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Title:
P2Y12 receptor: platelet thrombus formation and medical interventions

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Abstract
There are a wide range of receptors and proteins on platelets playing essential roles in thrombus formation. Among them P2Y₁₂ receptor, a member of the G protein-coupled receptor family, has attracted lots of attention. Stimulation of P2Y₁₂ receptor by ADP results in activation of various signaling pathways involved in amplification of platelet activation and aggregation. There have been extensive attempts to design an ideal antithrombotic agent to block P2Y₁₂, which has a selective expression, as an intervention for cardiovascular disease. Current inhibitors of P2Y₁₂ receptor include indirect inhibitors or thienopyridines family (ticlopidine, clopidogrel and prasugrel), and direct P2Y₁₂ inhibitors (ticagrelor, cangrelor and elinogrel). Among them clopidogrel is the most common prescribed P2Y₁₂ blocker however this product has not met the ideal therapeutic standards. The main limitations of clopidogrel administration include slow onset, prevention of recovery of platelet functions, and interindividual variability. Hence, advanced studies have been carried out to achieve more efficient and safer P2Y₁₂ blockade. In this review we provide a comprehensive, yet brief, report on the overview of P2Y₁₂, its role on platelet thrombus formation and targeting this receptor as an intervention for cardiovascular disease, for the benefit of basic science and clinical researchers.

Key words
Platelet . P2Y₁₂ receptor . Signaling pathways . Indirect inhibitors . Direct inhibitors
1 Introduction

Platelets play an essential role in both the normal hemostasis maintenance and the pathological thrombus formation development [1]. For example, within atherosclerotic arteries subject to high shear stress platelets in conjunction with other factors are responsible in vascular occlusion, a crucial mechanism in myocardial infarction and stroke [1]. Platelet thrombi can lead to clinical consequence of cardiovascular or cerebrovascular disease that is associated with 36% of all deaths in Australia [2].

After vascular injury, platelets translocate and rapidly adhere to exposed subendothelial matrix components including von Willebrand factor (vWF) and collagen through adhesive receptors including GPIb-IX-V complex and GPVI-FeR γ-chain complex, respectively. Platelet membrane GPIbα recognizes the activated conformation of vWF, initiating intracellular signaling events that lead to integrin αIIbβ3 activation. In the process of platelet adhesion, signals are also generated that lead to platelet activation. Platelet activation involves activation of integrin αIIbβ3 leading to platelet shape change and spreading, formation of stable platelet adhesion, release of granule contents, generation of lipid mediators and accumulation of platelet aggregates to form a thrombus [3]. Activation of platelets is achieved through a variety of cell surface receptors such as G-protein coupled receptors, integrins and glycoprotein receptors. A positive feedback loop by physiological agonists such as thrombin and collagen is also initiated by secreted products and secondary mediators of platelet activation. These secondary mediators include adenosine diphosphate (ADP: released from platelet granules) and thromboxane A₂ (generated within platelets) which activate other platelets and amplify the recruitment of platelets to a growing thrombus. Secreted ADP is important for platelet activation, as patients with defects in dense granule storage or specific ADP receptors have bleeding abnormalities [4].

ADP mechanism of action is through two G-protein coupled receptors, the Gq-coupled P2Y₁ receptor and the Gi-coupled P2Y₁₂ receptor. The P2Y₁ receptor contributes to ADP-induced platelet shape change while the P2Y₁₂-coupled G, signaling is essential for potentiation of dense granule secretion, thromboxane A₂ generation, irreversible aggregation and stabilization of a platelet thrombus [5, 6]. Concomitant signaling through both ADP receptors is necessary and sufficient for fibrinogen receptor (integrin αIIbβ3) activation [5, 6]. Parallel to these events, collagen-induced platelet activation through GPVI and induces integrin αIIbβ3 activation, resulting in adhesion and aggregation.

The main purpose of this review article is to provide a comprehensive and concise review on P2Y₁₂ receptor on a wide range from its discovery, its role in platelet thrombus formation, deficiency of this receptor and pharmacological inhibition of this receptor with different drugs to overcome cardiovascular disease.

2 Overview of P2 receptors

Nucleotide receptor family known as P2 receptors mediate the actions of extracellular nucleotides to intercellular signaling [7]. Membrane-bound P2-receptors consist of two classes of membrane receptors; P2X ligand-gated cation channels and G protein–coupled P2Y receptors [1, 8]. Mammalian P2X receptors are classified into 7 distinct subtypes (P2X₁ - P2X₇) that are distributed throughout the body and response to endogenous agonist of adenosine triphosphate (ATP) [9]. P2X receptors are expressed abundantly in the nervous system, underlie fat purinergic synaptic transmission, and play important roles in nervous system and peripheral diseases [9].

To date, 8 subtypes of P2Y receptor (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃, P2Y₁₄) have been cloned and characterized [1, 10, 11]. The jump in sequence of the P2Y numbering is due to the mistake in
identification of certain receptors which are assumed to belong to this family such as P2Y₅ and P2Y₇ [8] or nonmammalian P2Y receptors (chicken p2y3 and Xenopus laevis p2y8 receptors) [8, 12]. P2Y₉ and P2Y₁₀ receptors are not nucleotide receptors, either [8]. P2Y receptors are widespread with a different range of physiological roles [7]. They are distributed through cells and tissues such as platelets (P2Y₁ and P2Y₁₂) to epithelia (P2Y₂ and P2Y₄), placenta (P2Y₄), heart, blood vessels and brain (P2Y₆), immunocytes (P2Y₁₁) and neural cells (P2Y₁ and P2Y₁₂) [7]. Based on their pharmacological aspects, P2Y receptors can be subclassified into adenine nucleotide sensitive receptors mainly responding to adenosine diphosphate (ADP) and ATP (P2Y₁, P2Y₁₁, P2Y₁₂ and P2Y₁₃), the uracil nucleotide sensitive receptors responding to uridine triphosphate (UTP) or uridine diphosphate (UDP) (P2Y₄ and P2Y₆), the mixed sensitive receptor (P2Y₂), and UDP-glucose sensitive receptor (P2Y₁₄) [10]. Furthermore, according to their functional coupling to specific G proteins and effector proteins, P2Y receptors can be subdivided to Gₐ-coupled subtypes; P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁, and Gᵣ-coupled subtypes; P2Y₁₂, P2Y₁₃ and P2Y₁₄ [13].

Among P2 receptors only P2X₁, P2Y₁ and P2Y₁₂ are expressed at significant levels in platelets of healthy volunteers, with the P2Y₁₂ is expressed at the highest extent followed by P2X₁ and P2Y₁, respectively [14]. Two of P2Y receptors play essential roles in platelet thrombus formation. The P2Y₁ receptor initiates platelet activation in response to ADP and participates in platelet aggregation mediated by collagen [15]. The P2Y₁ receptor is coupled to Gq and initiates calcium mobilization from internal stores inducing platelet shape change and weak and transient aggregation mediating by ADP [16-18]. In general, the P2Y₁ receptor mediates weak responses to ADP and is crucial in the early steps of platelet activation mediated by ADP or collagen [1]. Whilst, the P2Y₁₂ receptor completes and amplifies platelet activation and aggregation [1].

3 P2Y₁₂ Receptor

P2Y₁₂ receptor has been shown to be expressed in human, bovine, rat and mouse tissues [7]. The identity of the platelet P2Y₁₂ receptor was characterized in 2001 by using expression cloning [19] and ligand screening methods [20, 21]. P2Y₁₂ is highly expressed in human platelets and to a smaller extent in brain [19]. Some of the essential roles of P2Y₁₂ receptor are platelet aggregation in addition to inhibition of neural cells [7]. P2Y₁₂ receptor is activated by ADP (a natural agonist stored in platelet dense granules) and very potently by ADP analogue 2-methylthio-ADP (2-MeSADP) [7]. ATP and its triphosphate analogues (e.g. 2MeSATP and 2C1ATP) are P2Y₁₂ antagonists [22, 23]. Other nucleotide antagonists include N⁵-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-βγ-dichloromethylene-ATP (cangrelor; AR-C69931MX), and the nucleoside analogue AZD6140 [7]. In addition, active metabolites of the thiopyridine compounds, clopidogrel, ticlopidine and prasugrel block this receptor [7]. These P2Y₁₂ antagonists have been administered in pharmacotherapy to inhibit platelet aggregation [7].

3.1 Biochemistry of P2Y₁₂ Receptor

The P2Y₁₂ receptor structure contains the specific features of G-protein-coupled receptors including 7 hydrophobic transmembrane (TM) regions connected by 3 extracellular loops (EL) and 3 intracellular loops (Fig. 1) [7]. The human P2Y₁₂ receptor consists of 342 amino acid residues [1]. The human P2Y₁₂ amino acid sequence contains 10 cysteine residues. There are four cysteine residues at extracellular domains at position 17, 97, 175 and 270 which are proposed to form 2 disulfide bridges between N-terminal domain and EL3, and
between EL1 and EL2 [7, 24]. Ding et al., however showed that there is no essential disulfide bridge between cysteines 17 and 270 [25]. In addition, there are five cysteine residues at position 194, 208, 248, 292, and 302 located in the TM domains, and one intracellular cysteine residue is present at position 315 [24]. The importance of cysteine residues in the function of P2Y$_{12}$ has been proposed by the ability of thiol reagents, e.g. clopidogrel, to inhibit ADP responses in platelets [19]. Although N-linked glycosylation of the P2Y$_{12}$ receptor plays an essential role in signal transduction, it is not crucial for ligand binding or cell surface expression [26].

### 3.2 Overview of P2Y$_{12}$ signaling pathways in platelets

Guanine nucleotide-binding regulatory proteins, also known as G proteins, mediate the interaction between cell surface receptors and intracellular enzyme generation second messenger molecules during platelet activation [27]. The G proteins are multimers, e.g. G$_{i0}$, G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_{s0}$, G$_{s1}$, G$_{s2}$, G$_{s3}$ [27-29], consist of three distinct subunits including $\alpha$, $\beta$ and $\gamma$ existing in more than one form [27]. For example, two G proteins distributed in virtually all cells include, G$_{ai}$: the $\alpha$ subunit of the G protein that stimulate cyclic adenosine monophosphate (cAMP) formation by adenylyl cyclase, and G$_{ia}$: the $\gamma$ subunit of the G protein that inhibits adenylyl cyclase [30].

Human platelets expressed G$_{ai}$, four members of the G$_{i}$ family of G proteins including G$_{i0}$, G$_{i2}$, G$_{i3}$ and G$_{i4}$ [27-29, 31], G$_{s}$, G$_{s1}$, G$_{s2}$ and G$_{s6}$ [28, 32, 33]. It has been shown that G$_{ai}$ pathway plays a vital role in platelet aggregation [34]. Two of the most highly expressed G$_{i}$ proteins on platelets are G$_{i2}$ that couples to the $\alpha_{2A}$ adrenergic receptor for epinephrine [35], and G$_{i3}$ that is the target of the ADP receptor P2Y$_{12}$ [36].

The P2Y$_{12}$ receptor couples to $\alpha_{i2}$ subunit of G protein (Fig. 2) [19, 36, 37]. Stimulation of the heterotrimeric G protein, e.g. by ADP, results in dissociation of G$_{i}$ and G$\beta$$\gamma$ subunits which activate various signaling pathways [6]. The G$_{i2}$ inhibits production of adenylyl cyclase which results in reduction in cytosolic cAMP concentrations [36, 38, 39]. This decrease in cAMP reduces the activation of cAMP-dependent protein kinase (PKA) responsible for phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) [1]. VASP phosphorylation plays an essential role in fibrinogen receptor activation in response to ADP and platelet aggregation [40]. VASP is an actin regulatory protein that inhibits the integrin $\alpha$IIb$\beta$3 activation [41, 42]. Therefore, the levels of VASP phosphorylation/dephosphorylation can indicate P2Y$_{12}$ inhibition/activation as well as a selective assay for clopidogrel effects on patients, e.g. patient resistance to clopidogrel [43, 44].

On the other hand, it has been shown that stimulation of P2Y$_{12}$ by ADP, through G$\beta$$\gamma$ subunits, stimulates phosphatidyl inositol-3 kinase (PI-3K) activity (Fig. 2), resulting in late accumulation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P$_2$) induced by PAR1-activated peptide and rapid and transient accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P$_3$), with essential roles in sustaining platelet aggregation [45-47]. Various isoforms of PI3K are expressed in platelets. Two major isoforms of PI3K present in platelets include PI3K$\beta$, a member of the class Ia isoforms which consists of catalytic subunit of p110$\alpha$ and p110$\beta$ regulated by tyrosine kinases, and PI3K$\gamma$ belonging to the class Ib isoform which consists of catalytic subunit of p110$\gamma$ activated by G-protein coupled receptors [48-51]. Continual signaling through PI3K$\beta$ and PI3K$\gamma$ via P2Y$_{12}$ receptors stimulated by ADP is indicated to be required for stabilizing thrombus growth resulting in maintenance of $\alpha$IIb$\beta$3 activity and fibrinogen binding [48].

It has been also elucidated that P2Y$_{12}$ activates GTPase Rap1b via PI-3K dependent pathway with essential roles in platelet activation (Fig. 2) [31, 47, 52, 53]. Rap1b is the most abundant Ras GTPase in platelets [52]. Rap1b is located at membrane of resting platelets and associates with actin-based cytoskeleton after platelet
stimulation [52]. Rap1b cycles from inactive form (GDP-bound) to an active form (GTP-bound) regulated by guanine nucleotide-exchange factors, guanine nucleotide dissociation inhibitors and GTPase-activating proteins [54, 55]. Multiple pathways for Rap1b activation has reported in human platelets including Ca\(^{2+}\), cAMP, protein kinase C and tyrosine kinases after using different agonists [52, 55]. In response to ADP however it is speculated that PI3K\(\beta\) plays an essential role in a signaling pathway between \(\beta\gamma\) dimers released upon stimulation of the G\(\text{i}\) coupled receptors and activation of Rap1b whereas PI3K\(\gamma\) isoform was demonstrated to not be involved in this process [47, 56]. Rap1b is rapidly activated after stimulation with ADP, in a G\(\text{i}\)-dependent pathway through the action of the PI3K product PtdIns(3,4,5)P\(_3\) which is a rapid and transient product but not the PtdIns(3,4)P\(_2\) which forms mainly as a result of integrin \(\alpha\)IIb\(\beta\)3-mediated platelet aggregation [47]. This supports the role of Rap1b activation in the initial process of platelet activation resulting in activation of integrin \(\alpha\)IIb\(\beta\)3 and platelet aggregation [47].

In addition, activation of PI-3K results in dual phosphorylation (threonine 308 and serine 473) hence activation of Akt (Fig. 2) by phosphatidylinositol dependent kinases (PDKs), which is an important signaling intermediate in agonist-induced platelet activation [57, 58]. Akt or protein kinase B: PKB or RAC is a family of intracellular serine/threonine protein kinases, expressed in various cells, activated by different agonists, e.g. platelet derived growth factor, insulin and thrombin, and has multifunctional roles, e.g. prevention of apoptosis, regulation of glycolysis and controlling glucose uptake [57, 59]. Human platelets express Akt1 (PKB\(\alpha\)) and Akt2 (PKB\(\beta\)) [57, 59]. PI3K products including PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\) initiate phosphorylation of Akt [57, 60] by PDK 1 at threonine 308 and possibly PKD 2 [57, 60] or autophosphorylation [61] or integrin-linked kinase [62] at serine 473. Membrane attachment of Akt and dual phosphorylation are essential for activation of Akt [57, 59]. Akt activation in a PI3K dependent manner after thromboxane \(A_2\) or thrombin stimulation promotes ADP release from platelets dense granules that stimulates P2Y\(_{12}\) receptor signaling pathways resulting in enhancement of integrin \(\alpha\)IIb\(\beta\)3-mediated platelet aggregates and stabilization [63]. Therefore ADP amplifies Akt activity [63]. Also, it has been demonstrated that Akt2 plays a more essential role in this process in mice as compared with Akt1 [63].

Furthermore, the \(\beta\gamma\) dimers can activate the G-protein-gated inwardly rectifying potassium channels (GIRKs) via binding to their cytosolic regions mediating activation of Src tyrosine kinases downstream [64, 65]. Both GIRK channels and Src family of tyrosine kinases after stimulation of P2Y\(_{12}\) may play a role in ADP-induced cytosolic phospholipase \(A_2\) (cPLA\(_2\)) phosphorylation (serine 505) and thromboxane \(A_2\) generation hence platelet aggregation [64] (Fig. 2). Src family tyrosine kinases belong to cellular signal transduces that can be activated by different extracellular signals to modulate various cellular functions, e.g. proliferation, survival, adhesion and migration [66]. Platelets contain high amount of proto-oncogene product pp60\(^{src}\) which is suggested to be associated with platelet cytoskeletal proteins and important in platelet aggregation [67, 68]. It has been proposed that Src family tyrosine kinases stimulate the extent of \(\alpha\)IIb\(\beta\)3 activation but they are not essential in this process [69]. It is also speculated that both P2Y\(_1\) and P2Y\(_{12}\) can activate Src [69, 70].

Also, extracellular-signal-regulated kinase (ERK) is suggested to be downstream of Src family kinases [71] (Fig. 2). ERK is a subgroup of mitogen-activated protein kinases (MAPKs) family of serine-threonine kinase activated by various extracellular stimuli, such as growth factors and hormones [72]. It has been shown that platelets expressed two forms of ERK, ERK1 (p44\(^{mapk}\)) that remains intact after thrombin-mediated platelet activation whereas ERK2 (p42\(^{mapk}\)) becomes phosphorylated [73]. Similarly, ADP stimulation results in
phosphorylation of ERK2, predominantly [74]. It has been proposed that activation of cPLA2 is a downstream link between ERK and integrin activation [72, 73] (Fig. 2).

Signaling through both the P2Y12 and P2Y1 receptor has been shown to be important for ADP-induced ERK2 phosphorylation in platelets therefore generation of thromboxane A2 [71, 74] (Fig. 2). cPLA2 is a Ca2+-dependent lipase and cleave arachidonic acid containing phospholipids at their sn-2 position releasing arachidonic acid that is a precursor to lipoxins, thromboxanes, leukotrienes, prostaglandin eicosanoid and platelet activating factor [75]. Various agonists regulate cPLA2, including hormones, neurotransmitters and antigens, and MAPK and protein kinase C may activate cPLA2 [75]. Phosphorylation of cPLA2 at serine 505 has been proposed to be downstream of ERK-2 stimulation and essential for hormonally mediated release of arachidonic acid from membrane-bound phospholipids hence generation of thromboxane A2 [74, 76].

We have attempted to summarize signaling pathways responsible for P2Y12 activation in platelets in this review. However there are various paradoxical reports on the P2Y12 signaling pathways due to complexity of this network. Hence the precise signaling pathway has yet remained to be elucidated.

4 The P2Y12 receptor deficiency
Congenital P2Y12 deficiency is an autosomal recessive disorder [77, 78]. P2Y12 deficiency is associated with deletions of the nucleotide in the open-reading frame, and frameshift mutation leading to protein premature truncation (e.g., P2Y12 haploinsufficiency and a 378delC mutation in patients remaining allele [79]), or with substitution of a nucleotide in the transduction initiation codon (e.g. ATG to ACG [80]) [77]. Congenital dysfunctions of P2Y12 are associated with molecular malfunctions of the sixth trans-membrane domain (a G– to –A transition in one allele hence altering the codon for Arg-256 in the sixth trans-membrane domain to Gln) or the nearby third extracellular loop of the receptor (a C– to –T transition in the other allele hence altering the codon for Arg-265 in the third extracellular loop to Trp) [4]. The integrity of this region of the protein is crucial for normal receptor function [4]. A heterogeneous mutation, predicting a lysine to glutamate (Lys174Glu) substitution was reported to be associated with the impaired ligand binding to the P2Y12 receptor in patient with mild type 1 von Willebrand disease [77, 81]. Hence this mutation is suggested to play an essential role in disruption of the ADP-binding site of the P2Y12 receptor [77, 81].

Platelets with a moderate P2Y12 deficiency show similar characteristics to the primary secretion defect (PSD). The PSD which is characterized by abnormal secretion but normal granule stores, thromboxane A2 production and ADP-initiating aggregation, is the most common platelet congenital defect [78]. The P2Y12 deficiency results in dysfunctional platelets and bleeding diathesis, characterized by mucocutaneous bleeding and excessive post-surgical and posttraumatic blood loss [78, 82, 83]. The first patient with selective defect of platelet response to ADP was described in 1992 by Cattaneo et al. [84]. This patient (white origin, aged 49) had lifelong history of excessive bleeding (especially mucosal: nose bleeds), easy bruising, prolonged bleeding time, and abnormality in platelet aggregation the same as PSD, including reversible platelet aggregation in response to weak agonists and decreased aggregation induced by low concentrations of collagen or thrombin with the most defect at aggregation responses to ADP [78, 84]. This platelet aggregation is similar to platelet profiles after administration of thienopyridines (e.g. clopidogrel) to humans [78, 85]. More cases with a similar profile have been reported [77, 82, 83]. These cases also emphasize the pivotal role of platelet ADP receptors for normal platelet secretion and function [83].
5 P2Y12 inhibitors

P2Y12 receptor has a very limited and selective distribution on tissues [8, 19]. In addition, this receptor plays a crucial role in the thrombus formation and stabilization [1]. Therefore, P2Y12 receptor is a very good candidate for antiplatelet drugs [19]. Ideal antithrombotic agent is characterized by its predictable pharmacodynamics profile (avoid monitoring), rapid onset, rapid offset (and/or available antidote), compatible with adjunctive medicine, potent efficacy, low risk, low cost and easy administration [87].

Current inhibitors of P2Y12 receptor are categorized into indirect acting irreversible inhibitors (thienopyridines: ticlopidine, clopidogrel and prasugrel), and direct acting reversible P2Y12 inhibitors (ticagrelor, cangrelor and elinogrel) [87].

5.1 Thienopyridines family

Thienopyridines are the first family of P2Y12 inhibitor [87]. Thienopyridines are prodrug required to be metabolized by hepatic cytochrome P-450 (CYP) to form active metabolite [87]. The active metabolite then covalently (via formation a disulfide bond with cysteine residues) and irreversibly binds to the P2Y12 receptor hence inhibiting the P2Y12 receptor [87].

5.1.1 Ticlopidine

Ticlopidine is the first generation of thienopyridines [87]. This prodrug is administered orally twice a day [88] and reaches the maximum platelet inhibitory effect after 3 days of treatment [89]. Various clinical trials reveal that combination of ticlopidine with aspirin enhances platelet inhibitory effect and reduces cardiovascular events especially in patients undergoing placement of coronary artery stents [89-92]. This synergic effect is contributed to platelet inhibitory effect through blocking two different pathways including P2Y12 and cyclooxygenase (COX)-1 [93]. Ticlopidine administration however causes serious side effects including neutropenia, aplastic anaemia, thrombotic thrombocytopenic purpura, and gastrointestinal effect which are a drawback of using this medicine [94].

5.1.2 Clopidogrel

Clopidogrel (Plavix/Iscover [94]), the most commonly prescribed drugs worldwide [95], belongs to the second generation of thienopyridines, with fewer side effects and replaced ticlopidine [87, 96]. Anti-aggregating property of clopidogrel is also several times greater than ticlopidine [97]. Furthermore, various clinical trials, such as percutaneous coronary intervention - Clopidogrel in Unstable angina to prevent Recurrent Events (PCI-CURE), Clopidogrel for Reduction of Events During Observation (CREDO) and PCI- Clopidogrel as Adjunctive
Therapy (CLARITY) trials or Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial, have revealed cost-effective of pre-treatment and long-term treatment in percutaneous coronary intervention or prevention atherothrombotic events with clopidogrel [98-100].

Clopidogrel is also an inactive prodrug which becomes active after intravenous or oral administration with no trace of circulating activity in the plasma of treated animals or humans [97]. An intravenous formulation of clopidogrel (PM103) has been developed as an alternative dosage form to oral clopidogrel for administration in the acute care setting [101]. Absorbed clopidogrel undergoes two metabolic pathways: about 85 – 90% of the absorbed drug is hydrolyzed by esterases that generate inactive metabolite whereas only 10 – 15% is metabolized by CYP isoforms in the liver to form an active metabolite [102, 103]. The short lived active metabolite then binds irreversibly to the cysteine 17 and cysteine 270 in the extracellular domains of P2Y$_{12}$ receptor on platelets hence clopidogrel effects last for the whole platelet lifespan (7 – 10 days) [25, 102]. The antiplatelet effects of clopidogrel are time and dose dependent with the approximately 50 – 60 % inhibition of platelet aggregation [104]. The approved doses of clopidogrel are a 300 mg loading dose and a 75 mg of maintenance dose [94, 105, 106]. In addition, combination of clopidogrel with aspirin resulting in concurrent inhibition of ADP and thromboxane A$_2$ pathways of platelets hence causes additive/synergic antithrombotic effects in patients undergoing coronary stenting [94]. A loading dose of 600 mg of clopidogrel also reveals more potency and efficacy in clinical practice, e.g. percutaneous coronary intervention [107-109].

Although clopidogrel is the most popular antiplatelet drug with high efficacy, it does not have the main criteria of the ideal antithrombotic agent [87]. The antiplatelet effect of clopidogrel is delayed since this prodrug needs to undergo hepatic metabolism to generate an active metabolite. Maintenance daily dose of clopidogrel (75 mg) with no preload reaches the steady state levels of platelet aggregation within 4 – 7 days. This delayed onset of action of clopidogrel was overcome by administration of loading doses (300 – 600 mg) that reach the steady state level by 4 – 24 h [104, 110, 111].

Another disadvantage of administration of clopidogrel is the substantial variability between individual in platelet inhibition, e.g. a reduced efficacy of clopidogrel in some patients [110]. The high interindividual variability of the response and incidence of drug resistance can affect clinical outcomes extensively since poor responders may receive inadequate protection from major adverse cardiac effects, e.g. patients undergoing percutaneous coronary intervention [111, 112]. Several studies showed approximately 8 – 44% of prevalence of clopidogrel non-responsiveness/resistance [111-116]. This variable response may be due to differences between individual in the conversion of prodrug to active metabolite because of variable CYP3A4 metabolic activity [113]. Several studies also showed that common loss of function polymorphisms of CYP2C19 and CYP2C9 are concomitant with reduced exposure to the active metabolite of clopidogrel [117, 118]. Different extent of absorption of prodrug or clearance of the active metabolite may also play a role in clopidogrel resistance [110]. Clopidogrel efflux via P-glycoprotein ATP-dependent pump encoded by the ABCB1 gene (also known as MDR1) [95]. Patients with genetic variants in ABCB1 (ABCB1 3435 TT homozygotes) are more likely to experience adverse cardiovascular outcomes after clopidogrel treatment [95]. Furthermore, P2Y$_{12}$ receptor variability, such as elevated level of receptors, enhanced level of ADP or upregulation of other platelet activation pathways may cause interindividual variation is response to clopidogrel [110].

Further limitation of clopidogrel is its irreversible inhibition of P2Y$_{12}$ receptor resulting in a slow recovery of platelet function after drug withdrawal [119]. This may initiate bleeding risk within 5 – 7 days after
termination of drug administration especially in patients who need urgent surgical revascularization [119] hence increased transfusion requirements and extended intensive care unit and hospitalization [87, 119]. Therefore, new P2Y₁₂ antagonists with predictable and efficient inhibition of platelet function in all patients and minor adverse effects were required to be designed.

5.1.3 Prasugrel
Prasugrel is a third-generation member of oral thienopyridine [93]. Prasugrel should also undergo metabolism to generate an active form that blocks P2Y₁₂ selectively and irreversibly [120]. Prasugrel is completely and rapidly absorbed and extensively metabolized in humans [121]. The active metabolite is detectable in plasma after 15 min and reaches maximum concentration at 30 min [121]. The cytochrome isoenzymes that are responsible for the generation of the active metabolite are mainly CYP3A and CYP2B6, and to a lesser extent CYP2C9, CYP2C19 and CYP2D6 [122]. Hence the common loss-of-function mutations in CYP2C9 and CYP2C19 which affect clopidogrel, hardly interfere with the formation of the prasugrel active metabolite [117]. Furthermore, intestinal CYP3A contributes more in the generation of an active metabolite as compared with hepatic CYP3A which can explain the rapid appearance of active metabolite in plasma [103]. In addition, prasugrel has been demonstrated to be more rapid, potent and consistent in inhibition of platelet function than clopidogrel [120]. It is transformed into its active metabolite more efficiently as compared with clopidogrel [120]. The maximal effect of prasugrel (60 mg loading dose) began to plateau approximately 1 h after administration compared to about 4 h for clopidogrel (300 mg loading dose) [120]. A single oral administration of prasugrel results in a dose-related inhibition of binding 2-MeSADP to platelets in rats about 10 - fold more potent than that of clopidogrel [123].

In stable aspirin-treated patients with coronary artery disease, prasugrel administration also resulted in greater inhibition of platelet aggregation and a lower rate of drug-resistance compared with clopidogrel [124]. Furthermore, it has been reported that active metabolite of prasugrel enhances the inhibitory effect on platelet aggregation of agents that acts via raising cAMP (e.g. prostaglandin I₂, adenosine and forskolin) [125]. The antiaggregation effects of prasugrel are observed at 30 min, and lasts until 72 h after administration which demonstrates fast onset and long duration of action of this product [93].

Prasugrel (60 mg loading dose and 10 mg/day maintenance dose) was more potent in inhibition of platelet than clopidogrel (a 600 mg loading dose and 150 mg/day maintenance therapy) [126]. The Trial to Assess Improvemnt in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolyis in Myocardial Infarction (TRITON–TIMI) 38, a phase 3 trial involving patients with acute coronary syndromes with scheduled percutaneous coronary intervention, showed that prasugrel administration reduced the rates of ischemic events (e.g. stent thrombosis) compared to clopidogrel however increased the risk of major bleeding with fatal consequences [127]. Prasugrel also inhibits P2Y₁₂ irreversibly hence the limitation of slow offset of action remains as for clopidogrel [87]. Therefore, prasugrel administration may not efficiently replace clopidogrel.

5.2 Direct P2Y₁₂ inhibitors
In order to cover these gaps for inhibiting aggregation of platelets by fast-acting and reversible antagonists with short half-lives, direct P2Y₁₂ antagonists have been recently developed.
5.2.1 Ticagrelor

Ticagrelor (AZD6140) is the first oral reversible ADP P2Y12 receptor antagonist and a member of cyclopentyltriazolopyrimidine class [128, 129]. It has been reported that the P2Y12 receptor is targeted by ticagrelor through a mechanism that is not competitive with ADP suggesting the presence of an independent receptor binding site [130]. Iyú et al. have also revealed that ticagrelor mainly affect P2Y12 receptors [131] with the ability to enhance the inhibitory effect of natural (e.g. vascular prostaglandins I2, D2 and adenosine) and other (e.g. forskolin) modulators of platelet functions which are raising intracellular cAMP through interaction with Gs-coupled receptor [125]. This may be a great advantage for humans [125].

Further metabolic conversion is not required for ticagrelor as it directly and dose-dependently inhibits P2Y12 receptor with about 95% inhibition of platelet aggregation within 2 – 4 h [128]. Ticagrelor has an early onset of action within 2 h with no loading dose [132, 133]. Due to a short half-life of about 12 h [128, 134], this product requires to administer twice-daily [132, 133]. Another advantage of ticagrelor compared to thienopyridines family is shorter time for drug offset after ticagrelor withdrawal [128, 132, 133].

Ticagrelor dose of 90 mg twice daily has demonstrated higher and less fluctuated levels of platelet inhibition compared with standard-dose regimens of clopidogrel [133]. Also, single oral dose of ticagrelor up to 400 mg daily was safe and well-tolerated in healthy subjects [128]. While ticagrelor shows similar safety and tolerability to clopidogrel in patients with non-ST-segment elevation acute coronary syndrome, its reversible inhibition of P2Y12 receptor is beneficial in rapid initiation of coronary bypass and surgical procedures after drug discontinuation [134]. Furthermore, PLATElet inhibition and clinical Outcomes (PLATO) trial: phase 3, randomized, double blinded, parallel-group multinationals clinical study, revealed that treatment with ticagrelor as compared to clopidogrel in patients who had an acute coronary syndrome, with or without ST-segment elevation, significantly reduced the mortality rate from vascular causes (e.g. myocardial infarction or stroke) [135]. While the rate of overall bleeding remained the same for both treatments, there was an enhancement in the rate of non-procedure related bleeding in ticagrelor-treated group [135]. However, three subgroups in PLATO trial, including patients enrolled in North America, males < 82 kg or females < 71 kg and patients not on lipid-lowering medications, did not benefit from ticagrelor treatment [135]. The controversial reports in patients enrolled in North America may be due to geographical differences between populations of patients [135] or pattern of medications practice as aspirin maintenance dose was identified as a potential explanation for the regional differences in North American patients [136]. However this aspect requires more investigation.

Ticagrelor administration (180 mg load and 75 mg per day maintenance dose) also results in more rapid and superior platelet inhibition than high loading dose of clopidogrel (600 mg load and 90 mg twice a day maintenance dose) in patients with stable coronary artery disease [137]. This inhibition was sustained during the maintenance phase and was faster in offset after discontinuation of treatment [137]. In addition, in patients with acute coronary syndrome managed a non-invasive treatment strategy, more intense P2Y12 receptor inhibition with ticagrelor achieved a clinically relevant reduction in ischemic events and mortality but with no major increase in bleeding compared with clopidogrel [138].

It has been also demonstrated that the greater antiplatelet effect of ticagrelor compared to clopidogrel is irrespective of CYP2C19 and ABCB1 polymorphism [139, 140]. Although CYP2C19 genotype affected the antiplatelet effect of clopidogrel, no effect was observed during ticagrelor therapy [139]. Hence administration of
ticagrelor instead of clopidogrel eliminates the need for genetic testing before dual antiplatelet treatment with aspirin [140]. Ticagrelor treatment also overcomes nonresponsiveness to clopidogrel since its antiplatelet effect is similar in responders and nonresponders [141].

There were however higher rates of dyspnoea, hypotension, and nausea in patients treated with ticagrelor [134]. The respiratory side effects occurring after oral ticagrelor or intravenous cangrelor may be due to the development of mild asymptomatic thrombotic thrombocytopenic purpura advancing to more acute scenario including fluid retention and dyspnoea because of the reversible nature of these drugs [142]. Dyspnoea is often arising during the first week of treatment with ticagrelor at mild or moderate level of severity however usually transient in spite of continuing therapy [143]. The more frequent incidence of dyspnoea may be due to modulation of adenosine metabolism [134]. However further studies are required to investigate ticagrelor side effects.

5.2.2 Cangrelor
Cangrelor (AR-C69931MX) is a potent, selective and competitive P2Y₁₂ receptor antagonist that is administered intravenously [129, 144]. Cangrelor is an analog of ATP, natural P2Y₁₂ receptor antagonist, with inhibitory effect in a dose-dependent manner [129, 145]. It has been also reported that cangrelor enhances platelet cAMP level through unidentified platelet G₁ protein-coupled receptor mediating inhibition of platelet function [146]. Whereas, similar pattern of action to ticagrelor in promoting the inhibitory effect of natural modulators of platelet functions or other agents that act via increasing cAMP with the main effect of cangrelor on P2Y₁₂ receptors was reported by another group [125, 131].

Cangrelor acts directly on the P2Y₁₂ receptor with a rapid onset of action (approximately 15 [144] to 30 min), rapidly achieves steady-state inhibition of platelet aggregation with a half-life of approximately 2 – 5 min [145] and clearance at steady state of 12.7 ml/min per kg [147]. In addition, cangrelor does not require to be metabolized to form active product and directly inhibits the P2Y₁₂ receptor. Therefore cangrelor has a rapid reversal effect, as 70% of patients recovered more than 60% of baseline aggregation response after 1 h of termination of administration [145]. This is a great advantage in patients with hemorrhagic complications or required surgical intervention [145]. In patients undergoing percutaneous coronary intervention, intravenous cangrelor (4 µg/kg per minute) also compares favourably with abciximab (a glycoprotein IIb/IIIa receptor antagonist) with acceptable bleeding risk and adverse cardiac events while reaching rapid, reversible inhibition of platelet aggregation with less side effect of prolonged bleeding time [144]. In addition, acceptable safety, tolerability and efficacy of adjunctive cangrelor administration with fibrinolysis suggested the potential of this combination in the treatment of acute myocardial infarction [148].

Following acute in-hospital, patients receive clopidogrel treatment to prevent further cardiovascular complications [149]. To achieve sustained platelet P2Y₁₂ inhibition in patients treated with cangrelor, clopidogrel should be administered when the cangrelor treatment is terminated since simultaneous administration of both drugs prevent the effect of a 600 mg loading dose of clopidogrel 4 – 6 h later [149]. An in vitro study showed that cangrelor modulates the platelet function inhibitory effect of the active metabolites of clopidogrel or prasugrel hence careful consideration should be given for co-administration of these drugs [150].

Periprocedural cangrelor during percutaneous coronary intervention followed by 600 mg of clopidogrel was not superior to placebo followed by 600 mg of clopidogrel in reducing primary end point of death from any
cause including myocardial infarction, or ischemia-driven revascularization at 48 h, whereas the prespecified secondary end points of stent thrombosis and death were lower in the cangrelor group, with no significant increase in the rate of transfusion [151]. Administration of cangrelor 30 min before percutaneous coronary intervention for 2 h after percutaneous coronary intervention however was not superior to an oral loading dose of 600 mg of clopidogrel, administered 30 min prior to the procedure, in decreasing the composite end point of death from any cause, myocardial infarction, or ischemia-driven revascularization at 48 hours [152]. Hence implication of cangrelor in routine practice may be questionable.

On the other hand, study of bridging antiplatelet therapy with cangrelor in patients undergoing cardiac surgery revealed that among patients who discontinue thienopyridine therapy prior to cardiac surgery, the use of cangrelor for at least 48 h compared with placebo resulted in a higher rate of maintenance of platelet inhibition with low risk of thrombotic events and no significant excess bleeding complications [153]. Therefore, intravenous cangrelor may be a feasible management strategy in patients waiting for cardiac surgery who require prolonged platelet P2Y12 inhibition after thienopyridine discontinuation [153]. However more investigation is required to study cangrelor ADP blockade effects as an antiplatelet therapy.

5.2.3 Elinogrel
Elinogrel (PRT060128) is an investigational, potent, competitive, direct acting reversible P2Y12 receptor inhibitor with the fast onset and offset of action that can be administered both orally and intravenously [154, 155]. Elinogrel belongs to the family of quinazolinedione [155]. Administration of elinogrel intravenously showed well-tolerability and safety, and achieves immediate and high level of platelet inhibition [154]. The average terminal half-life at the 40 mg elinogrel administered intravenously was about 11 h and maximum platelet inhibition achieved at 20 min [154]. The inhibitory effect of platelet was completely reversible within 8 – 24 h of elinogrel administration [155, 156]. This is an advantage in reducing bleeding side effect in the setting of urgent surgery and avoiding unnecessary delay before nonurgent surgery [157]. Preliminary data on safety and tolerability of single dose intravenous loading doses (10 – 60 mg) of elinogrel have recommended this treatment as an adjunctive therapy for primary percutaneous coronary intervention for ST-elevation myocardial infarction [156].

Pharmacologically, the intravenous and oral forms of elinogrel are identical [157]. Therefore the potential for the vulnerability associated with the transition from one intravenous to a different oral P2Y12 receptor inhibitor may be avoided [157]. It has been suggested that one single 60 mg oral dose of elinogrel overcomes, reversibly, the high platelet reactivity due to CYP2C19*2 genotype on standard dual-antiplatelet therapy with aspirin and clopidogrel [158].

Currently, a phase II randomized, double-blind, clopidogrel-controlled trial is undergoing to assess the safety, tolerability and preliminary efficacy of elinogrel (intravenous and oral) administration compared with clopidogrel in patients undergoing nonurgent percutaneous coronary intervention [157].

Elinogrel has been indicated to possess greater therapeutic index (less bleeding) compared with that in P2Y12−/− mice which may be because of the reversible and competitive nature of this antiplatelet drug [159]. However, thienopyridines have decreased therapeutic index (increased bleeding) possibly due to P2Y12− independent off-targeting effects at the vessel wall [159]. Furthermore, animal studies have revealed that equivalent, maximal levels of platelet aggregation inhibition in response to ADP was achieved by elinogrel (60
mg/kg) whereas clopidogrel (50 mg/kg) failed to reproduce the phenotype associated with P2Y₁₂ deficiency [160]. In addition, clopidogrel is not able to block the inducible pool of P2Y₁₂ exists on platelets, which can be exposed upon platelet activation in response to strong agonist and contributes to thrombosis, whereas elinogrel can block this pool [160]. Hence pharmacological properties of elinogrel make this drug an attractive antiplatelet agent. However more extensive clinical investigations are required to determine the efficacy and safety of this product.

6 Concluding remarks

In summary, purinergic P2Y₁₂ receptor, a member of G protein-coupled receptors, is essential in normal hemostatic process and pathophysiological conditions. Understanding of the structure and the signaling pathways of the P2Y₁₂ receptor is pivotal to identify ideal targets for antithrombotic drugs. The 342 amino acid residues P2Y₁₂ receptor structure contains 7 hydrophobic transmembrane regions in conjunction with 3 extracellular loops and 3 intracellular loops with 10 cysteine residues. Stimulation of P2Y₁₂ by ADP results in coupling this receptor with α₁₂ subunit of G protein hence activation of various signaling pathways responsible for amplification of platelet activation and stabilization of platelet aggregates. Deficiency of this receptor results in platelet dysfunction and bleeding diathesis indicating the possibility of targeting this receptor as an antithrombotic drug. Selective tissue distribution of P2Y₁₂ receptor compared with other purinergic receptors, e.g. P2Y₁, also makes this receptor a great advantage as a target for anti-thrombotic therapy. Current inhibitors of P2Y₁₂ receptor are classified into indirect inhibitors or thienopyridines family (ticlopidine, clopidogrel and prasugrel), and direct P2Y₁₂ inhibitors (ticagrelor, cangrelor and elinogrel). Clopidogrel is the most commonly prescribed inhibitor of P2Y₁₂ receptor. However due to therapeutic limitations of this prodrug, such as slow onset and offset and interindividual variability, direct inhibitors of P2Y₁₂ receptor are currently under investigation.

Declaration of interest

The authors report no declarations of interest.
References


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Fig 1  Biochemistry of human P2Y\textsubscript{12} receptor. The human P2Y\textsubscript{12} receptor structure contains 7 hydrophobic transmembrane (TM) regions connected by 3 extracellular loops (EL) and 3 intracellular loops. The human P2Y\textsubscript{12} receptor contains 342 amino acid residues with 10 cysteine residues. Four extracellular cysteines are at position 17, 97, 175, and 270 which are likely to form 2 disulfide bridges between N-terminal domain and EL3, and between EL1 and EL2. There are five cysteine residues at position 194, 208, 248, 292, and 302 within the transmembrane domains, and one intracellular cysteine residue at position 315.

Fig 2  Overview of purinergic P2Y\textsubscript{12} receptor signaling in platelets. While the P2Y\textsubscript{1} receptor initiates platelet activation in response to ADP, P2Y\textsubscript{12} receptor completes and amplifies platelet activation and aggregation. The P2Y\textsubscript{12} receptor couples to Ga\textsubscript{i2} G protein subunit pathway. Agonists, e.g. ADP, stimulate the heterotrimeric G protein resulting in dissociation of Ga and Gb\gamma subunits. The Ga\textsubscript{i2} inhibits production of adenyl cyclase hence reduces cytosolic cyclic adenosine monophosphate (cAMP) concentrations, cAMP-dependent protein kinase (PKA) and phosphorylated vasodilator-stimulated phosphoprotein (VASP). On the other hand, Gb\gamma subunit stimulates phosphatidylinositol-3 kinase (PI-3K), Rap1b and Akt activities. In addition, the b\gamma dimers can activate the G-protein-gated inwardly rectifying potassium channels (GIRKs) that in conjunction with P2Y\textsubscript{1} signaling pathways mediating activation of Src, ERK, cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) and thromboxane A\textsubscript{2} generation.