$ in CHO
ticagrelor	6 minutes [n/a-19.16]	2 minutes [1.15-2.49]
ADP	1 minutes [n/a-2.31]	8 minutes [2.45-98.31]
2MeSADP	2 minutes [0.89-2.82]	Not calculated

Table3.1.2. Comparison of the calculated half-lives of ticagrelor, ADP and 2MeSADP at the $P2Y_{12}$ receptor in HEK293T cells and CHO cells, respectively.

Competition binding studies for ticagrelor, 2MeSADP and ADP were also performed, to establish the relationship between the activities of the three compounds at the $P2Y_{12}$ receptor (see Figure 3.1.3), confirming relevant literature (*van Giezen et al., 2009; Haghighi et al., 2021*). Ticagrelor, 2MeSADP and ADP (all 10 µM) were acutely incubated with either HEK293T or CHO cells overexpressing the $P2Y_{12}$ receptor and G proteins constructs for 5 minutes before BRET recordings were taken. Ticagrelor activity at the $P2Y_{12}$ receptor was significantly reduced by both 2MeSADP and ADP in HEK293T cells, with a considerably higher degree of significance for 2MeSADP (see Figure 3.1.3, Panel A). In CHO cells, ticagrelor activity could only be significantly reversed by 2MeSADP (see Figure 3.1.3, Panel B). The ability of 2MeSADP to significantly reverse ticagrelor signalling in wild-type HEK293T cells represents the reference point of the relationship between 2MeSADP and ticagrelor activities and their resistance to washout at different $P2Y_{12}$ receptor mutants, developed in Results section 3.6.

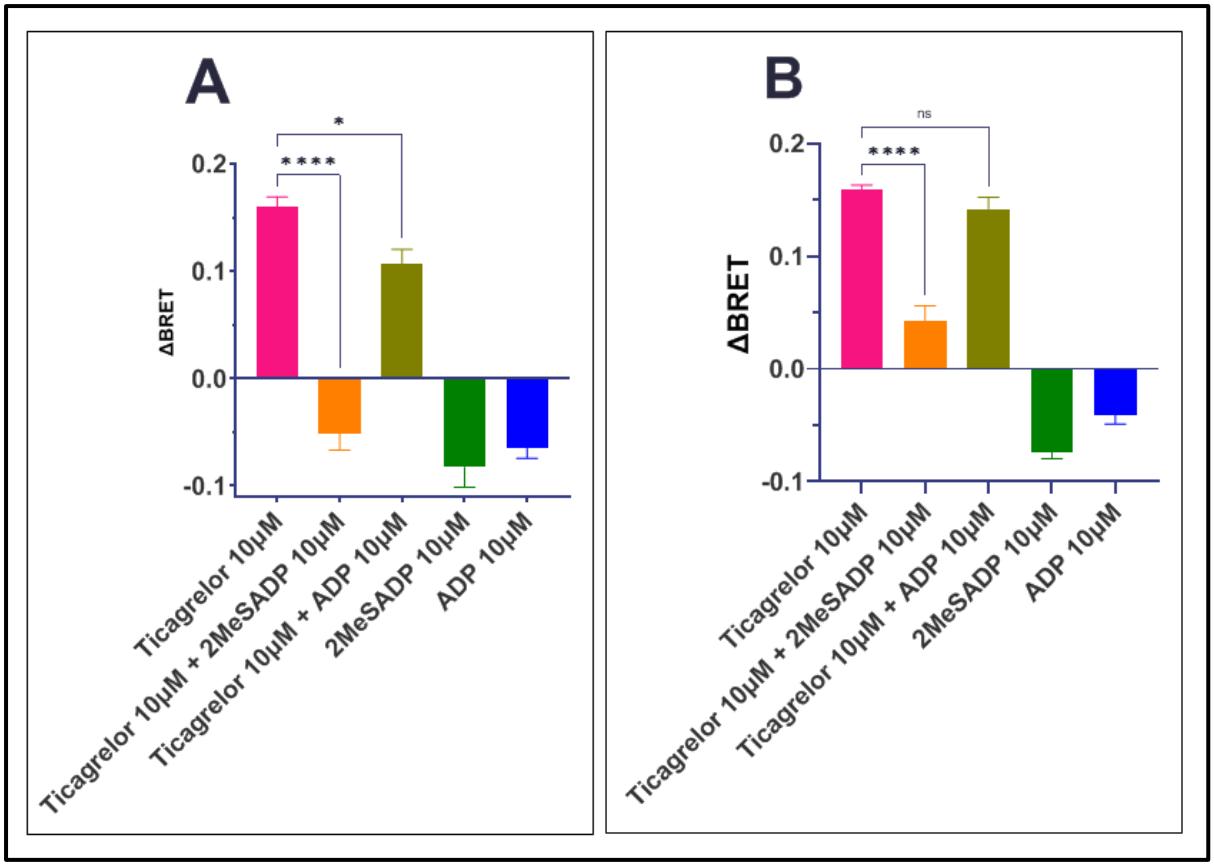


Figure3.1.3. Investigation of the reversibility of ticagrelor by 2MeSADP and ADP at the $P2Y_{12}$ receptor in HEK293T cells (A) and CHO cells (B). HEK293T (A) and CHO (B) cells transiently transfected with the $P2Y_{12}$ receptor and necessary G proteins plasmids. Prior to BRET recordings, cells were acutely treated with ticagrelor 10 μM, ADP 10 μM and 2MeSADP 10 μM, respectively, and $P2Y_{12}$ receptor activation was registered. Data (n=3, represented ± S.E.M) are represented as the difference in receptor activation produced by ticagrelor alone and in presence of either 2MeSADP or ADP, 2MeSADP alone and ADP alone, relative to recorded baseline receptor activity.

Studies were also performed in human platelets to confirm that, as expected, ticagrelor was able to antagonise platelet $P2Y_{12}$ receptor responsiveness (see Figure 3.1.4). Human platelets in platelet-rich plasma obtained from human blood samples were used to assess platelet aggregation induced by four extensively studied platelet agonists, i.e., ADP, U46619, a thromboxane A2 analogue, TRAP-6, an agonist at the protease-activated receptor (PAR), and collagen-related peptide (CRP), an agonist for the platelet activator glycoprotein (GP) VI. Ticagrelor fully inhibits ADP-induced platelet aggregation, partly blocks U46619- and TRAP-6-induced platelet aggregation, and elicits no clear effect on CRP-induced platelet aggregation (see Figure 3.1.4). Following these initial studies confirming that ticagrelor acts as an inverse agonist in both HEK293T and CHO cells and antagonises ADP responses in human platelets, subsequent studies focussed on potential regulators of $P2Y_{12}$ receptor activity.

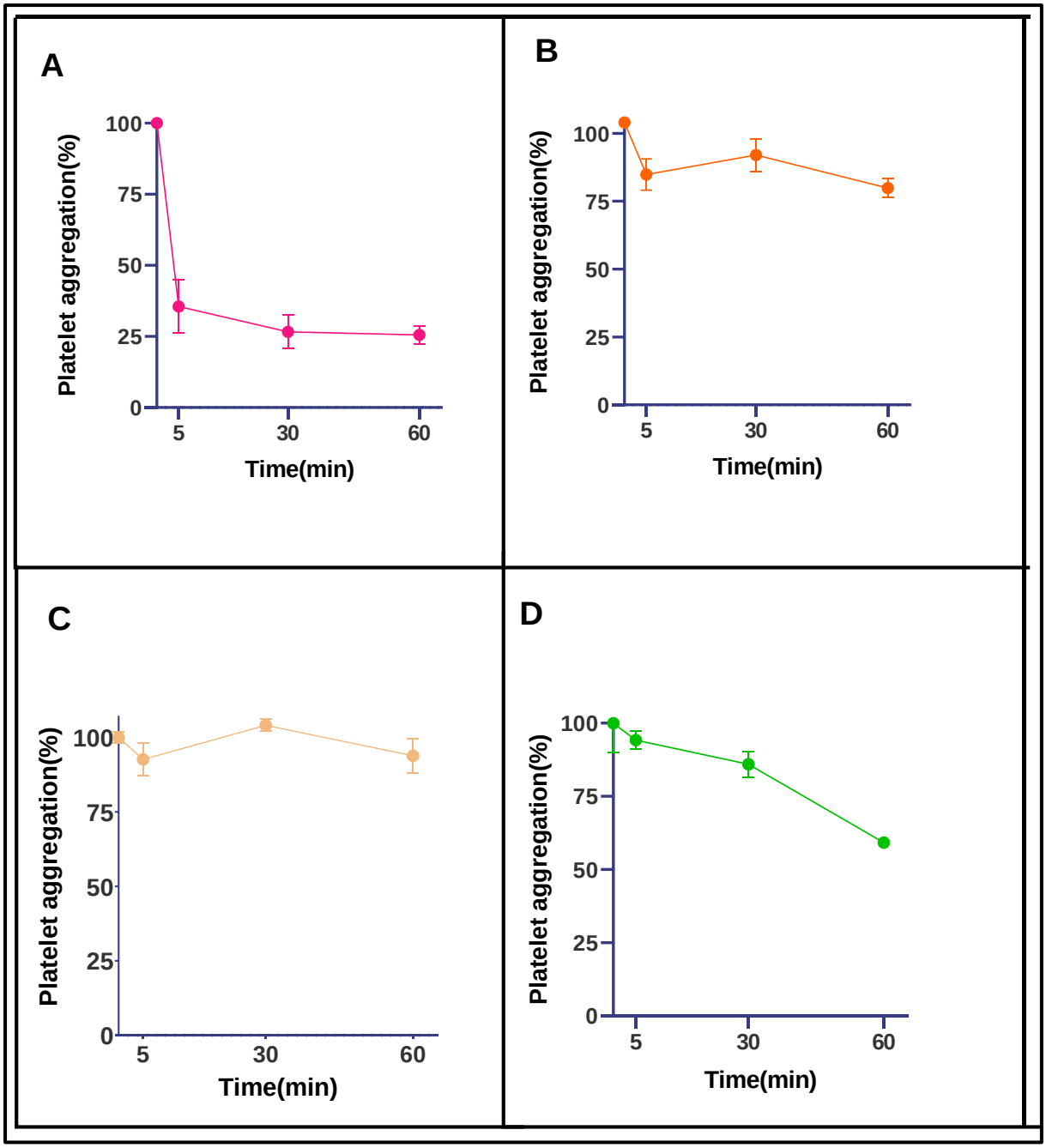


Figure3.1.4. Assessment of the effect of ticagrelor on ADP (A), U46619 (B), CRP (C) and TRAP-6 (D)-induced platelet aggregation. Whole blood samples were centrifuged at 1000 x g for 17 minutes for separation of the different blood components into red blood cells and platelet-rich plasma (PRP) as established in the laboratory-specific protocol**.** Platelet count in the resulting platelet-rich plasma was adjusted to approximately $1x10^9$ platelets. Then, the platelet preparation was incubated in a 37°C water bath for at least 30 minutes prior to experimentation. Platelet (PRP) samples were then incubated with ticagrelor 10µM for 1 hour. Platelet-induced aggregations to ADP 10 µM, U46619 10 µM, CRP 10 µM, TRAP-6 10 µM were recorded at 0, 5, 30 and 60 minutes of ticagrelor incubation. Data, n=3 (blood samples from three different human patients) represented ± S.E.M, are presented as platelet aggregation (relative to recording baseline) over time of ticagrelor incubation.

3.2. Assessment of the activity of various ions at the $P2Y_{12}$ receptor.

The effect of bivalent ions on surface receptors on platelets has been extensively studied (zinc: *Taylor and Pugh, 2016*; magnesium: *Ravn et al., 1996*; calcium ions: *Varga-Szabo et al., 2009*; copper: *Johnson, 2009*; iron: *Elstrott et al., 2022*). For example, zinc ions have been shown to act as platelet activators. Zn^{2+} is present in the plasma at concentrations of approximately 20-30 µM, most of which forms complexes with plasma proteins such as albumin, resulting in a free Zn^{2+} concentration in the plasma of 0.5 µM. However, Zn^{2+} is considered to abound (sixfold greater than surrounding tissues) in atherosclerotic plaques, potentially resulting in a potent priming factor for platelets in case of plaque rupture (*Ahmed et al., 2021*). Moreover, zinc ions, alongside copper ions, regulate the constitutive activity of the melanocortin-4 receptor, suggesting Zn^{2+} could potentially regulate $P2Y_{12}$ receptor activity (*Link et al., 2020*). Alongside Zn^{2+} ions, the effect of Mg^{2+} and Ca^{2+} ions on the $P2Y_{12}$ receptor were also assessed. Both Mg^{2+} and Ca^{2+} ions are reported to shift the conformation of the A_{2A} adenosine receptor towards an active state (*Ye et al., 2018*). In addition, Mg^{2+} ions were shown to have inhibitory effects on platelet aggregation (*Ravn et al., 1996*), whilst Ca^{2+} ions are known to play a critical role in platelet activation (*Varga-Szabo et al., 2009*).

In this section, HEK293T cells and ionic solutions were prepared for experimentation in PBS 1x supplemented with 0.1% glucose. Zn^{2+}, Mg^{2+} and Ca^{2+} were used as respective chloride salts and dissolved in purified water, to avoid crystallization. The effect of each of these ions on basal (A), ADP-stimulated (B) and ticagrelor-stimulated activity was assessed (see Figure 3.2.1). In the study, the rationale for the Zn^{2+} concentrations used was to mimic the plasma free Zn2+ concentration (1 µM) and the pathological Zn^{2+} concentration that may occur in plaque rupture (10 µM).

Overall, Zn^{2+} produced a concentration-dependent increase in basal activity, with little, not statistically significant changes to ADP-induced activation and to the inverse agonism of ticagrelor at the $P2Y_{12}$ receptor. Conversely, Mg^{2+} and Ca^{2+} did not produce significant changes in any of the basal activity, ADP-induced activation or ticagrelor activity at the $P2Y_{12}$ receptor.

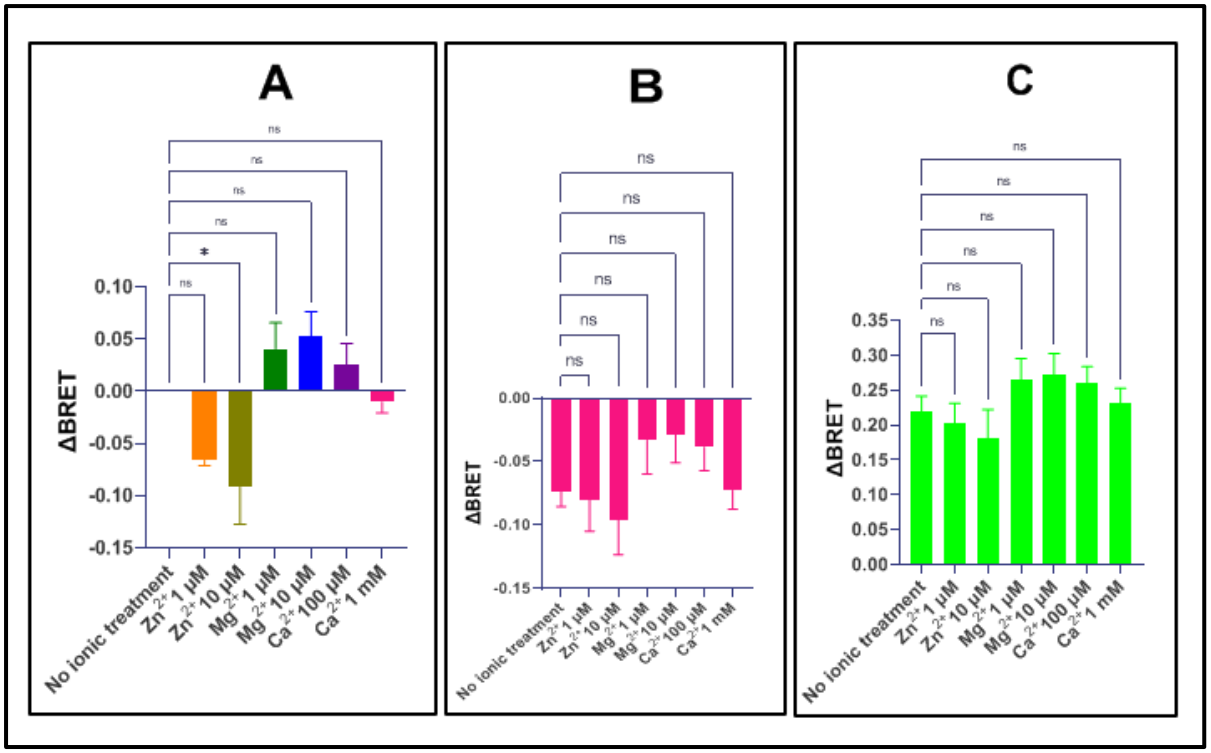


Figure3.2.1. Investigation of the effect of Zn^{2+}, Mg^{2+}, and Ca^{2+} ions on the agonist-independent activation of the $P2Y_{12}$ receptor (A), and on ADP (B) and ticagrelor (C)-induced $P2Y_{12}$ receptor activation in HEK293T cells. HEK293T cells transiently transfected with $P2Y_{12}$ receptor and necessary G proteins plasmids were incubated with Zn^{2+}, Mg^{2+} and Ca^{2+}. Ionic solutions of 10 µM and 1 µM were used for Zn^{2+} and Mg^{2+}. Ca^{2+} was used in concentrations of 100 µM and 1 mM to mimic homeostatic Ca^{2+} levels. Responses to ticagrelor 10 µM and ADP 10 µM were recorded after 5 minutes of incubation. Data (n=5), represented ± S.E.M, are depicted as the ionic-, ADP- and ticagrelor-mediated $P2Y_{12}$ receptor stimulation, relative to basal receptor activity.

Based on the significant difference produced by Zn^{2+} on basal $P2Y_{12}$ receptor activity in this experiment and considering the high degree of variability suggested by the large error bars in the study on ADP and ticagrelor activity, the effects of Zn^{2+} on the agonist-independent and agonist-dependent activation of the $P2Y_{12}$ receptor were further studied (see Figure 3.2.2). As depicted in Panel A of Figure 3.2.2, Zn^{2+} alone produced an increase in basal activity of the $P2Y_{12}$ receptor in a concentration-dependent manner. When incubated together with ADP or ticagrelor (see Figure 3.2.2, Panel B), Zn^{2+} produced a dose-dependent increase in ADP activity at the $P2Y_{12}$ receptor and a dose-dependent decrease in ticagrelor-induced stimulation of the $P2Y_{12}$ receptor.

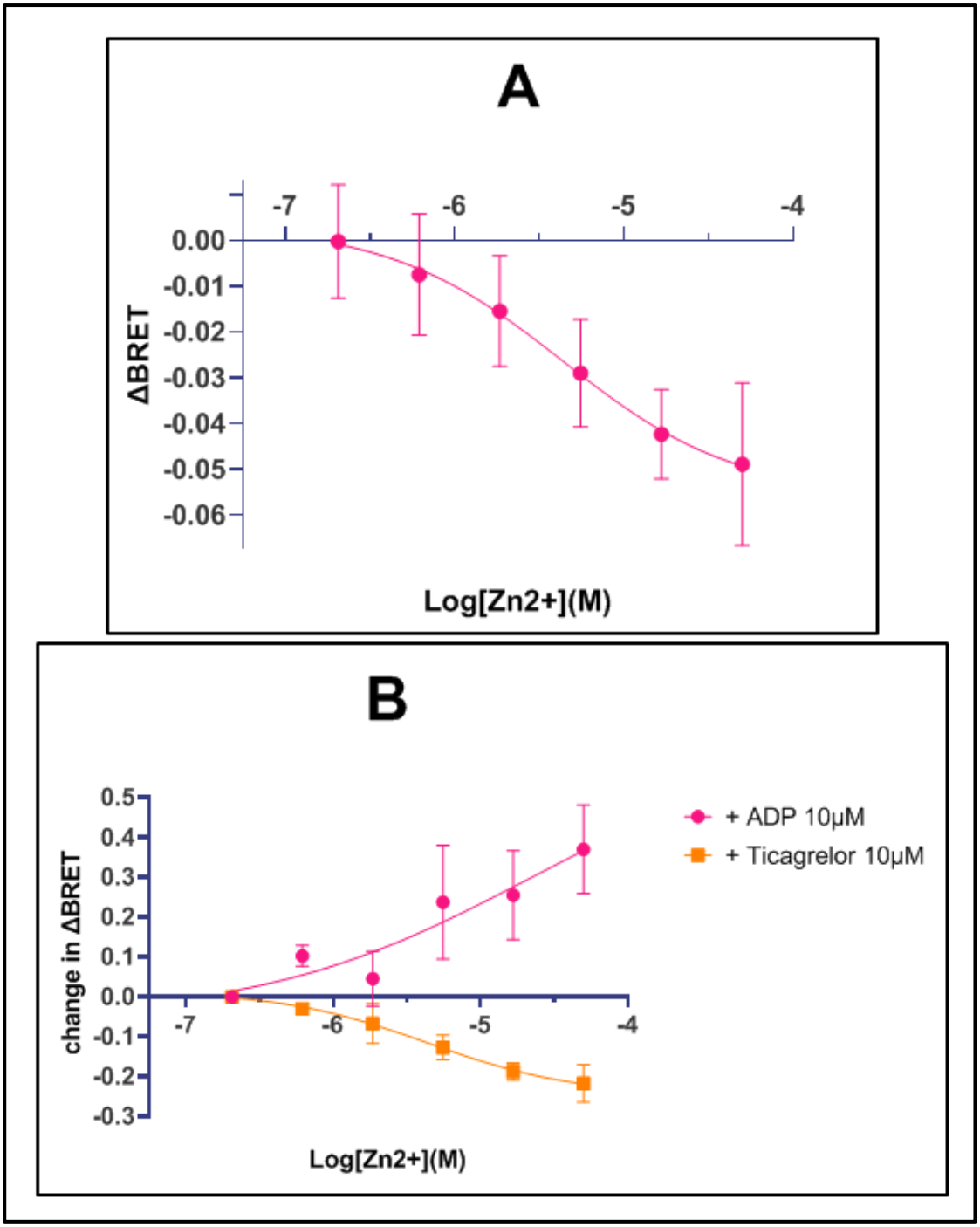


Figure3.2.2. Investigation of the effect of Zn^{2+} alone on the $P2Y_{12}$ receptor (A) and on the ADP 10 μM and ticagrelor 10μM-induced stimulation of the $P2Y_{12}$ receptor (B) in HEK293T cells. HEK293T cells transiently transfected with $P2Y_{12}$ receptor and necessary G proteins plasmids were acutely treated (5 minutes incubation) with increasing concentrations of Zn^{2+} to assess the effect of Zn^{2+} on the $P2Y_{12}$ receptor. The experiment was repeated in presence of ADP 10 μM and ticagrelor 10 μM to observe the effect of Zn^{2+} on agonist-induced stimulation. Data (n=4, represented ± S.E.M) are presented as concentration-response curves showing receptor activation (relative to baseline recording) produced by concentrations of Zn^{2+}. Panel B depicts the difference produced by increasing concentrations of Zn^{2+} on ADP 10 μM and ticagrelor 10 μM activity at the $P2Y_{12}$ receptor, relative to the effect recorded for ADP 10 μM and ticagrelor 10 μM alone at the receptor.

Given our results with the $P2Y_{12}$ receptor, we next examined whether the described effects of Zn^{2+} ions were receptor specific. Therefore, Zn^{2+} ions effects were also evaluated in HEK293T cells transiently transfected with another G_i-coupled GPCR, the μ-opioid receptor (MOR), $G_{\alpha i}$-Rluc, G_β and G_γ-GFP proteins (see Figure 3.2.3), and with pcDNA3.1 vector control only. Zn^{2+} had no apparent effect on pcDNA3.1 only-transfected cells. As with the $P2Y_{12}$ receptor, Zn^{2+} was found to increase G protein-subunit disassociation potentially by affecting the basal activity of the μ-opioid receptor. In addition, agonist-stimulated μ-opioid receptor activation with the μ-opioid receptor agonist DAMGO (10 μM) was also enhanced.

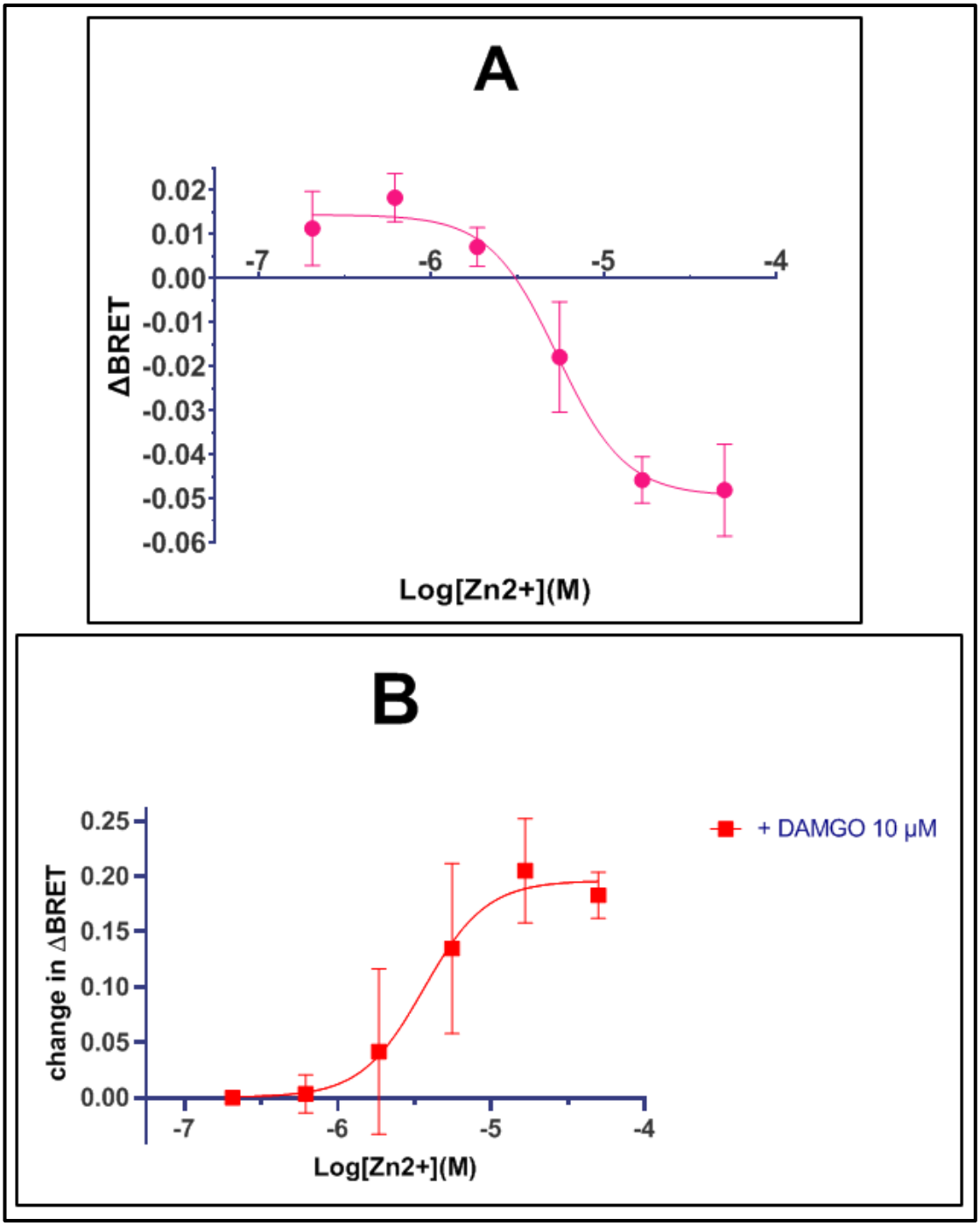


Figure.3.2.3. Investigation of the effect of Zn^{2+} on the µ-opioid receptor (A) and on the DAMGO 10 µM-induced stimulation of the µ-opioid receptor (B) in HEK293T cells. HEK293T cells transiently transfected with the µ-opioid receptor and necessary G proteins plasmids were acutely treated (5 minutes incubation) with increasing concentrations of Zn^{2+} to assess the effect of Zn^{2+} on the µ-opioid receptor. Next, the experiment was repeated in presence of DAMGO 10 µM to observe the effect of Zn^{2+} on agonist-induced stimulation. Data (n=4, represented ± S.E.M) are presented as concentration-response curves showing receptor activation (relative to baseline recording) induced by each concentration of Zn^{2+}. Panel B depicts the difference produced by increasing concentrations of Zn^{2+} on DAMGO 10 µM activity at the µ-opioid receptor, relative to the effect recorded for DAMGO 10 µM alone at the receptor.

Given these results, we next assessed the effect of Zn^{2+} on G protein activation in absence of a GPCR (see Figure 3.2.4). In this case, Zn^{2+} produced an increase in G protein activation. Therefore, rather than having a direct effect on the $P2Y_{12}$ receptor, Zn^{2+} appeared to be modulating G protein activation.

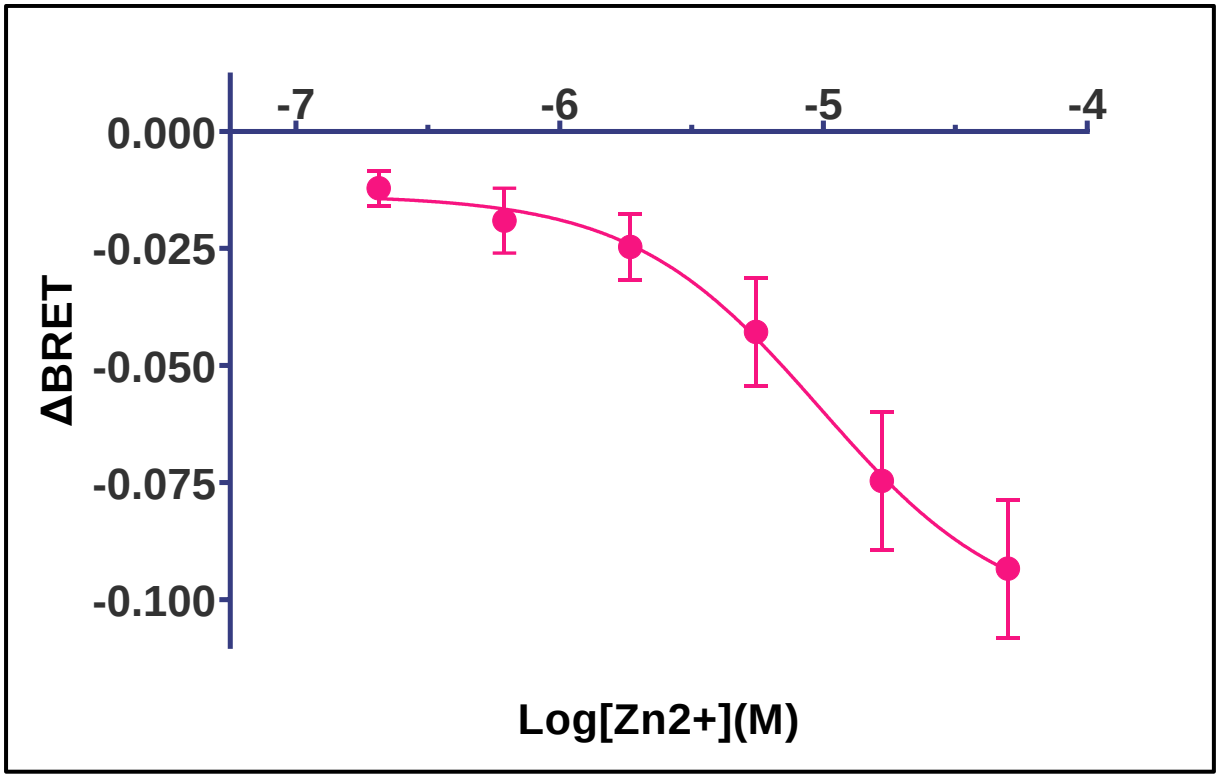


Figure3.2.4. Concentration-dependent Zn^{2+}-induced G protein activation in HEK293T cells. HEK293T cells transiently transfected with pcDNA3.1 viral vector control and necessary G proteins were acutely (5 minutes) incubated with increasing concentrations of Zn^{2+} to determine its effect on G protein activation. Data (n=4, represented ± S.E.M) are presented as a concentration-response curve showing G protein activation (relative to baseline recording) induced by each concentration of Zn^{2+}.

3.3. Study on the interaction between lipid rafts/BST-2 and the $P2Y_{12}$ receptor.

There has recently been an extensive focus on the interaction between cholesterol and G protein coupled receptors, as shown in computational studies by various research groups (*Kiriakidi et al., 2019; Sengupta & Chattopadhyay, 2015*). This interaction has also been studied at the $P2Y_{12}$ receptor. Several studies demonstrated the preferential localization of the $P2Y_{12}$ receptor in lipid rafts, the cholesterol-rich microdomains of the plasma membrane that work as a platform for inter-receptor signalling in platelets, and that such microdomains are required for proper function of the $P2Y_{12}$ receptor. One study by *Quinton et al.*, published in 2005, showed that lipid rafts are required for normal $P2Y_{12}$-$G_{\alpha i}$ activation following ADP-mediated platelet activation. A second study, published by *Savi et al.* in 2006, explained how clopidogrel, the gold standard of anti-platelet therapy before the advent of ticagrelor, exerts its role at the $P2Y_{12}$ receptor by disrupting $P2Y_{12}$ receptor oligomers present in lipid rafts.

In this study, the cholesterol chelator Methyl-β-cyclodextrin (MβCD) was used to assess the importance of cholesterol. Methyl-β-cyclodextrin (MβCD) is a water-soluble oligosaccharide which contains a central hydrophobic cavity, able to bind cholesterol strictly at membrane surface level in a 2:1 MβCD: cholesterol ratio for acute depletion (*Nishijo et al., 2003*). Following literature review, MβCD was used universally at a concentration of 10 mM (*Borisova et al., 2011*; *Bali et al., 2004*; *Grgurevich et al., 2003*).

The effect of MβCD treatment on ticagrelor, ADP and 2MeSADP activation (see Figure 3.3.1) of the $P2Y_{12}$ receptor were respectively studied in both HEK293T cells and CHO cells. In both cell lines, methyl-β cyclodextrin produced a non-significant decrease in agonist-dependent stimulation of the receptor. This was less noticeable for 2MeSADP. Intriguingly, there was a significant decrease in ticagrelor-dependent signalling at the receptor (see Figure 3.3.1). Despite the lack of statistical significance in the study of agonist stimulation, this study again suggests that the presence of cholesterol in the $P2Y_{12}$ receptor microenvironment is important for receptor function.

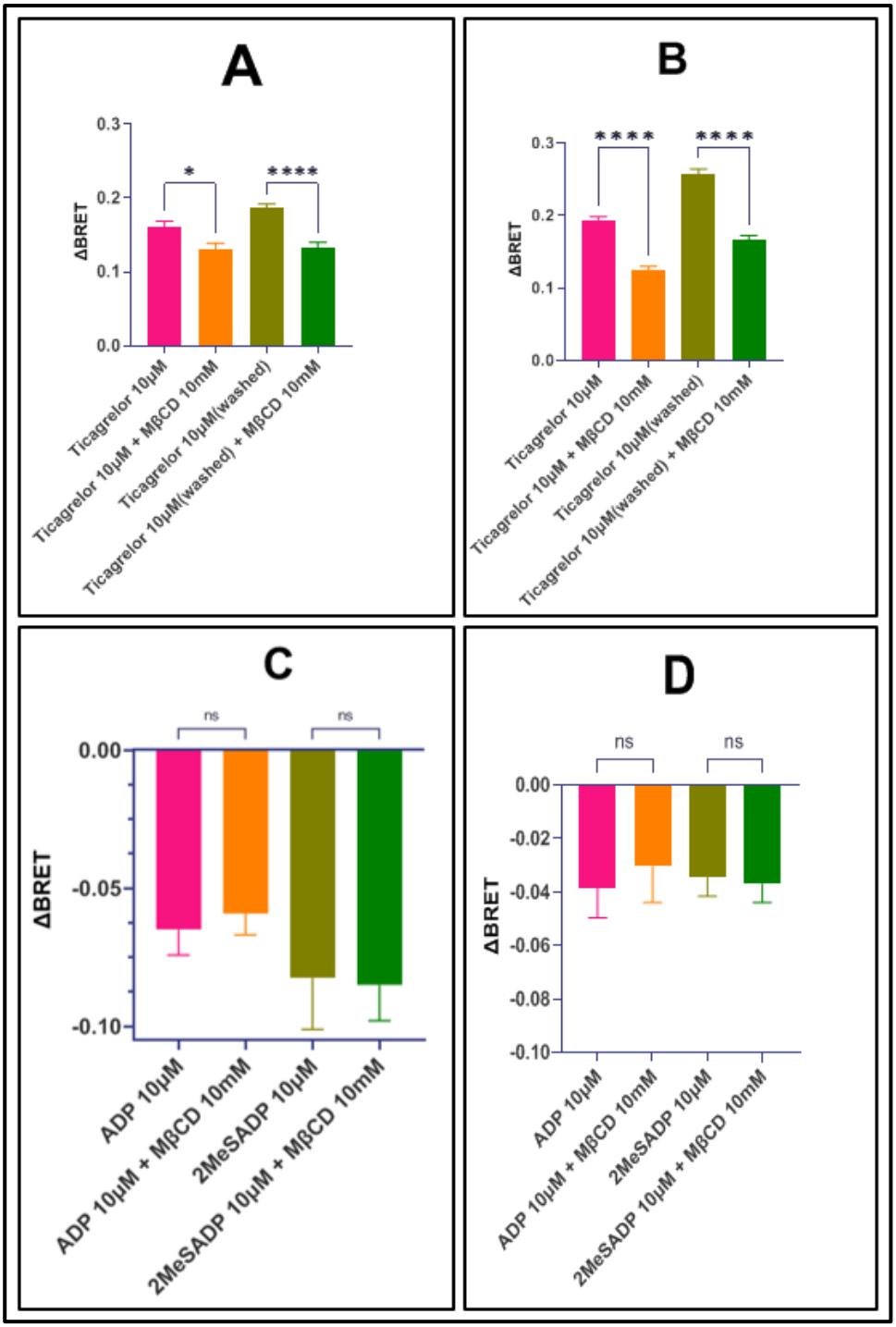


Figure3.3.1. Investigation of the effect of Methyl-β cyclodextrin (MβCD) on ticagrelor, ADP and 2MeSADP-mediated signalling at the $P2Y_{12}$ receptor in HEK293T cells (A, C) and CHO cells (B, D). HEK293T (A, C) and CHO (B, D) cells transiently transfected with the $P2Y_{12}$ receptor and necessary G proteins plasmids were incubated with Methyl-β cyclodextrin 10 mM for 30 minutes. Cells were next washed and prepared for experimentation as described above. Prior to BRET recordings, untreated cells were also acutely treated with ticagrelor 10μM, ADP 10 μM or 2MeSADP 10 μM and the effect of MβCD treatment on $P2Y_{12}$ receptor activation was registered. Data (n=3, represented ± S.E.M) are represented as the difference in receptor stimulation produced by ticagrelor, ADP or 2MeSADP alone and in presence of MβCD treatment relative to recorded baseline receptor activity.

The second part of this section focuses on tetherin, also known as Bone Marrow Stromal Antigen-2(BST-2). Tetherin is an integral membrane protein which was shown to regulate membrane microdomain organization (*Billcliff et al., 2013*). Therefore, the presence of tetherin in the $P2Y_{12}$ microenvironment may be important for the activity of the $P2Y_{12}$ receptor. A recent study from our research group on the effect of tetherin on the $P2Y_{12}$ receptor shows how tetherin plays a key role as a negative regulator of $P2Y_{12}$ receptor activity. Tetherin was shown to regulate $P2Y_{12}$ receptor trafficking and lateral movement within lipid rafts (*Zhao et al., 2021*). In the present study, the role of tetherin on ADP and ticagrelor-mediated $P2Y_{12}$ receptor stimulation was assessed (see Figure 3.3.2). In doing so, tetherin-lacking HEK293T cells were transiently transfected with 1 µg of tetherin plasmid, alongside the usual $P2Y_{12}$ receptor-G proteins complex.

The presence of transiently transfected tetherin/BST-2 increased ticagrelor-mediated signalling (see Figure 3.3.2, Panel B). The EC_{50} values for ticagrelor were $2.76x10^{-7}$ M [95% CI: $9.05x10^{-8}$-n/a] in absence of tetherin/BST-2 and $5.41x10^{-8}$ M [95% CI:$7.68x10^{-9}$-$4.81x10^{-7}$] in presence of tetherin/BST-2. Tetherin also decreased ADP-mediated activation of the $P2Y_{12}$ receptor (see Figure 3.3.2, Panel A). The EC_{50} values for ADP were $9.59x10^{-8}$ M [95% CI: $2.49x10^{-8}$-$6.03x10^{-7}$] in absence of tetherin/BST-2 and $2.28x10^{-7}$ M [95% CI: $9.89x10^{-8}$-$9.57x10^{-7}$] in presence of tetherin/BST-2. The changes produced by tetherin/BST-2 were concentration-dependent.

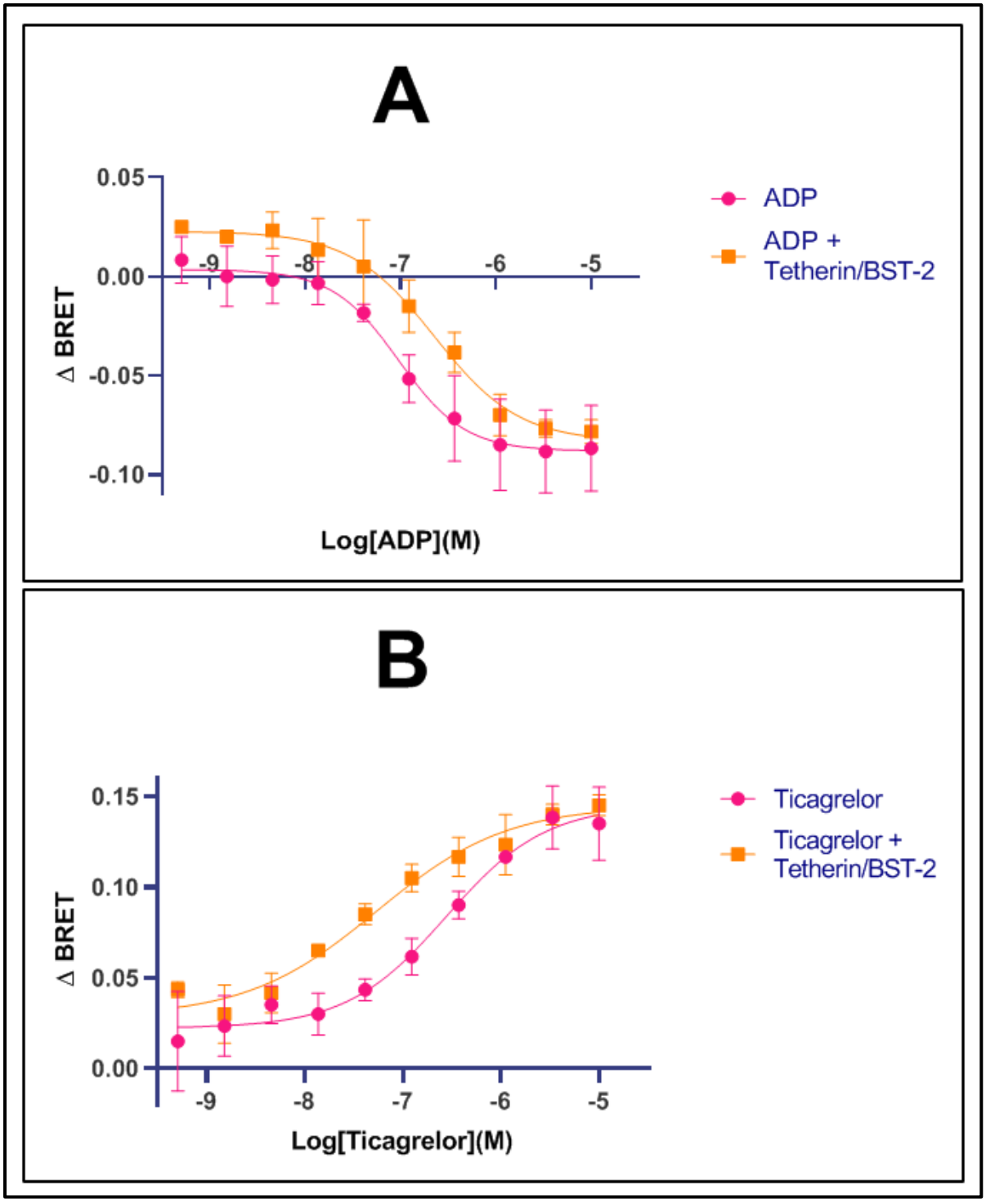


Figure3.3.2. Investigation of the effect of transiently transfected tetherin/BST-2 on ticagrelor and ADP-mediated $P2Y_{12}$ receptor signalling in HEK293T cells. HEK293T cells, transiently transfected with the $P2Y_{12}$ receptor, necessary G proteins and 1 µg tetherin/BST-2 plasmids, were acutely (incubation time of 5 minutes) treated with 10 3-fold serially diluted doses of ADP (A) and ticagrelor (B), with a highest concentration of 10 µM. Data (n=3, represented ± S.E.M) are represented as the difference in concentration-response curves for ticagrelor or ADP in presence and absence of tetherin/BST-2. The concentration-response curves were calculated relative to recorded baseline receptor activity in absence of tetherin/BST-2.

3.4. Study on the irreversibility by washout of ticagrelor-mediated signalling at the $P2Y_{12}$ receptor.

Ticagrelor was introduced in 2013 as a reversible antagonist at the $P2Y_{12}$ receptor. More recently, it was reported to show incomplete reversibility effects in patients in need of immediate surgical intervention (*Gerrits et al., 2017*; *Buchanan et al., 2015*). Recent unpublished observations from the Mundell laboratory also have observed that the effects of ticagrelor were resistant to washout. Initial experiments aimed to recapitulate those observations in both HEK293T and CHO cells and human platelets (see Figure 3.4.1).

In HEK293T cells (see Figure 3.4.1, Panel A) receptor transfected cells were incubated with ticagrelor (0.4 and 10 µM; 30 minutes) or ADP (10 µM; 5 minutes). Cells were spun down and then resuspended in fresh media for 10 minutes, three times, before experimentation. These were compared with cells acutely treated with ticagrelor (0.4 or 10 µM) or ADP (both 10 µM). As previously found in the Mundell laboratory, this extensive washing effectively attenuated ADP-stimulated $P2Y_{12}$ receptor activation. Ticagrelor-stimulated activity was not reduced by extensive washing. Similar experiments were also undertaken in CHO cells (see Figure 3.4.1, Panel B) following the exact protocol outlined above. Again, in these cells, extensive washing did not reduce ticagrelor-stimulated $P2Y_{12}$ receptor activity.

Studies in human platelets were also undertaken in collaboration with Dr. Jawad Khalil in the Mundell laboratory (see Figure 4.1 in *Chapter IV: Discussion*). These data demonstrate that ticagrelor appears resistant to removal by washing.

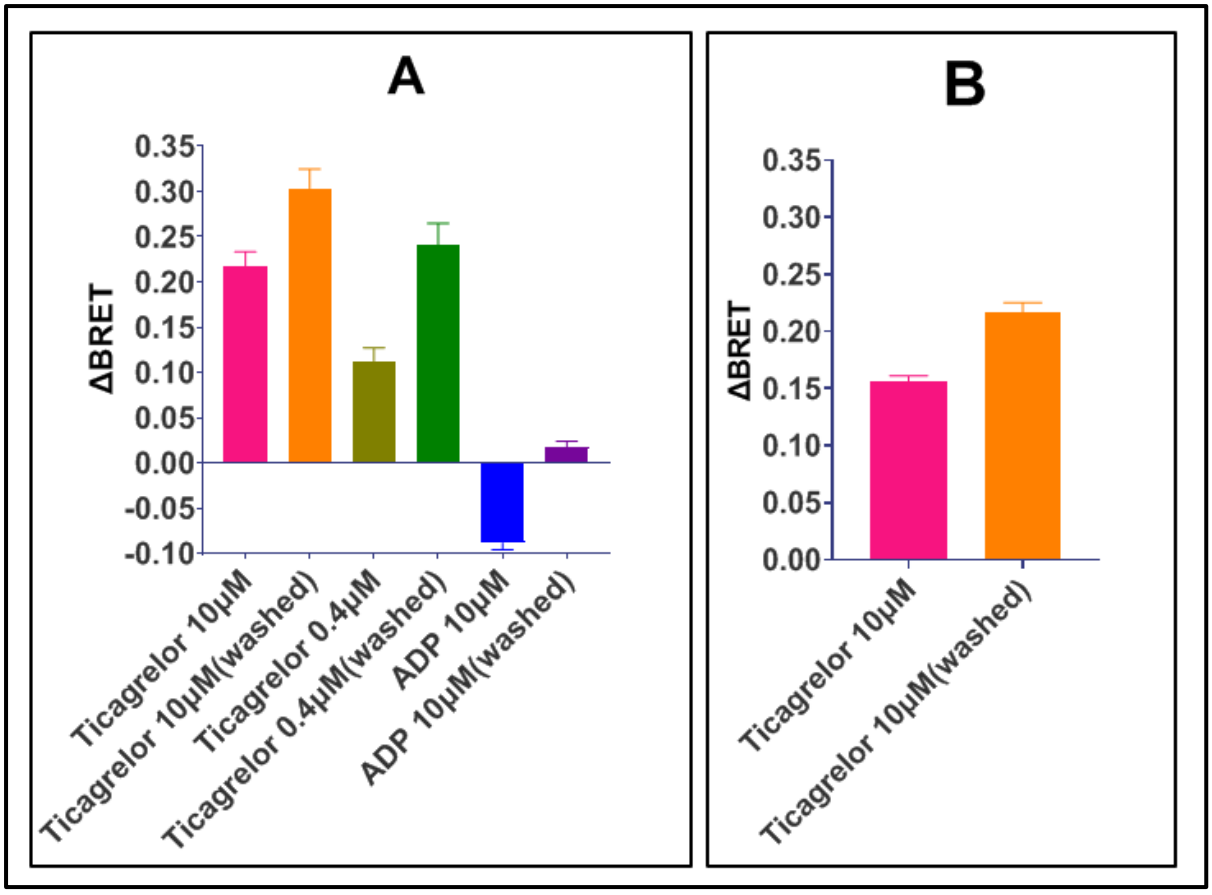


Figure3.4.1. Study on the reversibility of ticagrelor at the $P2Y_{12}$ receptor in HEK293T cells (A) and CHO cells (B) after washout. Panel A: Transiently transfected HEK293T cells with $P2Y_{12}$ receptor and G proteins plasmids were incubated with ticagrelor 10 µM and ticagrelor 0.4 µM for 30 minutes and ADP 10 µM for 5 minutes, respectively, before washout (representing *ticagrelor (washed)* and *ADP (washed)* datasets). Cells were next centrifuged for 2 minutes at 1000 RPM and the resulting supernatant discarded, followed by resuspension in fresh experimental media and incubated at room temperature for 10 minutes to equilibrate. The process was repeated three times before cells were finally plated for experimentation. Untreated cells were also acutely incubated with either ticagrelor 10 µM or ADP 10 µM before BRET recordings were taken (representing *ticagrelor* and *ADP* datasets). Panel B: CHO cells, transiently transfected with $P2Y_{12}$ receptor and G proteins plasmids were incubated with ticagrelor 10 µM for 30 minutes, before washout (representing *ticagrelor(washed)* dataset). Cells were next prepared for experimentation as described for HEK293T cells in Panel A. Acute (5 minutes) incubation with ticagrelor 10 µM for untreated cells was used for *ticagrelor* dataset.

2MeSADP, a synthetic agonist at the $P2Y_{12}$ receptor, has been proposed to bind to a similar site to ticagrelor. 2MeSADP was developed in response to the unstable nature of ADP in experimental assays and was subsequently described to possess superior efficacy at the $P2Y_{12}$ receptor, as well as a distinct binding site to ADP. Furthermore, competition radioligand binding assays at the $P2Y_{12}$ receptor using the antagonist ticagrelor, 2MeSADP and ADP discovered that ticagrelor displays classical competitive antagonism versus 2MeSADP, whilst showing apparent non-competitive antagonism versus ADP (*van Giezen et al., 2009*). This may be suggestive of a shared binding pocket between ticagrelor and 2MesADP at the $P2Y_{12}$ receptor. Interestingly, 2MeSADP, like ticagrelor, also appears resistant to washout (Figures 3.4.2 and 3.4.3). This effect appeared to be concentration dependent. At low agonist concentrations, 70-85% 2MeSADP activity was lost by washing. At higher concentrations, in the μM range, 2MeSADP remained relatively resistant to washout. The shared resistance to washout by ticagrelor and 2MeSADP is significant to the question posed in the following section.

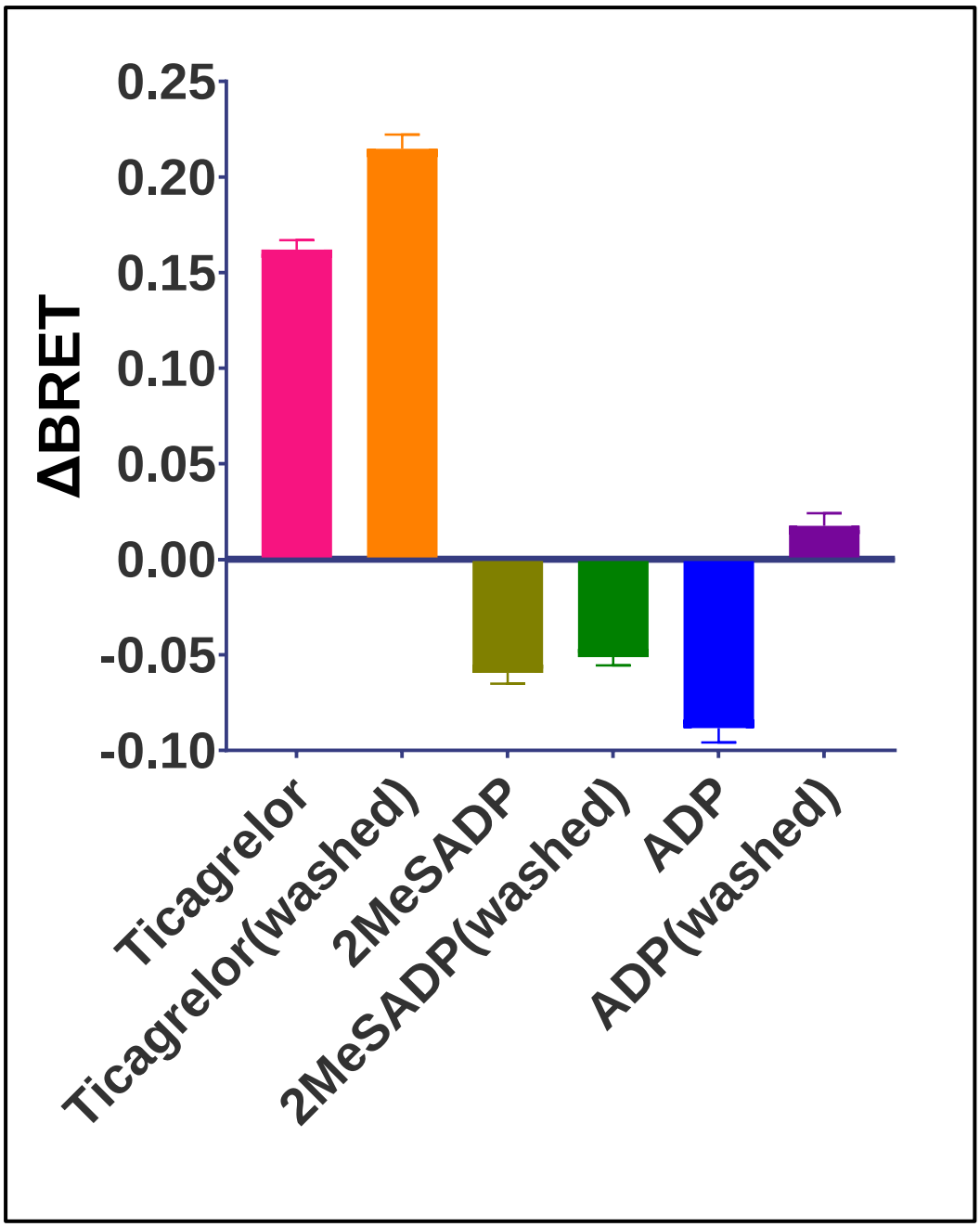


Figure3.4.2. Study on 2MeSADP reversibility relative to ticagrelor and ADP reversibility at the $P2Y_{12}$ receptor in HEK293T cells after washout. Transiently transfected HEK293T cells with $P2Y_{12}$ receptor and G proteins plasmids were incubated with Ticagrelor 10 µM for 30 minutes, 2MeSADP 10 µM and ADP 10 µM for 5 minutes, respectively, before washout (representing *ticagrelor(washed), 2MeSADP(washed)* and *ADP (washed)* datasets). Cells were next centrifuged for 2 minutes at 1000 rpm and the resulting supernatant discarded, followed by resuspension in fresh experimental media and incubated at room temperature for 10 minutes to equilibrate. The process was repeated three times before cells were finally plated for experimentation. Untreated cells were also acutely incubated with either ticagrelor 10 µM, 2MeSADP 10 µM or ADP 10 µM before BRET recordings were taken (representing *ticagrelor, 2MeSADP* and *ADP* datasets). Data (n=3, represented ± S.E.M) are depicted as the difference in receptor stimulation produced by ticagrelor, ADP or 2MeSADP post-acute incubation and post-washout relative to recorded baseline receptor activity.

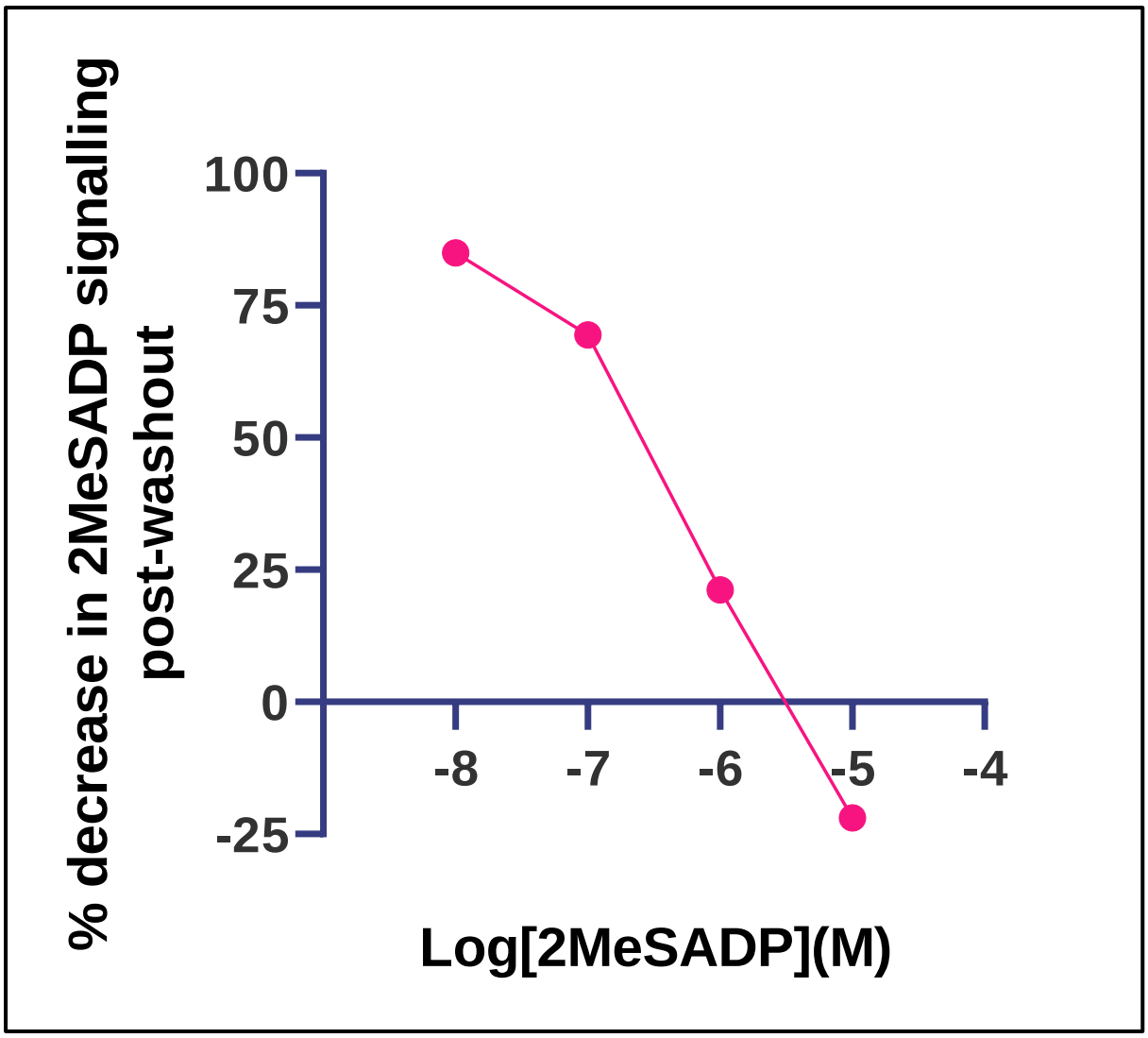


Figure3.4.3. Study on the concentration-dependent reversibility of 2MeSADP by washout at the $P2Y_{12}$ receptor in HEK293T cells. Transiently transfected HEK293T cells with $P2Y_{12}$ receptor and G protein plasmids were incubated with four concentrations of 2MeSADP (10 µM, 1 µM, 100 nM and 10 nM) for 5 minutes before cells were prepared for experimentation as described previously. Acute (5 minutes) incubation with the same four concentrations of 2MeSADP was used for the purpose of experimentation and the $P2Y_{12}$ receptor activation by 2MeSADP was registered. Data (n=3, represented ± S.E.M) are depicted as the ratio between receptor activation produced by four concentrations of 2MeSADP post washout and receptor activation produced by the same four concentrations of 2MeSADP following acute incubation at the receptor, relative to recorded baseline activity of the receptor.

3.5. Why is ticagrelor resistant to washout at the $P2Y_{12}$ receptor?

Both ticagrelor and 2MeSADP appeared resistant to washout. We next aimed to find an explanation as to why ticagrelor showed such washout resistance. Three theories were explored:

1. Since cholesterol and membrane microdomains regulate $P2Y_{12}$ receptor signalling, could they also play a role in preventing ticagrelor washout at the receptor?

2. The $P2Y_{12}$ receptor can be phosphorylated and regulated by protein kinase A (*manuscript in preparation by Dr. Jawad Khalil*). Ticagrelor has also been demonstrated to increase protein kinase A activity in cells. Could, therefore, PKA activation after ticagrelor addition modify the $P2Y_{12}$ receptor to increase ticagrelor binding and resistance to washout?

3. 2MesADP and ticagrelor are hypothesized to bind to a similar part of the binding pocket and both are resistant to washout. Could therefore specific residues within the ligand binding pocket of ticagrelor at the $P2Y_{12}$ receptor play a role in ticagrelor washout resistance?

3.5.1. Removal of cholesterol from the $P2Y_{12}$ receptor microenvironment does not reverse ticagrelor activity at the $P2Y_{12}$ receptor.

As shown above, cholesterol enriched microdomains play a key role in regulating $P2Y_{12}$ receptor activity. To assess their potential role in ticagrelor washout resistance, cholesterol was again depleted using methyl-β-cyclodextrin (MβCD) in both HEK293T and CHO cells overexpressing the $P2Y_{12}$ receptor. The effect of cholesterol depletion on ticagrelor-mediated signalling and ticagrelor washout are depicted in Figure 4.5.1.

As expected, treatment with methyl-β cyclodextrin produced significant decreases in ticagrelor signalling at the $P2Y_{12}$ receptor in both HEK293T and CHO cells. However, in cells treated with MβCD, washout still failed to reverse the activity of ticagrelor at the receptor (see Figure 4.5.1).

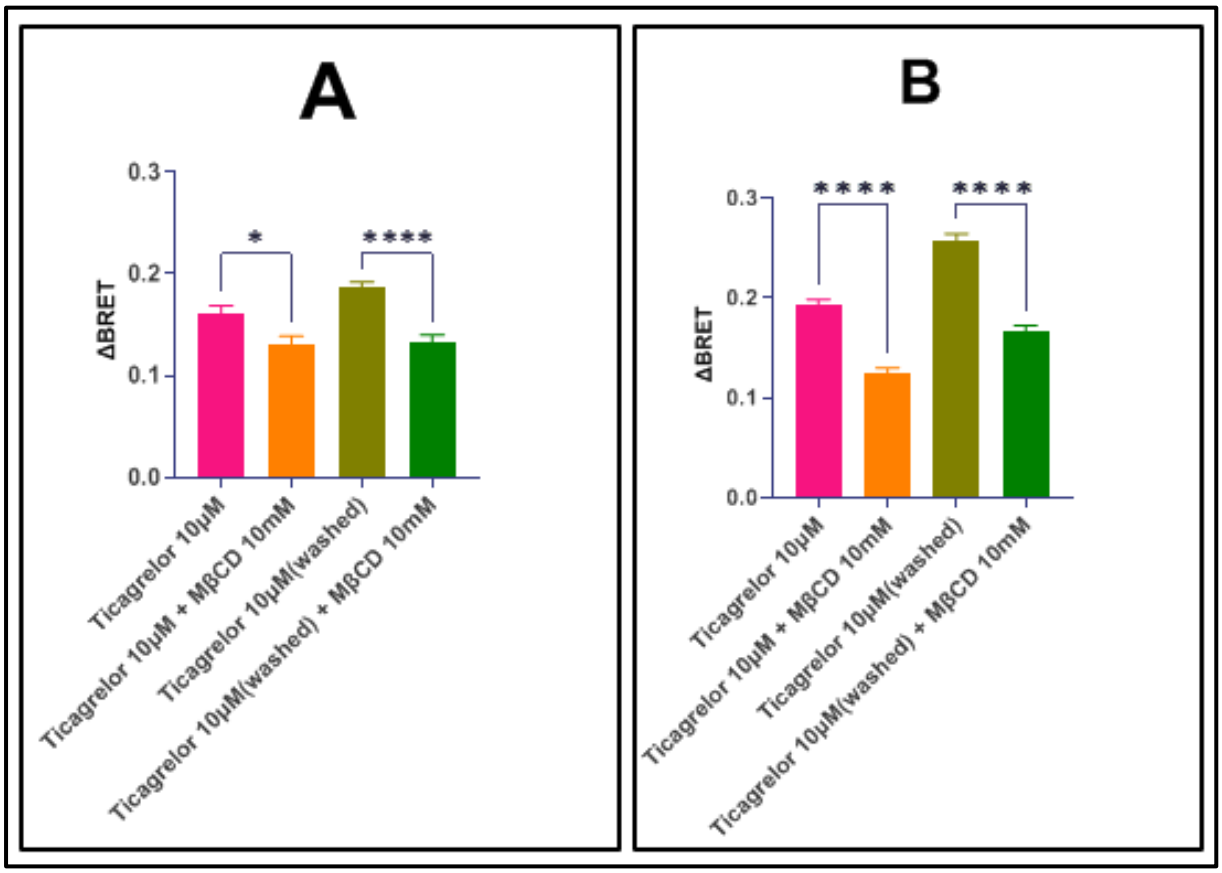
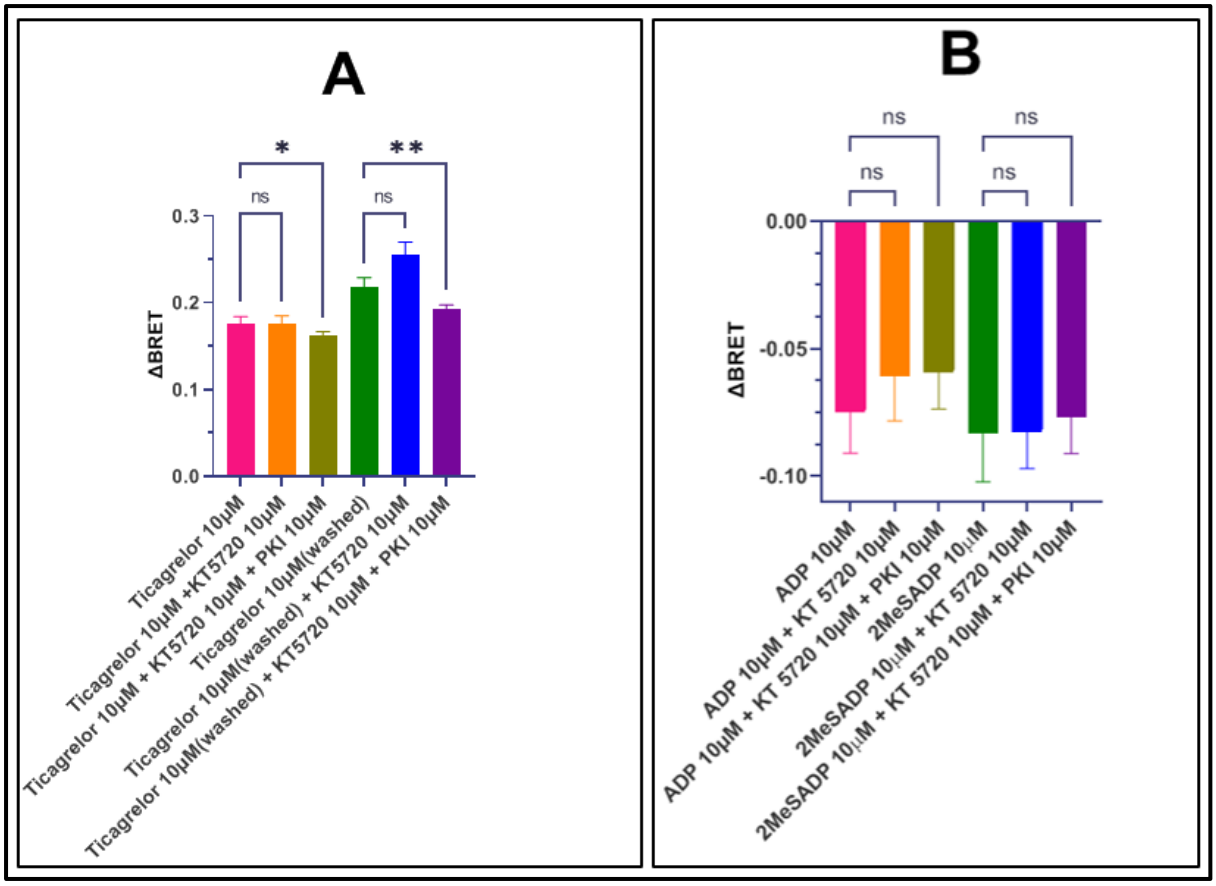


Figure3.5.1. Investigation of the effect of Methyl-β cyclodextrin (MβCD) on ticagrelor-mediated signalling at the $P2Y_{12}$ receptor in HEK293T cells (A) and CHO cells (B). Panel A: HEK293T (A) and CHO (B) cells transiently transfected with the $P2Y_{12}$ receptor and necessary G proteins plasmids were incubated with Methyl-β cyclodextrin 10 mM for 30 minutes prior to incubation with ticagrelor 10 μM for 30 minutes. Next, cells were washed and prepared for experimentation as described above. Acute treatment with ticagrelor 10 μM for untreated cells was also used for the purpose of the experiment, to assess irreversibility of ticagrelor. Data (n=3, represented ± S.E.M) are represented as the difference in receptor stimulation produced by ticagrelor alone and in presence of Methyl-β cyclodextrin relative to recorded baseline receptor activity

3.5.2. PKA-mediated phosphorylation of the $P2Y_{12}$ receptor by ticagrelor may play a potential role in ticagrelor reversibility at the $P2Y_{12}$ receptor.

It was previously shown in our lab (*Aungraheeta et al., 2016*) that ticagrelor leads to an increase in phosphorylated-vasodilator-stimulated phosphoprotein (VASP-P) levels following stimulation of the $P2Y_{12}$ receptor. VASP becomes phosphorylated by protein kinase A (PKA) upon $P2Y_{12}$ receptor activation. Moreover, the same study suggested that ticagrelor inhibits the Equilibrative nucleoside transporter 1 (ENT-1) on platelets and red blood cells, which is responsible for adenosine reuptake. The blockage of ENT-1 subsequently leads to an increase in extracellular adenosine that acts on the adenosine A_{2A} receptor, a G_s protein-coupled receptor which leads to an increase in PKA activity. Therefore, pharmacological inhibition of PKA activity could potentially explain the mechanism behind ticagrelor washout resistance.

To investigate this theory, the non-selective protein kinase inhibitor KT 5720 (10 μM) was incubated alone and in complex with the selective PKA inhibitor PKI (10 μM) for 30 minutes, before incubation with ticagrelor at the $P2Y_{12}$ receptor for 30 minutes, as described above (see Figure 4.5.2, Panel A). The effects of the two inhibitors were also assessed on the agonist-mediated receptor activation (see Figure 4.5.2, Panel B). The PKA inhibitor cocktail KT 5720/PKI produced a significant decrease in ticagrelor signalling as compared to KT 5720 alone treatment. The PKA inhibitors also decreased the activity of ADP and 2MeSADP at the $P2Y_{12}$ receptor, however not significantly.



3.5.2. Investigation of the effect of PKA inhibitors KT 5270 and PKA inhibitor cocktail KT 5270/PKI on ticagrelor-mediated signalling and ticagrelor reversibility at the $P2Y_{12}$ receptor in HEK293T cells (A), as well as on ADP and 2MeSADP-medicated $P2Y_{12}$ receptor activation in HEK293T cells (B). Panel A: HEK293T cells transiently transfected with the $P2Y_{12}$ receptor and necessary G proteins plasmids were incubated with either PKA inhibitor KT 5720 10 µM alone or the PKA inhibitor cocktail KT 5720 10 µM /PKI 10 µM for 30 minutes prior to incubation with ticagrelor 10 µM for 30 minutes. Cells were next washed and prepared for experimentation as described above. Acute treatment with ticagrelor 10 µM for untreated cells was also used for the purpose of the experiment, to assess irreversibility of ticagrelor. Panel B: HEK293T cells transiently transfected with the $P2Y_{12}$ receptor and required G proteins plasmids were incubated with either PKA inhibitor KT 5720 10 µM alone or the PKA inhibitor cocktail KT 5720 10 µM/PKI 10 µM for 30 minutes. Cells were next washed and prepared for experimentation as described above. Prior to BRET recordings, cells were acutely treated with either ADP 10 µM or 2MeSADP 10 µM and the effect of PKA inhibitor treatments on $P2Y_{12}$ receptor activation was registered. Data (n=3, represented ± S.E.M) are depicted as the difference in receptor stimulation produced by ticagrelor, ADP or 2MeSADP alone and in presence of experimental treatments relative to recorded baseline receptor activity.

3.6. Characterization of the effect of point mutations in the structure of the binding pocket of ticagrelor on the inverse agonism of ticagrelor and its resistance to washout at the $P2Y_{12}$ receptor.

Recent studies in the Mundell laboratory have begun to assess how ticagrelor is able to bind to the $P2Y_{12}$ receptor. Unpublished observations using molecular dynamics have revealed a network of potential residues in the orthosteric binding pocket of the $P2Y_{12}$ receptor that may regulate ticagrelor binding and/or activity. Of these, five were mutated to an alanine by Sukhvinder Bancroft: K80A, R93A, C97A, Y105A and N159A. In addition, two point-mutants previously shown to affect $P2Y_{12}$ receptor surface expression and trafficking, R122C (*Patel et al., 2014*) and P341A (*Cunningham et al., 2013*) were also tested. Surface expression of these mutants was previously undertaken in our laboratory (data not shown) showing that four of the five $P2Y_{12}$ receptor mutants, K80A, R93A, Y105A and N159A, express on the surface of HEK293T cells similarly to the wild-type $P2Y_{12}$ receptor, whilst C97A expression is still to be determined. In collaboration with Dr. Jawad Khalil, these mutants were tested. Interestingly, six of the seven mutants used in the experiment, K80A, R93A, C97A, Y105A, R122C and N159A, showed a significant decrease in ticagrelor activity (Figure 3.6.1), whilst P341A showed no effect. Therefore, P341A was disqualified from further study.

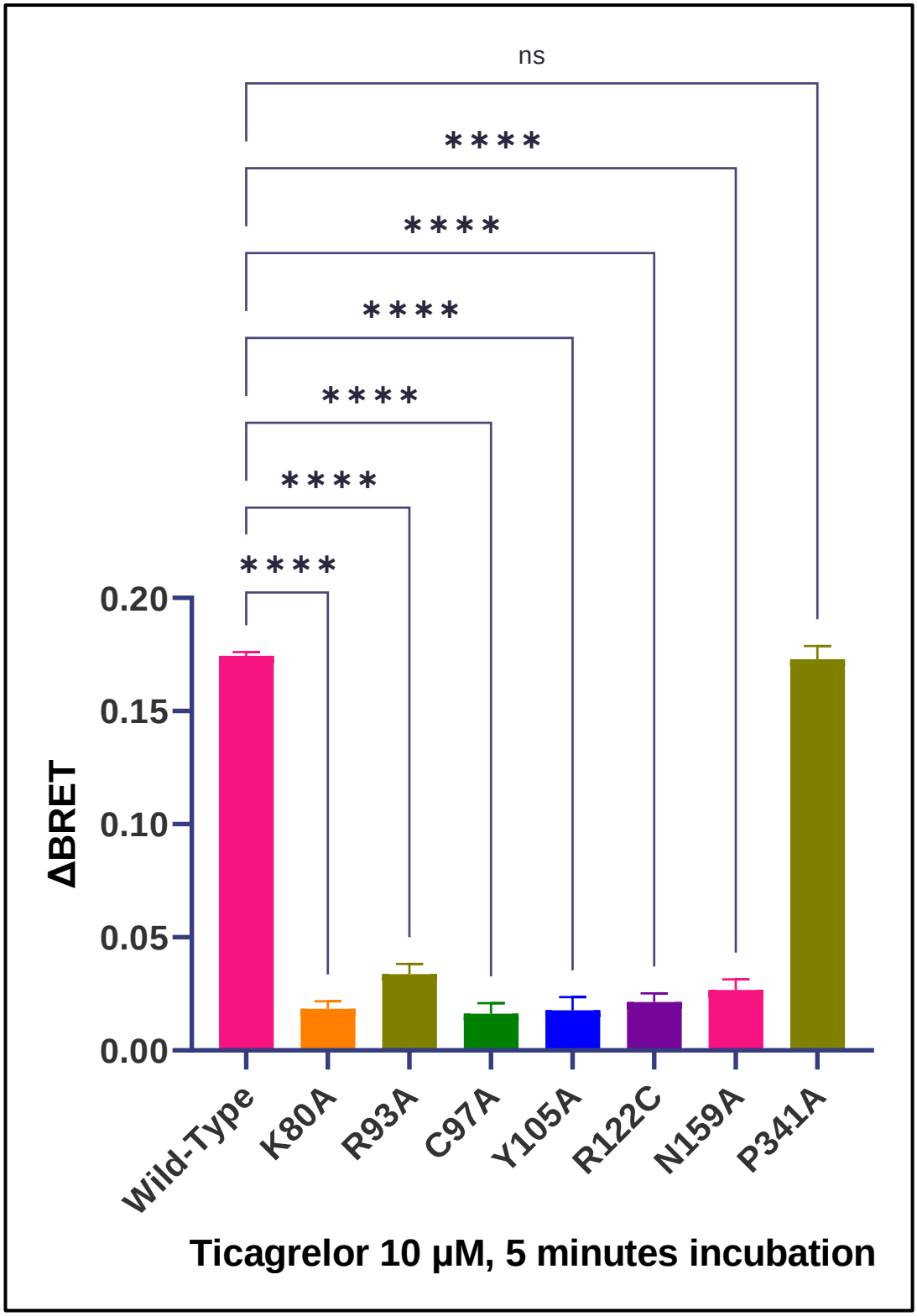


Figure3.6.1. Investigation of the effect of point-mutations in key residues in the binding pocket of ticagrelor on acute ticagrelor signalling at the $P2Y_{12}$ receptor in HEK293T cells. Transiently transfected HEK293T cells with mutant $P2Y_{12}$ receptor and G proteins plasmids were incubated with ticagrelor 10 µM for 5 minutes before BRET recordings were taken (representing *ticagrelor 10 µM, 5 minutes incubation*). Data (n=3, represented ± S.E.M) are depicted as the receptor stimulation produced by ticagrelor for each $P2Y_{12}$ receptor mutant used in the experiment.

Ticagrelor resistance to washout was also tested in each of these mutants (see Figure 3.6.2). Like the wild-type receptor, there was no appreciable decrease in ticagrelor activity at the mutated receptors following washout, however the C97A mutant recorded a significant increase in ticagrelor signalling of approximately 74%.

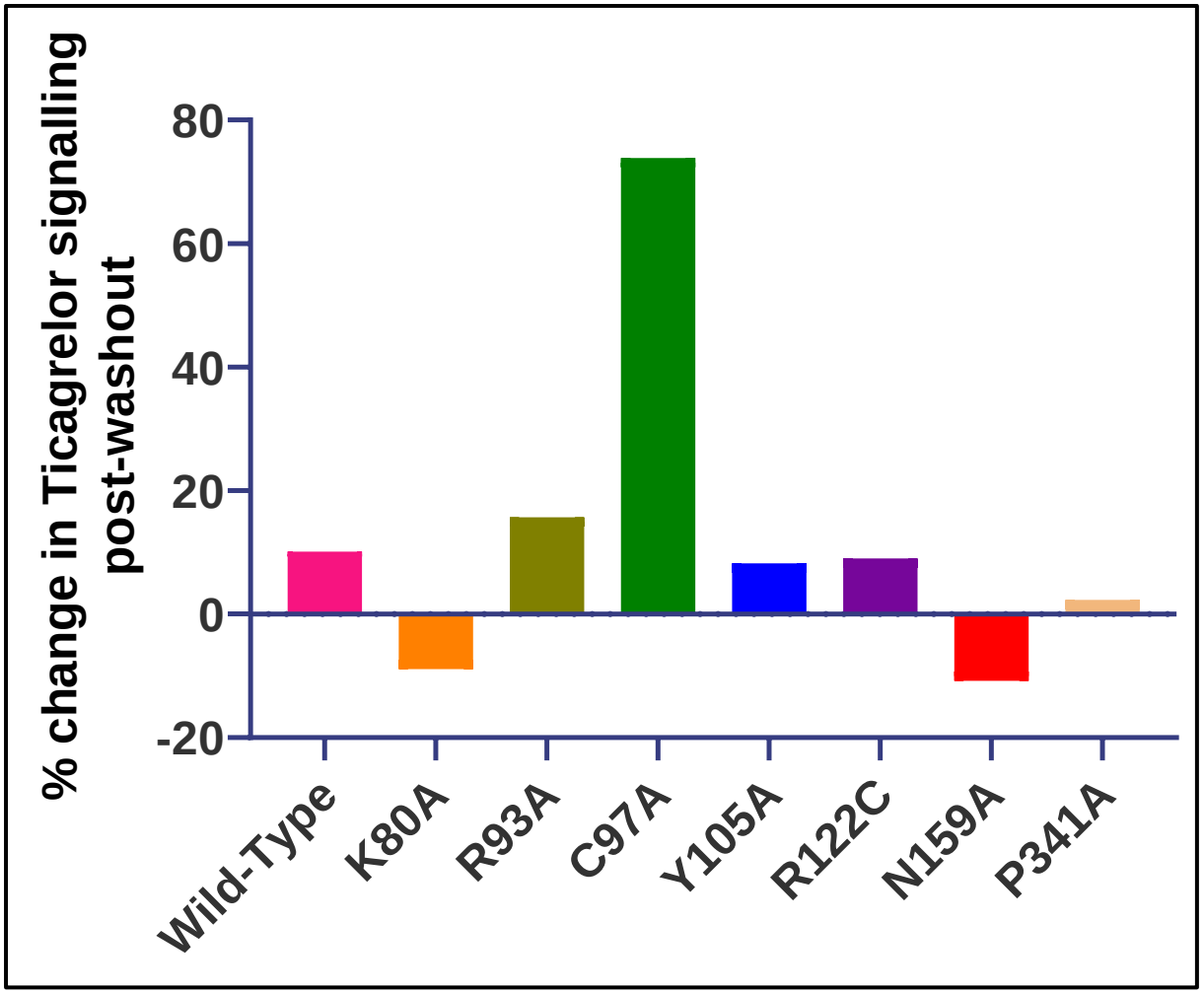


Figure3.6.2. Investigation of the effect of point-mutations in key residues in the binding pocket of ticagrelor on ticagrelor resistance to washout at the $P2Y_{12}$ receptor in HEK293T cells. Transiently transfected HEK293T cells with mutant $P2Y_{12}$ receptor and G proteins plasmids were incubated with ticagrelor 10 µM for 30 minutes before washout. Cells were next centrifuged for 2 minutes at 1000 rpm and the resulting supernatant discarded, followed by resuspension in fresh experimental media and incubated at room temperature for 10 minutes to equilibrate. The process was repeated three times before cells were finally plated for experimentation. Untreated cells were also acutely incubated with ticagrelor 10 µM before BRET recordings were taken. Data (n=3) are depicted as the percentage change in receptor stimulation produced by ticagrelor post-washout for each $P2Y_{12}$ receptor mutant used in the experiment. Positive values suggest an increase in stimulation, whilst negative values suggest a decrease in stimulation following washout.

Next, the effect of the $P2Y_{12}$ receptor mutants on the activity of 2MeSADP alone was assessed (see Figure 3.6.3). In four out of the six mutants, 2MeSADP-mediated receptor activation was significantly increased (K80A, R93A, Y105A and N159A), whilst the remainder showed markedly decreased 2MeSADP activity (C97A) or no significant difference in activity at the receptor (R122C).

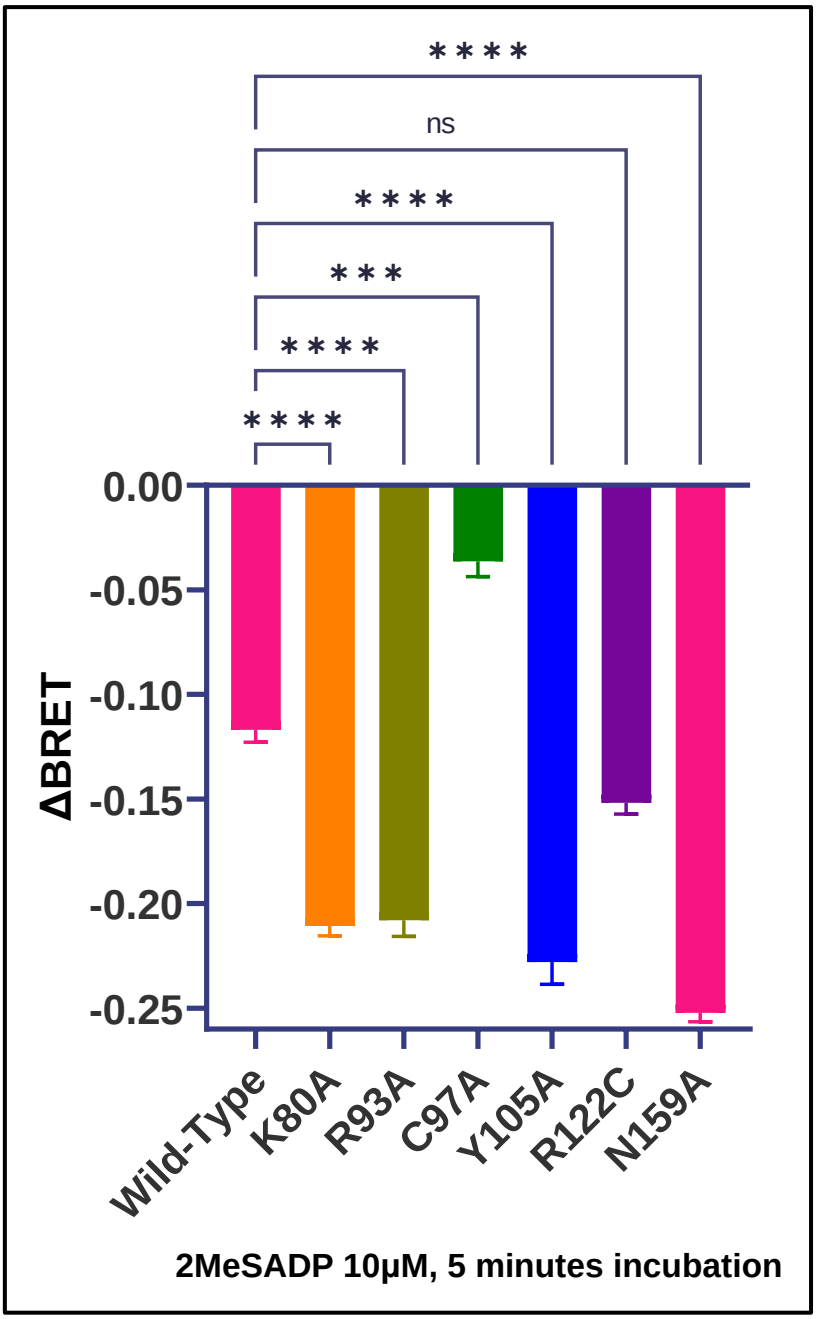


Figure3.6.3. Investigation of the effect of point-mutations in key residues in the binding pocket of ticagrelor on acute 2MeSADP signalling at the $P2Y_{12}$ receptor in HEK293T cells. Transiently transfected HEK293T cells with mutant $P2Y_{12}$ receptor and G proteins plasmids were incubated with 2MeSADP 10 μM for 5 minutes before BRET recordings were taken (representing *2MeSADP 10μM, 5 minutes incubation*). Data (n=3, represented ± S.E.M) are depicted as the receptor stimulation produced by 2MeSADP for each $P2Y_{12}$ receptor mutant used in the experiment.

Ultimately, the ability of 2MeSADP to reverse ticagrelor at each of the mutated receptors was examined (see Figure 3.6.4). Ticagrelor retained its antagonistic properties against 2MeSADP for K80A and R122C, with no significant changes relative to wild-type recordings, despite the highly significant opposing changes in ticagrelor and 2MeSADP activities. Ticagrelor antagonism was significantly reduced for R93A, Y105A and N159A, whilst the activity of 2MeSADP in presence of ticagrelor for C97A was significantly inhibited.

Despite the inability of the studied mutations of residues to affect ticagrelor reversibility, they produced differential effects on ticagrelor and 2MeSADP activity at the $P2Y_{12}$ receptor. These results will be explored in the discussion.

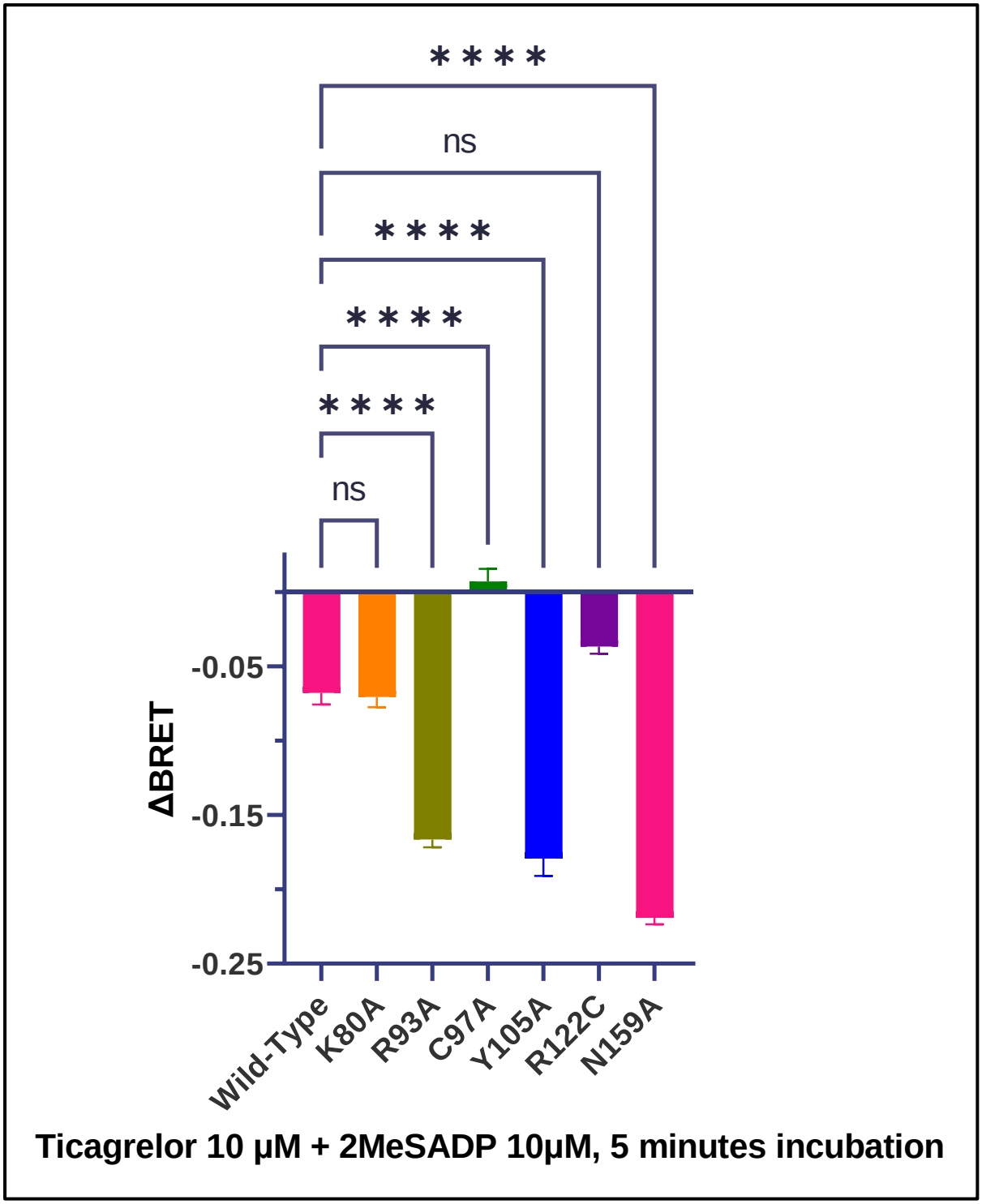


Figure3.6.4. Investigation of the effect of point-mutations in key residues in the binding pocket of ticagrelor on the binding competition between ticagrelor and 2MeSADP at the $P2Y_{12}$ receptor in HEK293T cells. Transiently transfected HEK293T cells with mutant $P2Y_{12}$ receptor and G proteins plasmids were incubated with ticagrelor 10 µM in presence of 2MeSADP 10 µM for 5 minutes before BRET recordings were taken (representing *Ticagrelor 10µM + 2MeSADP 10µM, 5 minutes incubation*). Data (n=3, represented ± S.E.M) are depicted as the receptor stimulation produced by ticagrelor in presence of 2MeSADP for each $P2Y_{12}$ receptor mutant used in the experiment.

Chapter IV: Discussion

Platelets play key roles in blood haemostasis and wound healing. However, in pathophysiological conditions, platelets are promoters of thrombosis, leading to potentially lethal coronary complications such as myocardial infarction. High platelet reactivity is a well-documented trigger for serious thrombotic events such as myocardial infarction in acute coronary syndrome patients (*Adamski et al., 2019*). Platelet activity is tightly regulated by their surface receptors, arguably the most important being the $P2Y_{12}$ receptor.

The $P2Y_{12}$ receptor is a purinergic receptor which determines further platelet responsiveness and aggregation upon activation. To counteract platelet hyperactivation, therapeutic agents such as the $P2Y_{12}$ receptor antagonist ticagrelor were introduced and became the gold standard in acute coronary syndrome patient care. More recently, the pathophysiology of platelets was attributed to abnormal $P2Y_{12}$ receptor activation in absence of bound agonist, termed constitutive activity (*Zhang J et al., 2012*). Further established clinically employed $P2Y_{12}$ receptor blockers such as ticagrelor and cangrelor have been shown to act as inverse agonists at this receptor lowering basal agonist-independent activity (*Garcia et al., 2019*). Therefore, there is a clear need to further understand the molecular regulators of the constitutive activity of the $P2Y_{12}$ receptor in order to greater understand its significance in regulating platelet reactivity. In addition, the establishment of therapeutically important drugs such as ticagrelor as inverse agonists at the $P2Y_{12}$ receptor has potentially significant impacts upon drug development, to minimize unwanted side effects (i.e., patient bleeding), as well as potentially improved efficacy for better clinical outcome.

The landmark discoveries of constitutive activity and inverse agonism by *Costa and Herz* in 1989 brought a new dimension to the pharmacological studies of diseases. Drugs previously considered antagonists were now relabelled as inverse agonists, allowing them to reduce the basal activity of the receptor, as well as block stimulation by endogenous ligands. The constitutive activity of platelet G protein-coupled receptors has recently begun to emerge as a potentially important area in the pathophysiology of thrombosis.

The present study is based upon the premise that the $P2Y_{12}$ receptor shows a high degree of agonist-independent activation due to its highly dynamic extracellular loops (*Zhang J et al., 2014*). This was later confirmed by Aungraheeta *et al.* in 2016 and by Garcia *et al.* in 2019. Aungraheeta and co-authors first described the potential mechanism of action of the antiplatelet drug ticagrelor in human platelets, showing its direct effect on the basal constitutive activity of the $P2Y_{12}$ receptor. Constitutive activity at the $P2Y_{12}$ receptor has been extensively studied in in platelets (*Zhang Y et al., 2012*; *Hu et al., 2017*). The $P2Y_{12}$ receptor plays a key coordinating role in regulating platelet reactivity. Platelets secrete and release the $P2Y_{12}$ receptor agonist ADP from dense granules upon their activation to further potentiate platelet reactivity in paracrine and autocrine manners. Therefore, extensive activation of the $P2Y_{12}$ receptor (high agonist-independent constitutive activity) could lead to hyperactive platelets and potential pathogenesis.

The aim of this study was twofold. First, it was to characterize the potential regulators of constitutive activity at the $P2Y_{12}$ receptor. Furthermore, it aimed to continue to develop our understanding of the pharmacology of ticagrelor. This study was predominantly based in HEK293T cells, with CHO cells and platelets used for comparison. As outlined above, a BRET-based assay was used in HEK293T and CHO cells to measure G protein subunit disassociation downstream of $P2Y_{12}$ receptor activation.

The time-course studies established that maximal responses were achieved after approximately 5 minutes of drug incubation. This time-point was therefore routinely used to measure receptor activation in subsequent studies. As expected, the agonists ADP and 2MeSADP increased receptor activation in a time- and concentration-dependent manner. In HEK293T cells, the calculated EC_{50} values of the ADP and 2MesADP, shown in Table 3.1.1 (EC_{50} for ADP= $3.91x10^{-7}$M [95% CI: $1.94x10^{-7}$-$2.09x10^{-6}$]; EC_{50} for 2MeSADP= $4.59x10^{-10}$M [95% CI: $2.84x10^{-10}$-$7.42x10^{-10}$]) are in concordance with previous literature, and the approximately 1000-fold difference in EC_{50} between the endogenous ADP and the synthetic 2MeSADP is suggestive of the enhanced potency of 2MeSADP at the $P2Y_{12}$ receptor(*van Giezen et al., 2009*; *Takasaki et al., 2001*; *Hollopeter et al., 2001*). In agreement with *Aungraheeta et al.*, *2016,* ticagrelor decreased basal $P2Y_{12}$ agonist-independent activity in a time- and concentration-dependent manner (EC_{50} for ticagrelor in this study= 0.55 µM [95% CI: 0.25-6.81]; EC_{50} for ticagrelor as reported in *Aungraheeta et al., 2016*= 0.27 µM [95% CI: 0.14-0.51]). Intriguingly, in CHO cells versus HEK293T cells, there was a larger ticagrelor response. This was accompanied by a reduced almost flatlined ADP response. These data are suggestive of a higher degree of constitutive activity of the $P2Y_{12}$ receptor in CHO cells or potential presence of a negative modulator of the agonist activity.

Platelet aggregation assays were also performed to confirm the effect of ticagrelor on ADP-induced platelet aggregation, as well as underline the synergistic activity between the $P2Y_{12}$ receptor and other key platelet surface receptors. As expected, ticagrelor was able to inhibit platelet aggregation nearly fully to ADP (*Husted and van Giezen, 2009*). It partially inhibited platelet aggregation to the PAR1/4 receptor agonist TRAP-6, suggestive of the association between $P2Y_{12}$ and PAR4 receptors (*Khan et al., 2014*) and decreased aggregation induced by thromboxane (TP) receptor agonist analogue U46619, suggestive of the association between P2Y12 and TP receptor, extensively studied in our laboratory. Ultimately, there was no clear effect of ticagrelor on CRP-induced platelet aggregation, potentially depicting the lack of interplay between $P2Y_{12}$ and GP VI.

Regulation of $P2Y_{12}$ receptor activity: a potential role for for Zn^{2+}?

The effect of cations, especially Zn^{2+}, has been extensively studied in platelets (*Ahmed et al., 2019*; *Watson et al., 2016*). The main source of Zn^{2+} for the human body comes from the diet, therefore Zn^{2+} deficiency is particularly common in under-developed countries. Zinc deficiency leads to impaired wound healing, bleeding, and platelet dysfunction (*Mammadova-Bach & Braun, 2019*). Recently, Zn^{2+} was shown to act as a positive regulator of the melanocortin-4 receptor activation (*Link et al., 2020*). This G_s coupled receptor displays

highly structural similarities to the $P2Y_{12}$ receptor. Zinc has also been demonstrated to be a potentiator of platelet activation (*Ahmed et al., 2019; Ahmed et al., 2020*). We therefore investigated the possible effect of several cations (Zn^{2+}, Ca^{2+}, Mg^{2+}) on the constitutive activity of the $P2Y_{12}$ receptor. Intriguingly, of these only Zn^{2+} was found to potentiate ADP-stimulated $P2Y_{12}$ receptor reactivity in a concentration-dependent manner. Further studies, however, revealed that Zn^{2+} also enhanced the activity of another G_i protein-coupled receptor, the μ-opioid receptor and, further, in the absence of any receptor, Zn^{2+} promoted G protein activation (i.e., independent of receptor stimulation). In 2005, Gao *et al.* suggested that Zn^{2+} inhibited $G_{\alpha s}$ protein function by reducing the binding of GTP to $G_{\alpha s}$, accompanied by a decrease in the release of GDP from the G protein. This is opposite to what we found in the current study with a potential increase in G_i protein activity due to the presence of Zn^{2+}. Further detailed study examining the effects of Zn^{2+} upon GTP/GDP G protein association is, therefore, warranted.

The current study and that of Gao *et al* brings a potentially interesting insight into the effect of ions on the activity of the $G_{\alpha i}$ and $G_{\alpha s}$. The two opposing effects of Zn^{2+}, potentiating G_i activation, whilst inhibiting $G_{\alpha s}$ function, may be suggestive of complex molecular pharmacology of Zn^{2+}. For example, in platelets, $G_{\alpha s}$ protein-coupled signalling pathways downstream of activation of receptors such as A_{2A} adenosine and IP prostanoid receptor increase protein kinase A activity and reduce platelet activation. The inhibition of this "inhibitory" pathway by Zn^{2+}, alongside the stimulation of the $G_{\alpha i}$ protein-coupled pathway, as suggested by the findings of this section, may shift the balance towards more readily active platelets. Therefore, a study on the effect of Zn^{2+} on $G_{\alpha s}$ proteins/$G_{\alpha s}$ protein-coupled receptors in platelets may be justified. Furthermore, a broader study on the potential overall effects of Zn^{2+} on G protein activity in platelets may also be required.

Regulation of $P2Y_{12}$ receptor activity: a potential role for membrane lipid microdomains?

The membrane lipid microenvironment of the $P2Y_{12}$ receptor has been shown to modulate receptor biology (*Haghighi et al., 2021*; *Quinton et al., 2005*). Lipid microdomains are cholesterol-rich portions of plasma membrane that facilitate the localization of signalling proteins. Disruption of such domains was shown to impair $P2Y_{12}$ receptor-stimulated platelet aggregation (*Quinton et al., 2005*). Furthermore, the mechanism of action of the $P2Y_{12}$ receptor antagonist clopidogrel has partially been shown to be due to $P2Y_{12}$ receptor oligomer disruption and receptor displacement from lipid microdomains (*Savi et al.*, *2006*). The current project further indicates the importance of cholesterol-rich domains in $P2Y_{12}$ receptor signalling. Acute cholesterol depletion through methyl-β-cyclodextrin (MβCD), a cholesterol chelator, produced a modest, although non-significant reduction in ADP-dependent stimulation of the receptor in HEK293T and CHO cells, with no change in 2MeSADP-mediated receptor activation. This difference between the two agonists, with 2MesADP classed as a potential "super agonist", is intriguing and suggests potential different conformational landscapes stabilized by cholesterol/receptor interaction. Intriguingly, in both HEK293T and CHO cells, ticagrelor signalling was significantly reduced by cholesterol depletion through MβCD, with the greater effect seen in CHO cells. The results are in concordance with previous

literature, as they suggest the importance of cholesterol in overall receptor signalling with cholesterol affecting agonist-mediated receptor responses. Potentially, cholesterol depletion also plays an important role in the inverse agonistic nature of ticagrelor at the receptor. The lack of effect of ticagrelor may in part be due to a loss of basal agonist-independent activity although this requires further, detailed investigation.

Recently, the transmembrane protein tetherin/BST-2, a critical regulator of membrane microdomain formation and required for lipid raft integrity (*Billcliff et al., 2013*), was shown to negatively regulate the activity of the $P2Y_{12}$ receptor (*Zhao et al., 2021).* In this study, the presence of tetherin/BST-2 was again shown to negatively regulate ADP-mediated $P2Y_{12}$ receptor activation, confirming the findings of Zhao *et al.* . Furthermore, the presence of tetherin/BST-2 enhanced the inverse agonistic effect of ticagrelor on the receptor.

What is the molecular mechanism behind ticagrelor acting as an "irreversible" $P2Y_{12}$ receptor blocker?

Ticagrelor is a widely prescribed medication in patients with thromboembolic episodes, either as a monotherapy or dual therapy, in combination with aspirin, yet it leads to increased bleeding risk in some patients (*Steg et al., 2019; Cannon et al., 2010*). Although labelled as a reversible antagonist, ticagrelor has been reported to irreversibly inhibit platelet activation post-administration (*Gerrits et al., 2017*). This proves particularly and clinically problematic in patients treated with ticagrelor who need immediate surgery. Therefore, in this study, the potential molecular mechanisms underlying irreversibility of ticagrelor were examined.

This current project further develops the discussion on the irreversible nature of ticagrelor. Initial studies confirmed that, as in platelets, as shown by the study performed in collaboration with Dr. Jawad Khalil presented in Figure4.1, ticagrelor is resistant to washout in both HEK293T and CHO cells. In human platelet samples, ticagrelor continued to antagonise ADP responses even after multiple platelet washes. ARC-66096 antagonism of ADP-stimulated platelet aggregation was reversed following platelet washes. Ticagrelor resistance to washout at the $P2Y_{12}$ receptor was recorded at both high concentration (10 μM), enough to bind 100% of the receptor population, in HEK293T cells, CHO cells and platelets, and the clinically physiological concentration (0.4 μM) in HEK293T cells.

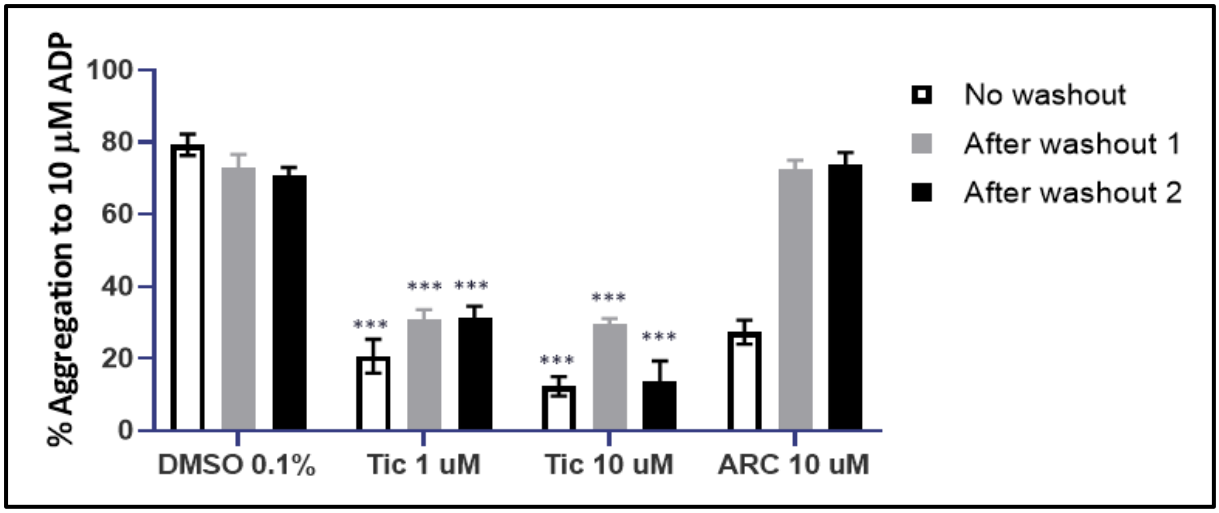


Figure4.1. Study on the reversibility of ticagrelor at the $P2Y_{12}$ receptor in human platelets after washout. Human platelet samples were prepared as described in the *Methodology* section above. The samples were incubated with DMSO 0.1%, ticagrelor 1 µM, ticagrelor 10 µM and ARC 10 µM for 30 minutes, respectively, before washout. The samples were next taken through two washout steps over 60 minutes and platelet aggregation to 10 µM ADP was recorded (*data provided following experimentation in collaboration with Dr. Jawad Khalil*)

Three potential mechanisms behind the irreversibility of ticagrelor were assessed:

1) **Post-translational PKA-mediated modifications, i.e., receptor phosphorylation.**

Ticagrelor has previously been shown to stimulate PKA activity through blockade of the Equilibrative Nucleoside Transporter 1 (ENT1). This, in turn, leads to an accumulation of extracellular adenosine which in turn activates the $G_{\alpha s}$-coupled A_{2A} adenosine receptor. Furthermore, work performed in our laboratory discovered that ticagrelor activity leads to phosphorylation of the $P2Y_{12}$ receptor through a PKA-mediated process and, therefore, ticagrelor reversibility may be due receptor phosphorylation. Unfortunately, pre-treatment with the PKA inhibitors KT 5720 and PKI (*Kase et al., 1987*; *Dalton et al., 2006*) failed to affect ticagrelor irreversibility. Neither KT 5720 alone, nor KT 5720 in combination with PKI could reverse ticagrelor activity at the $P2Y_{12}$ receptor, however the PKA inhibitor KT 5720/PKI mix significantly reduced ticagrelor signalling.

2) **The role of lipid rafts/membrane microdomains**

As outlined above, the cholesterol chelator methyl-β-cyclodextrin reduced ticagrelor activity in HEK293T and CHO cells. However, even after pre-treatment with methyl-β-cyclodextrin, ticagrelor "irreversibility" was maintained. Therefore, the lipid microenvironment does not appear responsible for the irreversible nature of ticagrelor.

3) **Ligand/receptor interaction**

An important finding in this project was that, alongside ticagrelor, 2MeSADP, a potent, synthetic agonist at the $P2Y_{12}$ receptor, was also resistant to wash-out.

Ongoing molecular dynamics studies in our laboratory, in addition to published data (*Zhang J et al., 2014*; *van Giezen et al., 2009*) have shown overlap between the binding pocket of 2MeSADP and ticagrelor at the $P2Y_{12}$ receptor. The discovery of the potentially irreversible nature of 2MeSADP binding at the $P2Y_{12}$ receptor led to the question of whether key interactions in the partially shared binding pockets of ticagrelor and 2MeSADP are responsible for the irreversibility of ticagrelor.

Previous studies (*Haghighi et al., 2021*; *Paoletta et al., 2015)*, as well as computational binding studies performed in our laboratory have identified key residues in the receptor structure which may be required for ticagrelor binding. Modelled alanine scanning mutagenesis showed that mutations in residues such as LYS80.2 (K80), ARG93.3 (R93), CYS97.3 (C97), TYR105.3 (Y105) and ASN159.4 (N159) could influence both ticagrelor binding at the receptor and the constitutive activity of the receptor. Furthermore, mutants of ARG122.3 (R122C), VAL234.6 (V234T) and PRO341.C-term (P341A) have already been documented to affect $P2Y_{12}$ receptor activity (*Patel et al., 2014*; *Cunningham et al., 2017; Cunningham et al., 2013*).

Given time constraints, an initial screen of a series of alanine point mutations revealed that ticagrelor inverse agonism at the receptor is significantly impaired at K80, R93, C97, Y105, R122 and N159. However, in the majority of those $P2Y_{12}$ receptor alanine mutants where ticagrelor activity was reduced, remaining ticagrelor activity was still resistant to washout. Therefore, although R93, Y105 and N159 are key residues in the binding of ticagrelor, they are not individually responsible for washout resistance. Furthermore, one big exception to the ticagrelor washout was the alanine mutation of C97, which showed an increase in ticagrelor signalling following washout, suggesting that C97 may help “destabilise” ticagrelor binding during washout. Therefore, mutations of a combination or all these residues maybe required to facilitate ticagrelor washout.

The activity of 2MeSADP at the receptor was also significantly affected in a number of these mutations with increases with K80A, R93A and Y105A. In contrast, the C97A mutant showed markedly decreased 2MeSADP signalling, making C97 a potential key residue in the binding of 2MeSADP. Although the mutant R122C increased 2MeSADP signalling, this did not reach statistical significance.

Competition studies between ticagrelor and 2MeSADP for each of the mutants were also undertaken. For the alanine mutants R93, Y105 and N159, ticagrelor was completely reversed by the addition of 2MeSADP. In contrast, for C97A, 2MeSADP agonist reversal was lost. The mutants K80A and R122C displayed no significant change relative to wild-type recordings, which may suggest that ticagrelor retains its antagonistic nature, despite losing its inverse agonism at the receptor.

Although these studies do reveal a complex relationship between different residues and ticagrelor binding and activity, there are obvious limitations in the alanine mutation approach and simply looking at ligand-stimulated activity as was used in these studies. To truly

understand ticagrelor binding at the receptor, radioligand receptor binding studies would be preferable. Unfortunately, there is currently no commercially available radioligand at the $P2Y_{12}$ receptor. In the absence of such a ligand, more detailed functional studies examining 2MeSADP reversal of ticagrelor inverse agonism could be performed. Further studies where clusters of residues are mutated would seem prudent. In addition, rather than simple alanine mutations, residues could potentially be mutated to change charge/ligand interaction to explore this complex relationship.

Future studies and implications

Although this body of work has revealed several interesting findings, it has also revealed several future research questions / avenues of research.

In this study, it was revealed that CHO cells, alongside HEK239T cells, could complement our research characterizing constitutive activity of the $P2Y_{12}$ receptor. Importantly, CHO cells endogenously express tetherin/BST-2, but lack another known regulator of $P2Y_{12}$ receptor activity NHERF-1 (*Wheeler et al., 2007*). HEK293T cells, meanwhile, endogenously express NHERF-1 but lack tetherin. Since NHERF-1 plays a major role in $P2Y_{12}$ receptor internalization by complexing with β-arrestin (*Nisar et al., 2012*), a direct comparison between CHO cells and HEK293T cells could provide information on the importance of NHERF-1 in $P2Y_{12}$ receptor signalling. Notably, this study struggled to fully optimize the CHO-$P2Y_{12}$ receptor-$G_{\alpha i}$ proteins system, with an inability to obtain clear, reproducible levels of receptor activity despite potentially better receptor expression relative to the HEK293T cell system (i.e., high level of receptor constitutive activity showed by elevated ticagrelor response and inability of ADP to overcome the constitutive activity and produce a consistent response).

Some of the findings in cell lines presented in this thesis require re-testing in platelets to measure endogenous $P2Y_{12}$ receptor activity and increase the impact of these present studies. Transient transfections in cell lines can lead to overexpression of the receptor-G protein complex, which could provide an overestimation on the impact of experimental findings. Despite the meaningful data that a BRET assay can provide, it can only measure G protein disassociation and, thus, further experimental assays which study the downstream signalling of receptor activation are necessary, e.g., cAMP assays. For example, the studies on the effect of Zn^{2+} on $P2Y_{12}/G_{\alpha i}$ signalling described interesting findings. These studies ought to be extended into platelets to confirm results. Western blotting to assess the effect of Zn^{2+} on the downstream signalling of the $P2Y_{12}$, as well as on ticagrelor signalling, could certainly bring more physiological and pharmacological relevance to of the current the study.

Additional work on the effect of methyl-β-cyclodextrin-mediated acute cholesterol depletion on $P2Y_{12}$ receptor signalling are also necessary. This study lacked experiments observing the effects of repletion of cholesterol following MβCD treatment on $P2Y_{12}$ receptor activity, as well as the effect of MβCD on receptor expression in cell lines. In addition, an investigation on the concentration-dependence of MβCD could also be significant. Ultimately, the effect of

MβCD on ADP or 2MeSADP-mediated platelet aggregation would have been a strong tool for the purpose of the study.

Importantly overall, this current study again demonstrates that ticagrelor/$P2Y_{12}$ receptor activity is resistant to washout. Ticagrelor signalling levels post-washout are similar to ticagrelor signalling at the $P2Y_{12}$ receptor following an equivalent incubation time (1 hour and 30 minutes) (data not shown). This finding, alongside the emergence of an 'antidote' for ticagrelor (*Buchanan et al., 2015, Pehrsson et al., 2017*) are suggestive of the causality between irreversible binding of the ticagrelor molecule at the $P2Y_{12}$ receptor and ticagrelor irreversible effects seen in patients. Therefore, the question of whether ticagrelor brings the receptor to a specific binding conformation, which in turn blocks the exit of ticagrelor from the receptor still needs to be addressed. Ultimately, more detailed molecular dynamics studies could potentially discover the reality behind the irreversible nature of ticagrelor.

Ultimately, further studies on the regulation of the $P2Y_{12}$ receptor constitutive activity are necessary to improve understanding and better the clinical outcome of cardiovascular disease patients. The $P2Y_{12}$ receptor displays a high degree of constitutive activity due to structural particularities which can be attributed to a highly dynamic molecular structure. This allows the receptor to easily and spontaneously shift from an inactive to an active conformation and lead to extensive, unwanted $P2Y_{12}$-mediated platelet activation and aggregation and further pathological consequences. For example, diabetes patients, who are at high risk of atherothrombosis, were shown to have highly constitutively active $P2Y_{12}$ receptors (*Hu et al., 2017*). Such a high degree of constitutive activity of the $P2Y_{12}$ receptor could be reversed by inverse agonists, compounds that can shift the conformation of a receptor towards inactivity. Ticagrelor, one half of the dual antiplatelet therapy deemed the gold standard in treatment of patients with acute coronary syndromes, alongside aspirin, does display inverse agonism at the $P2Y_{12}$ receptor and clinical superior efficacy relative to other $P2Y_{12}$ agents.

However, the side effects of bleeding on ticagrelor administration are significant. Despite being introduced as a reversible anti-platelet therapy agent in 2013, this does not seem to be the case (*Gerrits et al., 2017*). This brings serious issues for patients on ticagrelor treatment who need emergency surgery. Therefore, there is an absolute need to reverse the effects of ticagrelor on platelets and multiple groups have worked towards developing an "antidote" for ticagrelor irreversibility (*Pehrsson et al., 2017; Buchanan et al., 2015*).

Chapter V: Final remarks

Platelets play key roles in blood haemostasis and wound healing. However, in pathophysiological conditions, platelets are promoters of thrombosis and further, potentially lethal coronary complications such as myocardial infarction. Platelet activity is tightly regulated by their surface receptors. The $P2Y_{12}$ receptor is a purinergic receptor which determines platelet responsiveness and aggregation upon activation and is arguably one of the most important receptors on the surface of platelets. Furthermore, abnormal $P2Y_{12}$ receptor activation in absence of bound agonist, i.e., high constitutive activity, has been attributed to potential pathophysiology (*Hu et al., 2017*; *Zhang Y et al., 2012*). This thesis has further enhanced our understanding of the potential regulators of $P2Y_{12}$ receptor activity. There is still an obvious and clear need to describe the molecular regulators of the constitutive

activity of this receptor, which might prove useful in finding new drug targets for regulating platelet reactivity.

To counteract platelet hyperactivation, therapeutic agents such as the $P2Y_{12}$ receptor antagonist ticagrelor were introduced and have become the gold standard in acute coronary syndrome patient care. Although ticagrelor was initially classed as a reversible agent at the $P2Y_{12}$ receptor, studies from this thesis and from others have discovered that the effects of ticagrelor in patients are irreversible throughout the lifespan of platelets. Therefore, ticagrelor patients show a significant bleeding risk and are in danger when in need of emergency surgical interventions.

The question of how ticagrelor reversibility can be addressed is well documented and an antidote was already proposed. Whereas this thesis has started the discussion of why ticagrelor exhibits irreversibility at the $P2Y_{12}$ receptor, further, more detailed studies are required.

Ultimately, this project raises new, interesting questions on drug-receptor and receptor-microenvironment interactions in highly constitutively active receptor systems. The findings can now be extended to new research directions, potentially providing improved clinical outcome in patients with acute coronary syndromes.

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