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Progress in the development of antiplatelet agents: Focus on the targeted molecular pathway from bench to clinic

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ABSTRACT

Antiplatelet drugs serve as a first-line antithrombotic therapy for the management of acute ischemic events and the prevention of secondary complications in vascular diseases. Numerous antiplatelet therapies have been developed; however, currently available agents are still associated with inadequate efficacy, risk of bleeding, and variability in individual response. Understanding the mechanisms of platelet involvement in thrombosis and the clinical development process of antiplatelet agents is critical for the discovery of novel agents. The functions of platelets in thrombosis are regulated by two major mechanisms: the interaction between surface receptors and their ligands, and the downstream intracellular signaling pathways. Recently, most of the progress made in antiplatelet drug development has been achieved with P2Y receptor antagonists. Additionally, the usage of GP IIb/IIIa receptor antagonists has decreased, because it is associated with a higher risk of bleeding and thrombocytopenia. Agents targeting other platelet surface receptors such as PARs, TP receptor, EP3 receptor, GPIb-IX-V receptor, P-selectin, as well as intracellular signaling factors, such as PI3Kβ, have been evaluated in an attempt to develop the next generation of antiplatelet drugs, reduce or eliminate interpatient variability of drug efficacy and significantly lower the risk of drug-induced bleeding. The aim of this review is to describe the pathways of platelet activation in thrombosis, and summarize the development process of antiplatelet agents, as well as the preclinical and clinical evaluations performed on these agents.

Contents

Abbreviations: 12-LOX, 12-(S)-lipoxygenase; AA, Arachidonic acid; ACS, Acute coronary syndrome; ADME, Absorption, distribution, metabolism, and excretion; ADP, Adenosine diphosphate; ALX-0081, Caplacizumab; ATP, Adenosine triphosphate; CABG, Coronary artery bypass graft; CAD, Coronary artery disease; cAMP, Cyclic adenosine monophosphate; CD40L, CD40 ligand; cGMP, Cyclic guanosine monophosphate; COX, Cyclooxygenase; ECM, Extracellular matrix; EP3 receptor, Prostaglandin EP 3 receptor; ERp57, ERp57 is a lumenal protein of the endoplasmic reticulum (ER); Fab, Fragment Antigen-Binding; FcR γ-chain, Fc receptor γ-chain; GP (GPIb-IX-V, GPIbα,GPIbβ,GPIIb/IIIa,GPIX, GPV), Glycoprotein; GPVI-Fc, Glycoprotein VI fragment crystallizable region; IKKβ, IκB Kinase β; IPA, Independent practice association; IV, intravenous; IκBα, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; KGD sequence, Lys-Arg-Gly (KGD) sequence; LMWH, low molecular weight heparin; mAbs, monoclonal antibodies; Mac-1, Macrophage-1 antigen; MI, Myocardial infarction; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NIT family, Nitrilase 1 (NIT1) antibody; NOS, Nitric oxide synthase acute; NSTE ACS, Non-ST-segment elevation coronary syndrome; NSTEMI, Non-ST segment elevation myocardial infarction; P2Y (P2Y1, P2Y12), Purinergic receptors; p65 (RELA), Rel-like domain-containing protein A; PAD, Peripheral arterial disease; PARs, Protease activated receptors; PCI, Percutaneous coronary intervention; PDE (3A), Phosphodiesterase (3A); PI3K (PI3Kα, PI3Kβ), Phosphoinositide 3-kinase; PK, Pharmacokinetic; PSGL-1, P-sel-glycoprotein-ligand-1; PTCA, Percutaneous Transluminal Coronary Angioplasty; PTP1B, Protein tyrosine phosphatase 1B; PTP1B, tyrosine phosphatase 1B; Rac-1, Ras-related C3 botulinum toxin; RGD, Arginine-glycine-aspartic acid; Rho, Ras homologous protein; ROS, Reactive oxygen species; sCD40L, soluble CD40L; ST, stent thrombosis; STEMI, ST-segment elevation MI; STEMI, ST-segment elevation MI; TIA, Transient Ischemic Attack; TIME, Tirofiban in Myocardial Evaluation; TP, thromboxane A2 prostanoid; TP, Thromboxane (TxA2) receptor; TTP, Thrombotic Thrombocytopenic Purpura; Tx (TxA2, TxB2), Thromboxane; TxS, Thromboxane synthase; UA/NSTEMI, Unstable Angina/NSTEMI; vWF, von Willebrand factor..

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1. Introduction

Antiplatelet agents serve as the main therapeutic agents to manage acute thrombo-occlusive ischemic events and prevent secondary complications in vascular diseases. Although the outcomes of patients have been significantly improved owing to numerous antiplatelet therapies developed, the agents currently available are still with limitations including limited efficacy, increased risk of bleeding and variability in individual response. Developments of new treatment agents targeting various molecules involved in platelet function during thrombosis are still ongoing (Di Minno, Momi, Di Minno, & Russolillo, 2013). The process of developing a new agent involves target selection, a preclinical assessment of agent activity and specificity, as well as clinical trials to test the agent's efficacy and safety. Understanding the functional mechanism of the agents and the risks that may arise at different stages of preclinical and clinical studies are crucial in designing new agents. Previous reviews of antiplatelet agents usually focused on sharing the specific perspectives on the drug developed. In this review, we attempt to summarize the antiplatelet agents currently available and being developed, focusing on the targeted molecular pathways involved in platelet activation, as well as the experimental and clinical studies that have led to their therapeutic application.

2. Functional mechanism of platelet in thrombosis

Platelets are the smallest blood components that act as central mediators in thrombosis and hemostasis. Hemostasis, a critical process in preventing hemorrhage caused by damaged vessel walls, was suggested to be comprised of platelet accumulation (adhesion and aggregation) and subsequenent blood coagulation cascades, the two classical platelete-related waves of thrombin generation.

Injury or damage to the blood vessels results in the exposure of subendothelial matrix proteins such as collagen, vWF, fibrinogen, fibronectin, and laminin, to the circulation. These extracellular matrix ECM proteins interact with membrane glycoprotein (GP) receptors on the surface of platelets to trigger adhesion. Platelet adhesion is usually initiated when vWF binds to the GpIb-IX-V complex on the platelet membrane. This binding triggers further interactions between collagen and the GPVI and GpIIb/IIIa receptors. Besides stabilizing adhesion, the binding of ECM proteins to platelet receptors induces the release of platelet activation agonists such as thromboxane A2 (TxA2)and ADP. Activated platelets undergo shape changes into a pseudopodal form to clump into aggregates. In addition, these activated platelets enhance surface expression and conformational changes in the GPIIb/IIIa receptors. The transport of P-selectin to the receptor's surface to support platelet aggregation, is also enhanced. Upon activation, platelets also function as a harbor for coagulation factors and enhance cell-surface associated generation of thrombin; the central mediator in the blood coagulation cascade (Fig. 1). (See Tables 1–4).

3. Pathways involved in platelet adhesion

3.1. Targeting the interaction between GPIb-IX-V and vWF

The GPIb-IX-V complex contains one GPIbα subunit that is disulfide-linked to two molecules of GPIbβ and noncovalently-linked to GPIX and GPV (Andrews, Du, & Berndt, 2007). Initial platelet attachment to subendothelium is mediated by the binding of immobilized vWF to GPIb-IX-V. The interaction induces “out-inside” signaling, activates cell surface glycoprotein receptors (also known as intergrin), including GPIIa/IIb and GPVI, leading to stable adhesion and aggregation. The GPIb-IX-V complex also has a high affinity for thrombin, which can further activate platelets via GPIbα signaling and by accelerating PAR-1 cleavage. After activation through binding to vWF, GPIbα also interacts with other ligands to play a pivotal role in thrombus formation, such as regulating platelet activation through interaction with thrombospondin and P-selectin, promoting coagulation through interaction with factors XI, XII, VIIa, and kininogen, inducting inflammatory responses through interaction with P-selectin and integrin αMβ2, and facilitating arterial remodeling.

Owing to the critical function of GPIb-IX-V and vWF interaction in thrombosis, agents against GPIbα are attractive targets in the development of antiplatelet drugs. ALX-0081 (Caplacizumab), a humanized nano-antibody against vWF, binds to the A1 domain with high affinity. Direct inhibition of vWF by ALX-0081 has been shown to significantly improve peripheral endothelial function in patients with stable single vessel disease and undergoing percutaneous coronary intervention (PCI) (Muller et al., 2013). Phase I and II clinical trials in PCI patients with stable angina and high risk have shown promising antiplatelet effects and relatively safe profiles. Recently, the efficacy of ALX-0081 on acquired thrombotic thrombocytopenic purpura (TTP) have been demonstrated in phase II clinical trials (Peyvandi et al., 2016).

ARC1779, an anti-vWF aptamer, blocks the binding of the vWF A1-domain to GPIb. The function of ARC1779 has been examined in several preclinical and clinical studies (Arzamendi et al., 2011; Bae, 2012; Markus et al., 2011). In patients with CAD receiving double antiplatelet therapy, ARC1779 has been proven to significantly affect platelet adhesion; however, this result does not occur on platelet aggregation, P-selectin expression, and platelet leukocyte binding. Unfortunately, the clinical trial for ARC1779 was not successful and was, therefore, prematurely terminated.

Anfibatide, an anti-GPIbα drug purified from the snake (*Agkistrodon acutus*) venom, inhibits both vWF and α-thrombin binding to GPIbα. The platelet inhibitory activity of anfibatide has been proven in experimental models (Zheng et al., 2016), with the obtained results supporting another study where the agent was used to treat ischemic stroke.

Other agents targeting GPIbα such as h6B4-Fab, GPG-290, and anti-GPIbα NIT family monoclonal antibodies (Andrews et al., 2007) have been mainly studied in the preclinical stage. Binding of GPIbα with partners such as Mac-1 (Wang et al., 2017), which regulates leukocyte-platelet interaction, may however be considered as a target for further development of antiplatelet agents.

For future development of antiplatelet therapies targeting the interaction between the GPIb-IX-V complex and vWF, it must be noted that previous studies in this direction were mainly conducted at relatively early stages. In addition, the absence of an oral administration formula limited the use of these agents to reduce thromboembolism during surgeries. Due to the high specificity of the vWF factor, recent clinical studies have mainly concentrated on the application of these agents as treatments for acquired TTP; the pathology observed was an

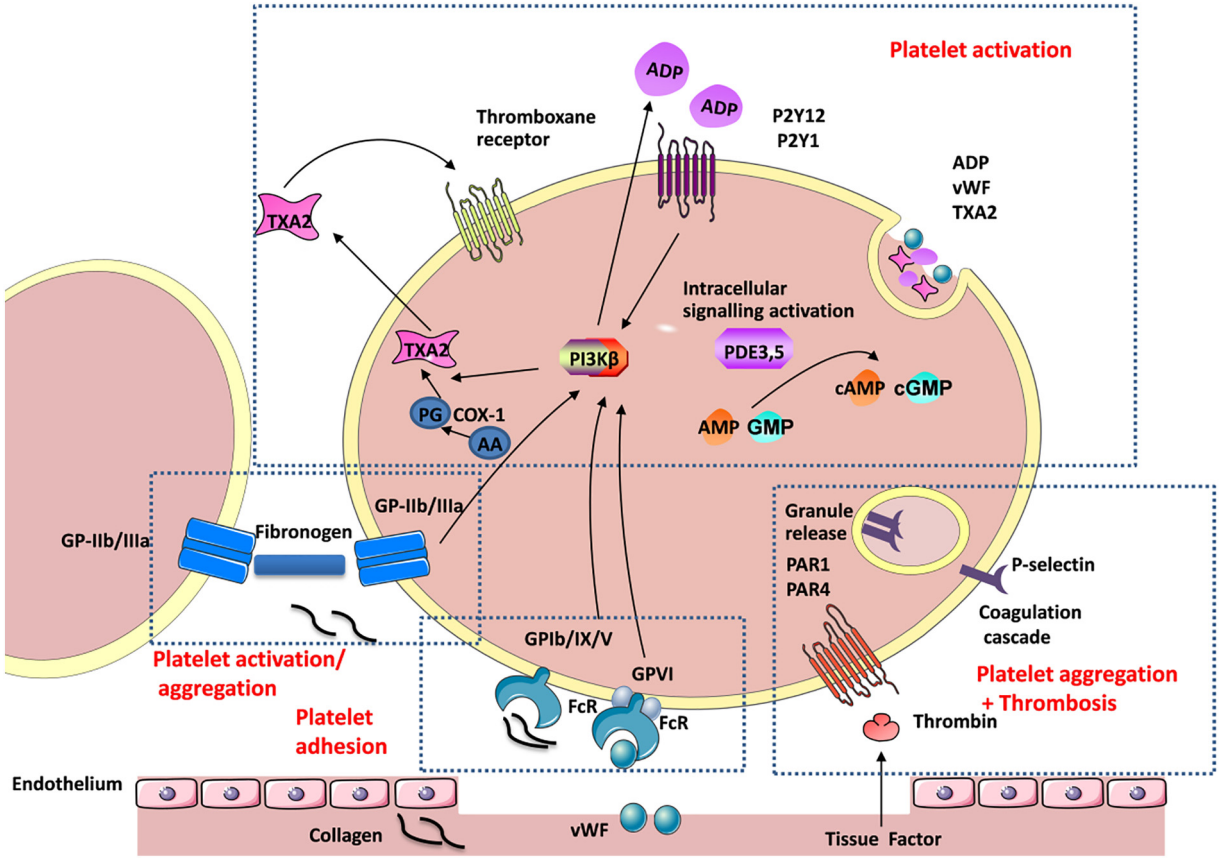

Fig. 1. Illustration of the platelet-related processes in thrombosis. Damage to blood vessels results in the exposure of ECM proteins vWF, collagen, fibrinogen, and fibronectin) to circulation. ECM proteins can then interact with platelet surface GP receptors (GPIb/IX/V) to trigger adhesion. Platelet activation occurs by the interaction of released agonists (TxA2, and ADP) with surface receptors (P2Y receptors, thromboxane receptor), and with intracellular signaling pathways (PDE/cAMP, PI3K, and TxA2 synthesis). The activated platelets undergo shape changes and clump into aggregates. The receptor complexes on the platelet surface (GPIIb/IIIa, PARs, and P-selectin) are involved in platelet aggregation, as well as the initiation of the coagulation cascade.

accumulation of ultra-large vWF multimers. However, the results of earlier studies that inhibiting vWF-mediated platelet adhesion had a higher efficacy and favorable results in reducing bleeding risk when compared to commonly applied antithrombotic drugs, suggesting that it is worth testing agents targeting this pathway in a variety of clinical settings.

3.2. Targeting the interaction between GPVI and collagen

The interaction between GPIb–V–IX and vWF induces GPVI dimerization and a high affinity to collagen. The interaction of GPVI as well as integrin with collagen, then mediates firm adhesion (Varga-Szabo, Pleines, & Nieswandt, 2008). GPVI is a transmembrane glycoprotein and is exclusively expressed on platelets and megakaryocytes. When GPVI couples with the Fc receptor γ-chain, a heterogeneous immunoglobulin-like receptor is formed. GPVI has been demonstrated as the major receptor for collagen to induce platelet activation (Nieswandt & Watson, 2003). The binding of collagen to GPVI induces the downstream tyrosine phosphorylation cascade, as well as other signaling pathways such as Rac1 and changes in intracellular Ca^{2+} level, leading to cytoskeletal rearrangements and changes in the shape of platelets, which is required for platelet aggregation (Vidal, Geny, Melle, Jandrot-Perrus, & Fontenay-Roupie, 2002). GPVI-collagen interaction also triggers the release of ADP and TXA2, which are soluble factors that activate and recruit circulating platelets (Jiang et al., 2015). GPVI-stimulated platelets then release thiol oxidoreductase ERp57 and inorganic polyphosphates to trigger the coagulation pathway (Schulz et al., 2010). It has been also suggested that GPVI-mediated platelet activation leads to the release of inflammatory cytokines that may promote thrombosis (Hitchcock et al., 2015). Besides collagen, GPVI may also interact with laminin and fibronectin which may affect platelet adhesion (Dutting, Bender, & Nieswandt, 2012). Due to its critical role in collagen-induced platelet activation, GPVI appears to be an ideal target for antiplatelet therapy. The advantages of developing a GPVI blockade treatment include interference at the early platelet activation stage, high specificity due to the exclusive expression profile, and lower risk of bleeding.

Multiple strategies have been explored for GPVI antagonist development. GPVI mimics such as GPVI-Fc, which is composed of GPVI extracellular domains fused to the Fc domain of IgG1, interfere with

Table 1
Antiplatelet agents that target platelet adhesion.

Target	Reagent	Representative studies
GPIbα	Caplacizumab (ALX-0081)	Phase 2, (Peyvandi et al., 2016) (Muller et al., 2013)
	ARC1779	Phase 2, (Markus et al., 2011)
	Anfibatide, H6B4-Fab, GPG-290	Preclinical, (Andrews et al., 2007; Lei et al., 2014; Zheng et al., 2016)
GPVI complex	Revacept	Phase 1, (Ungerer et al., 2011); Phase 2 (NCT01645306)
	9O12, 204–11, 5C4, 1G5, OM2	Preclinical, (Jamasbi et al., 2015; Jung et al., 2012; Lecut et al., 2003; Li et al., 2007; Mangin et al., 2012; Matsumoto et al., 2007; Moroi et al., 2003; Ohlmann et al., 2008)
	DZ-697b	Phase 1, (Zafar et al., 2010)
	(Small compound antagonists) Losartan, aptamers	Preclinical, (Flierl et al., 2015; Jiang et al., 2015; Ono et al., 2010)

Table 2
Antiplatelet agents that target platelet activation.

Target	Reagent	Representative studies
P2Y12 (irreversible prodrug)	Clopidogre, clopidogrel thiolactone	Phase 3, CAPRIE study ("A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee," 1996); CURE Trial (Mehta & Yusuf, 2000); PCI-CURE Trial (Mehta et al., 2001); Preclinical, (Chen & Wang, 2016; Shan et al., 2012); Phase 1, (Cushing et al., 2012)
	PLD-301, vicagrell	
	Prasugrel	Phase 3, (JUMBO)-TIMI 26 trial (Serebruany, Midei, Meilman, Malinin, & Lowry, 2006; Wiviott et al., 2005); FABOLOUS PRO Trial (Valgimigli et al., 2010)
	Ticlopidine	Phase 3, CATS Study (Gent et al., 1989); TASS study (Hass et al., 1989)
P2Y12 (reversible direct bingind)	Ticagrelor	Phase 3, RESPOND study (Gurbel et al., 2010); ONSET/OFFSET (Gurbel et al., 2009; Toma, 2010); DISPERSE studies (Husted et al., 2006); DISPERSE2 (Cannon et al., 2007; Juneja et al., 2013); PLATO study (James et al., 2009; Wallentin et al., 2009); TIME trial (Park, Baek, et al., 2014); Pegasustimi 54 trial (Bonaca et al., 2015; Cohen, 2016); FABOLOUS PRO Trial (Valgimigli et al., 2010)
	Cangrelor	Phase 3, CHAMPION PLATFORM(Bhatt et al., 2009); CHAMPION PCI trials (Harrington et al., 2009); CHAMPION PHOENIX (O'Donoghue et al., 2016); Phase 2, BRIDGE trial (Angiolillo et al., 2012);
	Elinogrel (PRT060128)	Phase 2, ERASE-MI Trial (Berger et al., 2009); INNOVATE-PCI Study (Leonardi et al., 2010)
	Regrelor (INS50589), AZD1283, SAR216471	Phase 1, (Johnson et al., 2007); Preclinical, (Bach et al., 2013); Preclinical, (Boldron et al., 2014)
P2Y1	BMS-884775	Preclinical, (Yang et al., 2014)
Phosphodiesterase inhibitor (PDE3)	Dipyridamole	Phase 1/2 TRinity AntiPlatelet responsiveness (TrAP) study (Tobin et al., 2011); Phase 3 ESPS2 (Sivenius et al., 1999), and the Phase 4 ESPRIT trial (Halkes et al., 2006); PRoFESS study (Diener et al., 2008)
	Cilostazol	Phase 4 (Dawson et al., 1998); (Regensteiner et al., 2002); (Shinohara et al., 2010; Uchiyama et al., 2014); (Chen et al., 2013; Geng et al., 2012); (Han et al., 2014; Jeng et al., 2015; Lim et al., 2016; Matsuda et al., 2016; Park, Baek, et al., 2014; Tai, Chen, Chien, & Yang, 2017)
	Anagrelide	IIS (Y. G. Chen, Lin, Shen, & Chian, 2012; Dombi et al., 2017)
	Milrinone	Preclinical (Anfossi et al., 1996; Kondo, Umemura, Miyaji, & Nakashima, 1999)
	DG-401	Preclinical (Fox et al., 2013; Tilly et al., 2014)
Phosphodiesterase inhibitor (PDE5)	Sildenafil (Vigra), Vardenafil (Levitra), Tadalafil (Cialis)	Preclinical and IIS (Akand et al., 2015; Aziret et al., 2014; De Bon et al., 2010; Pingarron-Martin & Arias-Gallo, 2014; Toque et al., 2008)
PDE	BF066, TA-993	Preclinical (Pan et al., 2012); (Katoh, Karasawa, Doi, & Odawara, 2001)
PI3Kβ	AZD6482; TGX-221	Preclinical, (Bird et al., 2011; Nylander et al., 2012)
Cox-1	Aspirin	Phase 3, CARDIFF (Elwood, 1983); (Breddin, Loew, Lechner, Oberla, & Walter, 1980; Breddin, Loew, Lechner, Uberla, & Walter, 1980); Phase 1 Formulation Modification (Loprete et al., 2014; Voelker & Hammer, 2012)
	Acetoxy quinolone 6-AQ, KBT-3022	Preclinical, (Priya et al., 2010) (Matsuo et al., 1997)
Thromboxane synthase	KC-764	Phase 1, (Nakashima et al., 1992)
	Ozagreal	Preclinical, (Myou et al., 2002); IIS, (Zhang et al., 2012)
Thromboxane receptor	Ramatroban, Seratrodast	IIS, (Dogne et al., 2002; Ishizuka et al., 2004; Pieters et al., 1993); Phase 1(Pieters et al., 1993)
	Terutroban (S18886)	Phase 2, TAIPAD study (Fiessinger et al., 2010); Phase 3, PERFORM study (Bousser et al., 2011)
Dual TxS inhibitor and TP antagonist	Picotamide	Phase 3, DAVID study (Neri Serneri et al., 2004)
	Ridogrel	Phase 3, RAPT trial ("Randomised trial of ridogrel, a combined thromboxane A2 synthase inhibitor and thromboxane A2/prostaglandin endoperoxide receptor antagonist, versus aspirin as adjunct to thrombolysis in patients with acute myocardial infarction. The Ridogrel Versus Aspirin Patency Trial (RAPT)," 1994)
	EV-077, NP-184, J78, TRA-418, DG-041, XC386	Preclinical (Fontana et al., 2011; Jin et al., 2005; Kuo et al., 2010; Rollini et al., 2014; Sakariassen et al., 2012; Tello-Montoliu et al., 2012; Tilly et al., 2014; Umemura, Tsukada, Kakiuchi, Yamada, & Matsuura, 2005; Wang, Hsu, Tsai, Huang, & Teng, 1984)
	Hydroxychloroquine,	Phase 1, Hydroxychloroquine (Achuthan et al., 2015)

collagen binding to reduce thrombus formation (Takayama et al., 2008). Phase I clinical trials have shown that humanized GPVI-Fc (Revacept) inhibited collagen-induced human platelet aggregation, without affecting general hemostasis (Ungerer et al., 2011). The efficacy and safety of Revacept in patients with carotid artery stenosis, TIA, or stroke, are currently being evaluated in Phase II studies (NCT01645306). Treatment with Revacept is, however, limited, as the amount of exposed collagen, dosage of product, and risk of immunization require careful evaluation. Other GPVI mimics have also been tested; however, no significant inhibitory effect was detected (Jiang & Jandrot-Perrus, 2014).

Small molecule compounds have also been explored as GPVI antagonists, with the blockage of GPVI interaction with collagen described for the small molecules, aptamers (Flierl et al., 2015). Losartan has also been suggested to inhibit collagen-induced platelet adhesion and activation (Ono et al., 2010). However, the therapeutic value of these reagents requires further examination. Compounds that interfere with GPVI dimerization (Ungerer et al., 2011) or the interaction with downstream signaling factors may be considered as antiplatelet reagents as they facilitate GPVI blockade.

GPVI antibodies are good candidates owing to their specificities and the limited GPVI copies expressed in platelets. Inhibitory antibodies block the interaction of GPVI with collagen, while activating antibodies bound to GPVI can induce the depletion of GPVI from the surface of platelets. The latter results can occur by metalloproteinase-mediated shedding since sustained activation of GPVI can trigger proteolytic attack near the transmembrane domain, resulting in a soluble ectodomain fragment (Chen, Locke, Liu, Liu, & Kahn, 2002). Several GPVI antibodies and the Fab fragments have been shown to inhibit GPVI-mediated platelet activation. Most studies have been performed using 9O12, the results indicated a good antithrombotic efficacy, with absence of the side effect of bleeding (Mangin et al., 2012). However, the efficacy and safety of these antibodies need to be further evaluated in arterial thrombosis models and clinical settings.

FcR γ-chain may be coupled with either GPVI or GPIb. Tyrosine phosphorylation of the FcR γ-chain is critical for the signal transduction downstream of these receptors, to induce platelet adhesion and activation (Quek et al., 2000). DZ-697b is a direct inhibitor of collagen- and ristocetin-mediated phosphorylation of FcR γ-chain. A phase I study (Zafar et al., 2010) showed that the oral agent, DZ-697b, had greater antiplatelet potency, comparable antithrombotic effects and significantly shorter bleeding time compared to clopidogrel.

The inhibition of GPVI has mainly been studied in pre- and early clinical settings for use as a novel direction in the development of antiplatelet agents. The efficacy and application of these agents, however, require

Table 3
Antiplatelet drugs that target Gp IIb/IIIa (platelet activation/aggregation).

Target	Reagent	Representative studies
GPIIb/IIIa	Tirofiban	Phase 3, PRISM and PRISM-PLUS Trials ("A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina", 1998; "Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction", 1998); TARGET trial (Doggrell, 2001)
	Abciximab	Phase 3, EPIC trial (Popma & Satler, 1994); EPILOG trial (van de Werf, 1996); EPISTENT trial ("Randomised placebo-controlled and balloon-angioplasty-controlled trial to assess safety of coronary stenting with use of platelet glycoprotein-IIb/IIIa blockade," 1998); TENACITY trial (Moliterno, 2011); MULTISTRATEGY study (Iavelov, 2008); PRISM-PLUS trial (Theroux et al., 2000; Zhao et al., 1999); SAVI-PCI study NCT01522417; GUSTO IV-ACS trial (Ottervanger et al., 2003); ISAR REACT trial (Gratsianskii, 2004) and later ISAR REACT 2 trial (Kastrati et al., 2006); TARGET trial (Mukherjee et al., 2005);
	Eptifibatide	Phase 3, SAVI-PCI study (NCT01522417); EARLY-ACS trial (Giugliano et al., 2009)
	Roxifiban (DMP754), elarofiban, NQ304,	Phase 1/2, (Murphy et al., 2003); Preclinical, (Y. H. Zhang et al., 2001)
GPIIb	RUC-1, RUC-4	Preclinical (Blue et al., 2009; Li et al., 2014)

further assessment. Interestingly, it has been shown that type 2 diabetes patients have an enhanced platelet surface expression of GPVI. Hyperglycemia, oxidative stress, and elevated vascular shear forces in these patients may also interfere with GPVI signaling, and may be associated with an increased risk of occurrence of thrombotic events (Arthur, Jandeleit-Dahm, & Andrews, 2017). Therefore, the possibility that agents targeting GPVI have a specific efficacy in type 2 diabetes patients is worth examining.

4. Pathways involved in platelet activation

4.1. Targeting ADP receptor (P2Y) pathway

Binding of ADP to the purinergic receptors (P2Y) in platelets, is a major step in the "out-inside" signaling of platelet activation. This binding also plays a critical role in thrombosis, with two families of ADP receptors existing on the surface of platelets. P2Y1 receptors mainly function in stimulating changes in platelet shape and aggregation; these occur by upregulating intracellular calcium (Gachet, 2006). Ligand-coupled P2Y12 induces signaling pathways that amplify and stabilize the aggregates, mainly by down-regulating the intracellular adenylyl–cyclase activity, which results in thrombin generation and stabilization (Cattaneo, 2015).

Table 4
Antiplatelet agents that target platelet aggregation/coagulation initiation.

Target	Reagent	Representative studies
PAR-1	Atopaxa (E-5555)	Phase 2, (Goto, Ogawa, et al., 2010)
	Vorapaxar (SCH530348)	Phase 3, TRACER study (Held et al., 2014; Leonardi et al., 2013; "The Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRA*CER) trial: study design and rationale," 2009); TRA 2 P-TIMI 50 trial (Bonaca et al., 2013; Bonaca et al., 2014; Morrow et al., 2009);
	KC-A0Y	Preclinical, (Lee, 2011)
PAR-4	BMS986120	Phase 1, (Wilson et al., 2018)
P-selectin	PSI-697	Phase 1/2, (Japp et al., 2013)

P2Y12 has been the most attractive target of antiplatelet drug development owing to its central role in platelet activation and aggregation. The clinically-available P2Y12 antagonists can be categorized into two groups based on their mechanism of action. The first group comprises the irreversible binding prodrugs such as ticlopidine, clopidogrel, and prasugrel. Active metabolites of these reagents bind irreversibly to the P2Y12 receptor, inhibiting ADP signaling. The second group includes ticagrelor and cangrelor, which directly bind to P2Y12, without being metabolized, in a reversible manner. The reversible antagonists of P2Y12 have the advantage of platelet function recovery and a lower risk of bleeding, without affecting antithrombotic efficacy.

The thienopyridine prodrugs are the most widely studied antiplatelet drugs that irreversibly inhibit ADP binding to the P2Y12 receptor (Robert, Miller, & Fagan, 1991). Clopidogrel was the first P2Y12 thienopyridine prodrug approved for medical use in 1998, and to this day, has remained the standard choice in antiplatelet treatment. The long period of application has raised the issue of resistance, with recent efforts directed to identifying other forms of drugs to overcome this issue. Examples of such drugs include the phosphate prodrug of clopidogrel thiolactone, PLD-301 (Chen & Wang, 2016), and vicagrel (Shan et al., 2012), which have been developed to improve the oral bioavailability and resistance of antiplatelet agents. A different formulation of clopidogrel has also been tested (Cushing et al., 2012).

Ticagrelor (formerly known as AZD140) was the first approved antagonist that reversibly interacts with the P2Y12 receptor (Goel, 2013). In July 2011, the Food and Drug Administration (FDA) approved ticagrelor administration in combination with aspirin. The main indication of this administration is the secondary prevention of thrombotic events during PCI and CABG management, in patients with ACS. Ticagrelor does not require activation via metabolic processes; thus, enabling a rapid onset and offset action. The risk of renal impairment is also low, owing to hepatic elimination; thus, a dose adjustment is not required and less individual variation exists. The clinical efficacy and safety of ticagrelor were demonstrated in a series of clinical trials. Early-stage trials such as the RESPOND study (Gurbel et al., 2010) revealed superior platelet inhibition by oral ticagrelor therapy, and explored the pharmacokinetic and pharmacodynamic features of the drug. A number of phase II trials, including the ONSET/OFFSET (Toma, 2010) and DISPERSE studies (Husted et al., 2006), further demonstrated the rapid onset and offset action of ticagrelor, and its efficacy in inhibiting platelet aggregation. The efficacy and safety of ticagrelor were further examined in trials such as the DISPERSE2 studies (Juneja, Gupta, & Kaushal, 2013), PLATO study (Wallentin et al., 2009), TIME trial (Park, Baek, et al., 2014) and Pegasustimi 54 trial (Bonaca et al., 2015). The results from these studies showed that ticagrelor significantly improved platelet inhibition and ischemic outcomes (including mortality, MI and stroke) when compared to clopidogrel, in patients with ACS. Another trial proved the effect of ticagrelor in reducing systemic inflammation (Thomas et al., 2015), including platelet-monocyte aggregate formation and the release of pro-inflammatory cytokines. Generally, ticagrelor has been shown to be well tolerated, with bleeding as the most common observed adverse effect (Danielak, Karazniewicz-Lada, & Glowka, 2018). The monitoring of hemoglobin concentration and renal function is recommended with this therapy (Juneja et al., 2013).

Prasugrel, first known as CS-747 or LY640315, is a third-generation thienopyridine prodrug (Niitsu, Jakubowski, Sugidachi, & Asai, 2005). The active form of prasugrel, generated via hepatic metabolism, binds irreversibly to P2Y12 to inhibit platelet aggregation (Robinson, Das, Koshy, & Das, 2009). Previous pre- and early-phase clinical trials have mainly focused on assessing its effect on platelet aggregation. Although the pharmacokinetic profile of prasugrel has not been clearly defined, a phase I study demonstrated that the onset of prasugrel is more rapid than that of clopidogrel, and the effects of platelet inhibition are more significant and consistent (Asai et al., 2006). The efficacy of prasugrel has also been observed in individual cases such as in a patient with

clopidogrel allergy (Chopra, Verma, & Klaustermeyer, 2011). The (JUMBO)-TIMI 26 trial which consisted of patients undergoing PCI (Wiviott et al., 2005) demonstrated that administering prasugrel resulted in a greater platelet inhibition and decreased incidence of ischemic events compared to clopidogrel administration; however, an increased incidence of bleeding events was observed. Prasugrel was approved for use in both Europe and USA in 2009 to reduce thrombotic cardiovascular events in PCI management of individuals with ACS. However, a recommended standard regimen has yet to become available for this agent as it is still under investigation. Phase I studies are also ongoing to evaluate the pharmacokinetics, pharmacodynamics and platelet reactivity in various patient groups (Jeon et al., 2015).

Cangrelor was generated with a chemical modification of ATP to enhance its affinity and resistance to ectonucleotidase degradation. Like ticagrelor, cangrelor does not function as a prodrug which requires metabolic conversion to generate its active form. Cangrelor reversibly binds and inhibits P2Y12. This chemical characteristic results in an almost immediate onset of antiplatelet effects (within 2 min of administration) and longer duration (Akers et al., 2010). A series of clinical trials have been performed to evaluate the efficacy and safety of cangrelor; however, it is mainly represented by 3 major phase III trials. One of these trials is the CHAMPION PLATFORM which was performed in P2Y inhibitor-naïve patients in a placebo-controlled setting (Bhatt et al., 2009). A composite endpoint of ischemic events including death, MI, and ischemia-driven revascularization in the first 48 h following PCI was used as the primary outcome. Bleeding events according to TIMI, GUSTO or ACUITY standards and other complications such as dyspnea, were used as secondary and safety outcomes. The CHAMPION PCI trials (Harrington et al., 2009) evaluated cangrelor within a similar outcome setting; however, a loading dose of clopidogrel was used as the control. Both trials failed to identify any statistically significant improvement in the groups treated with cangrelor for any of the outcomes. The lack of efficacy resulted in the premature terminations of both trials. The non-significant results of these trials were suggested to be related to the MI definition used. Therefore, in the third trial of this series, CHAMPION PHOENIX (O'Donoghue et al., 2016), a universal definition of MI [83] was used to adjudicate the MI events in the outcome assessment. The trial compared the efficacy of cangrelor to clopidogrel in P2Y12 inhibitor-naïve patients with stable angina, NSTEMI, or STEMI, who required PCI. In contrast to the previous 2 trials, CHAMPION PHOENIX identified significant decreases in the primary endpoint events in the cangrelor group; more ACUITY minor bleeding was also observed. Subsequently, the data from CHAMPION PLATFORM and CHAMPION PCI were re-evaluated using the universal definition of MI (Leonardi et al., 2018). This analysis showed a reduction in ischemic events (death, MI, or ischemia-driven revascularization) at 48 h in the cangrelor-administered groups. Cangerlor was approved by the FDA in 2015, and this is mainly attributed to the results of the CHAMPION PHOENIX trial. The main indication of cangrelor is an adjunct to PCI in an effort to reduce the risk of periprocedural MI, repeat coronary revascularization, and stent thrombosis. Cangrelor treatment should not be administered simultaneously with a P2Y12 inhibitor or a GbIIb/IIIa inhibitor, since cangrelor may attenuate the efficacy of these drugs through competitive binding (Siller-Matula, Trenk, Krahenbuhl, Michelson, & Delle-Karth, 2014). As expected, bleeding is the most common adverse effect with cangrelor administration, with dyspnea and worsening of renal function observed in patients with severe renal impairment (Bhatt et al., 2013). The cost of cangrelor may limit its clinical application; however, it may serve as a useful option for patients who are unable to take oral medications. More studies have been performed to test the efficacy and safety of cangrelor in various situations for ACS management. The BRIDGE trial (Angiolillo et al., 2012) is one such study, which evaluated the efficacy and safety of cangrelor in patients requiring a discontinuation of thienopyridine prior to CABG. A higher platelet reactivity was observed in the cangrelor-treated group, without any significant difference in excessive CABG-related bleeding between the two groups. Therefore, cangrelor is worth considering for patients requiring discontinuation of thienopyridine use before surgery.

Elinogrel (also known as PRT060128) is a direct-acting reversible P2Y12 inhibitor. The reversibility of elinogrel may decrease the risk of bleeding in the urgent surgery setting, and may reduce the delay before nonurgent surgery (Ueno, Rao, & Angiolillo, 2010). Elinogrel competes directly with ADP for binding to the P2Y12 receptor. This may be associated with a decrease in spontaneous major bleeding in the lower-flow, lower-shear environment of a bleed where ADP concentrations are higher, than in a high-flow, high-shear stenosis site. Finally, a direct-acting drug may decrease interpatient variability caused by varying degrees of metabolism. Elevating the dosing test of feasibility and tolerability has been performed in phase II studies, including the ERASE-MI pilot trial in STEMI patients before primary PCI (Berger et al., 2009), and the INNOVATE-PCI study in patients undergoing non-urgent PCI (Leonardi et al., 2010).

Regrelor (INS50589) is an earlier-developed reversible P2Y12 antagonists (Johnson et al., 2007). Testing the tolerability, pharmacokinetics, and pharmacodynamics of INS50589 has shown that it is well-tolerated; however, safety concerns have led to the cessation of clinical trials. Screening of new P2Y12 antagonists with novel chemical scaffolds are still in process, and AZD1283 (Bach et al., 2013) and SAR216471 (Boldron et al., 2014) have been tested as drug candidates.

Although P2Y1 generally considered as a redundant factor of P2Y12 with similar function, the antithrombotic efficacy of P2Y1 inhibition is similar to P2Y12 with better safety, and more importantly, a reduced risk of bleeding (Gachet, Leon, & Hechler, 2006). Therefore, a P2Y1 antagonist appears as an attractive target for new antiplatelet drugs. At present, no clinical trial exists for a P2Y1 antagonist, although some drug candidates are being studied (Jeon et al., 2014). One study compared the candidate, BMS-884775, to clopidogrel in thrombus inhibition and bleeding time. The results demonstrated the advantages of BMS-884775, with a higher percentage of thrombus formation inhibition observed and lower level of increase in bleeding time, compared to clopidogrel (Yang et al., 2014).

P2Y12 receptor inhibitors are currently the standard treatment drugs for ACS patients, with several clinical studies performed to validate their use. For the clinical application of these agents, the major concern is the complication of bleeding. Considerable rates of bleeding have been observed in clinical trials with P2Y12 receptor inhibitors; therefore, de-escalation or drug switching regimens should be addressed by future studies. The pharmacokinetics of these agents, which are closely associated with bleeding, vary at the individual level and are related to the individual's genotype. Therefore, these factors should be considered in the future development of agents targeting the P2Y12 receptor. Pharmacogenetics and tailored antiplatelet regimen may also require further studies.

4.2. Targeting intracellular signaling during platelet activation

Besides blocking surface receptors, inhibiting intracellular signaling during platelet activation may exert an inhibitory effect and a protective effect on thrombus (Gresele, Momi, & Falcinelli, 2011). The cyclic nucleotides, cAMP and cGMP, are two major secondary messengers that inhibit almost all known signaling pathways involved in platelet activation including cytoskeletal rearrangement, fibrinogen receptor activation, degranulation and expression of pro-inflammatory mediators. Early efforts to explore the inhibitors of phosphodiesterases (PDEs) as antiplatelet drugs have led to the identification of agents such as CCT62 (Liao, Tzeng, & Teng, 1998), and the natural agent, dicentrine, isolated from the Chinese herb, *Lindera megaphylla* (Yu et al., 1992).

4.2.1. Targeting PDEs

PDEs catalyze hydrolysis, inactivate cAMP and cGMP, and may lead to platelet activation (Gresele et al., 2011). Inhibitors of PDEs may therefore be considered for use as antiplatelet therapies. Platelets express

three PDE isoenzymes: PDE2, PDE3, and PDE5. PDEs 2 and 3 hydrolyze both cAMP and cGMP; however, the efficiency of PDE3 in cGMP hydrolysis is much lower than that of PDE2, whereas PDE5 specifically hydrolyses cGMP (Ito et al., 1996). The antiplatelet activities of nonselective PDE inhibitors, as well as inhibitors of PDE2, PDE3 and PDE5 have been tested. The effect of increasing intracellular cAMP level and platelet aggregation have been observed for some nonselective PDE inhibitors (Buerke, Cyrus, & Darius, 1997), including the vasoactive drug, Pentoxifylline, which is used in patients with intermittent claudication (Ueno et al., 2011). However, the clinical effects of PDEs on ischemic cardiovascular events have not been evaluated. In addition, studies using PDE2 inhibitors have mainly occurred in laboratory settings (Abbott & Thompson, 2004).

PDE3 is the main subtype of PDE expressed in platelets; therefore, the PDE3 inhibitor is the main target in the development of antiplatelet agents (Mawhin, Tilly, & Fabre, 2015). Cilostazol is a prodrug that was initially registered in Asian countries, and was approved by the FDA in 1999 for the treatment of intermittent claudication (Cheng, 1999). As a specific inhibitor of PDE3 in platelets and smooth muscle cells, cilostazol inhibits platelet activation. In addition, cilostazol inhibits adenosine uptake, which further enhances the intracellular level of cAMP (Sun et al., 2002). The antiplatelet activity of cilostazol results in various effects, including the inhibition of platelet aggregation, and suppression of P-selectin expression and TxB2 production (Kariyazono et al., 2001; Momo et al., 1992). Inhibition platelet activation by cilostazol has been proven in vitro and in human experimental settings (Liu et al., 2004; Nomura, Inami, Iwasaka, & Liu, 2004; Tanigawa et al., 2000). As cilostazol treatment has a relatively short recovery time in platelet function, the bleeding risk did not increase when administered and compared to conventional antiplatelet drugs. The efficacy and safety of cilostazol have been tested in many clinical studies; cilostazol was observed to increase the walking distance (Dawson, Cutler, Meissner, & Strandness Jr., 1998) and improve the quality of life of peripheral arterial disease patients (Regensteiner et al., 2002). Trials with recurrent ischemic stroke as the primary outcome, such as the CSPS 2 study, showed that cilostazol is effective in preventing stroke recurrence and other ischemic events, without significantly increasing the risk of bleeding (Shinohara et al., 2010). A meta-analysis of trials in patients with atherothrombotic disease has also shown that cilostazol reduced the risk of cerebrovascular events, with no increase in bleeding (Uchiyama et al., 2009). The efficacy of combined therapy of cilostazol to aspirin plus clopidogrel has also been demonstrated (Chen, Qian, Chen, Ma, & Ge, 2013). More studies have been performed to test the efficacy of cilostazol in various clinical situations (Han et al., 2014; Jeng et al., 2015; Park, Baek, et al., 2014), and currently, cilostazol is used as an antiplatelet drug worldwide.

Several other PDE3 inhibitors have been tested for their antiplatelet activities. Anagrelide has been shown to inhibit platelet aggregation and is also clinically used in patients with essential thrombocythemia (Dombi et al., 2017). Milrinone, a specific PDE3A inhibitor, is currently used in a clinical setting for congestive heart failure. Its effects on cAMP level and platelet aggregation have also been proven (Anfossi et al., 1996). DG-041 is another PDE3 inhibitor that has been developed, and is currently being assessed in a Phase II clinical trial for its use as an antiplatelet agent (Tilly et al., 2014).

Dipyridamole, a PDE3–PDE5 inhibitor, has been used as a coronary vasodilator for several years. Its activity in inhibiting platelet aggregation has been tested in the laboratory (Nakamura, Uchiyama, Yamazaki, & Iwata, 2002), and with animal models (Choudhury et al., 1996). Results from the TrAP study (Tobin et al., 2011) demonstrated ex vivo the effects of platelet inhibition by dipyridamole addition to aspirin administration. However, clinical evidence of the antithrombotic effect of dipyridamole remains lacking. In the trials such as ESPS2 (Sivenius et al., 1999) and the ESPRIT trial (Halkes, van Gijn, Kappelle, Koudstaal, & Algra, 2006), where the combination of dipyridamole with low-dose aspirin was evaluated, dipyridamole was shown to reduce the risk of stroke in patients with ischemic cerebrovascular disease. Based on the meta-analysis results, combination therapy is more effective than aspirin-only treatment (Verro, Gorelick, & Nguyen, 2008). The ACCP 2008 guidelines recommend dual therapy with extended-release dipyridamole plus aspirin, to prevent stroke, following the first transient ischemic attack or stroke (Hirsh, Guyatt, Albers, Harrington, & Schunemann, 2008). The later PRoFESS study where the combination of aspirin and extended-release dypiridamole was compared to clopidogrel showed that the rate of stroke recurrence was similar between the two treatments (Diener et al., 2008).

Various studies have examined the effect of three PDE5 inhibitors, sildenafil (Viagra), vardenafil (Levitra) and tadalafil (Cialis), on platelet aggregation, bleeding and other endpoints including GPIIb/IIIa receptor activation and P-selectin expression (Akand et al., 2015; Aziret et al., 2014; De Bon, Bonanni, Saggiorato, Bassi, & Cella, 2010; Pingarron-Martin & Arias-Gallo, 2014; Toque et al., 2008). A lack of studies evaluating the outcomes of clinical events however, remain. Another reported agent that may function in PDE inhibition and the phospholipid pathway is BF066. This agent was shown to inhibit platelet aggregation and thrombus formation in a dose-dependent manner (Pan et al., 2012).

PDE inhibitors can function in platelet activation and regulate the interaction between platelet and vascular cells; therefore, they can be effectively used as antiplatelet agents. However, since PDEs are widely distributed and are involved in many physiological functions, the side effects of these agents can be concerning. Continued platelet inhibition is critical in preventing thrombosis; thus, temporary reversibility may increase the risk of ischemic events. Since the effect of currently available PDE inhibitors may be reversible, their clinical effectiveness may be limited. In future developments of antiplatelet drugs, it is suggested that the specificity and irreversibility of agents targeting PDE be given high priority.

4.2.2. Targeting the PI3K tyrosine kinase pathway

Upon activation, P2Y12 receptors induce downstream intracellular signaling to activate platelets.

PI3Kβ, an isoform of Class I lipid kinases, is a crucial mediator in signal transduction downstream of ADP/P2Y12 binding. Therefore, PI3Kβ is a potential candidate in the development of antiplatelet drugs. AZD 6482, a PI3Kβ inhibitor has been tested for its effects on platelet-rich plasma aggregation and is currently being evaluated in a preclinical study (Nylander et al., 2012). TGX-221, a selective PI3Kβ inhibitor, has been tested at the clinical phase. When administered to patients, TGX-221 significantly reduced arterial thrombosis with no increase in BT, when compared to the combination of aspirin and clopidogrel (Bird, Smith, Bostwick, Shipkova, & Schumacher, 2011). A critical requirement in PI3Kβ inhibitor development is specificity. This is because other isoforms such as PI3Kα are involved in many essential physiological pathways such as insulin signaling.

Other agents such as the inorganic salt, sodium tungstate (Na_2WO_4) can function via the tyrosine kinase pathway. Na_2WO_4 functions as a protein tyrosine phosphatase 1B (PTP1B) inhibitor and may also function as a potential antiplatelet agent. Its inhibitory effect on platelet adhesive and cohesive properties has also been demonstrated in vitro (Fernandez-Ruiz et al., 2015). The G protein G13, a linker between receptor and Rho/Rho-kinase pathway, has been suggested to be involved in platelet activation. Therefore, this protein may also function as a potential target of antiplatelet drugs (Moers, Wettschureck, & Offermanns, 2004).

In the future development of agents targeting tyrosine kinase, one should consider that since PI3K is a central signaling factor involved in numerous cellular and physiological functions, the specificity of agents targeting this pathway is key to developing an antiplatelet treatment. Clinically, these agents may be more suitable as an adjunct, by combining it with other antiplatelet drugs.

4.2.3. Targeting the TxA2 pathway

TxA2, a member of the icosanoids family, is an unstable platelet-aggregating factor generated by phospholipids (Bhagwat, Hamann, Still, Bunting, & Fitzpatrick, 1985). Adhesion of platelets to an injured vascular surface triggers the elevation of intracellular Ca^{2+} level in platelets; thus, prompting phospholipases to release arachidonic acid (AA) from the cell membrane (Sohn et al., 2000). TxA2 is synthesized from AA through a cascade of enzyme catalyzed reactions, where the consequent actions of COX and thromboxane synthase (TxS) play critical roles (Smith, Marnett, & DeWitt, 1991). The released TxA2 functions as an inducer of platelet activation by interacting with the TxA2 receptors (TP) on platelets, in an autocrine and a paracrine manner. The most commonly targeted pathway by antiplatelet treatment is that of TxA2, to reduce ischemic events in cardiovascular patients. COX-1, TxS, and the TP receptor have been the major targets in the development of antagonist agents.

Aspirin (acetylsalicylic acid) exhibits its effect by irreversibly inhibiting the COX-1 enzyme in the AA pathway. The early CARDIFF trials in the 1970s demonstrated the efficacy of aspirin as the first line of antiplatelet treatment (Elwood, 1983). The benefits of aspirin in the secondary prevention of cardiovascular events have also been assessed in numerous trials (Breddin, Loew, Lechner, Oberla, & Walter, 1980; Breddin, Loew, Lechner, Uberla, & Walter, 1980). After decades of aspirin usage, efforts are still being dedicated to modifying the formulation to improve the onset of action (Loprete, Leuratti, Scarsi, & Radicioni, 2014). The function of other agents in inhibiting COX-1 has also been explored. A study has shown that the acetoxy quinolone, 6-AQ, which inhibits COX-1 by activating NOS activity through inducing its acetylation, inhibits platelet aggregation (Priya et al., 2010). KBT-3022 has also been explored as a potential antiplatelet drug; however, a lack of effects on platelet aggregation has been observed (Matsuo, Yokota, Yamashita, & Oda, 1997).

Several TxS inhibitors have been evaluated in different clinical settings. In previous years, the antiplatelet potential of pyrazolopyridine derivatives, such as KC-764, which inhibit TxS, has been evaluated. Although studies have examined the pharmacokinetics (Nakashima et al., 1992), effects on platelet aggregation (Momo et al., 1992), and protective effect of TxS inhibitors on myocardial damage using animal models (Ohmuro, Tsukada, Taga, Ohkubo, & Uchida, 1992), these results did not lead to the clinical application of this drug. Ozagreal is the only TxS inhibitor that is registered for clinical use, on asthma and neurological impairment in stroke patients.

TP antagonists may be a more effective antiplatelet and antithrombotic treatment target as they block TxA2 and the effects of other TP ligands. TP antagonists have also shown potential effects on endothelial function and plaque formation (Davi, Santilli, & Vazzana, 2012). Many TP antagonists have been explored in clinical studies; however, only a few reached the phase III stage. Examples include ramatroban and seratrodast, which were registered to treat allergic rhinitis and asthma (Dogne, de Leval, Benoit, Delarge, & Masereel, 2002; Ishizuka, Matsui, Okamoto, Ohta, & Shichijo, 2004). Terutroban (S18886) is a competitive TP antagonist and the phase II TAIPAD study (Fiessinger et al., 2010), demonstrated its tolerability and potential effectiveness. Another study, a phase III PERFORM study, which was aimed at demonstrating the efficacy of terutroban in reducing the number of cerebrovascular and cardiovascular events in patients with cerebrovascular diseases, did not show terutroban to have any superiority over aspirin. Therefore, terutroban was terminated for futility (Bousser et al., 2011). It is however suggested that the beneficial effects of terutroban be further evaluated in specific patients such as diabetics.

Agents with the dual activities as a TxS inhibitor and a TP antagonist have also been explored as a form of antiplatelet therapy. The efficacy of the dual TxS inhibitor and TP antagonist, picotamide, compared to aspirin, was demonstrated by the DAVID study (Neri Serneri, Coccheri, Marubini, & Violi, 2004) to reduce the number of all-cause mortalities among patients with PAD and diabetes. Although more trials in larger populations are required to further confirm its superiority over aspirin, it has been registered in Italy to treat PAD. Antiplatelet activity has also been detected with the use of another dual antagonist, ridogrel (Naran & Chetty, 1997); however, the RAPT trial ("Randomized trial of ridogrel, a combined thromboxane A2 synthase inhibitor and thromboxane A2/prostaglandin endoperoxide receptor antagonist, versus aspirin as adjunct to thrombolysis in patients with acute myocardial infarction. The Ridogrel Versus Aspirin Patency Trial (RAPT)," 1994) failed to demonstrate its efficacy over aspirin (at the dose examined) in patients undergoing thrombolysis for acute MI. EV-077, which was developed to treat vascular complications in patients with type 2 diabetes, has been proven to result in a rapid onset effect on platelet aggregation (Tello-Montoliu et al., 2012). However, clinical trial (NCT01551381) was terminated at Phase II in 2013 (data from the NIH Trials Site). The potential of these compounds as antithrombotic agents may also be worth further exploring.

The TxA2 pathway has been the most targeted pathway in the development of antiplatelet drugs, and aspirin was the first antiplatelet therapeutic drug. Recent clinical studies have mainly concentrated on situations that require a specific application of aspirin, such as in PAD and diabetic subjects. For further clinical development of these agents, an assessment of platelet response during treatment should be performed, since it may be influenced by individual characteristics as well as drug-drug interactions. An inadequate response may cause "aspirin resistance"; therefore, the clinical effectiveness of future developed agents targeting TxS should be further examined to verify if they can be translated to diabetes, or other diseases more closely related to the signaling pathways.

4.2.4. Targeting other intracellular signaling pathways involved in platelet activation

Other mechanisms involved in the regulation of cAMP levels may be explored as targets for antiplatelet activity, and can be accompanied by a lower risk of bleeding (Fuentes & Palomo, 2014b). Protease activated receptors (PARs) can exert their effects on multiple signaling pathways involved in antiplatelet activation, including COX-1, protein kinase C-alpha, calcium mobilization, thromboxane A2, sCD40L, platelet microparticles, cAMP- PDE, and proteins kinases G and A. Therefore, PARs may be considered as therapeutic targets for the treatment and prevention of cardiovascular diseases (Fuentes & Palomo, 2014a). Other factors involved in the regulation of cAMP homeostasis, such as multidrug resistance protein 4 (MRP4 or ABCC4) may also be tested for its antiplatelet effects and therapeutic potential in atherothrombotic disease (Belleville-Rolland et al., 2016).

Platelets do not have a nucleus; thus, it can be presumed that transcription regulation may not play an important role in platelet regulation. However, the central transcription regulator in an inflammatory response, NF-κB, has been reported to be involved in the regulation of platelet activation. It is therefore reasonable to consider events in the NF-κB pathway, such as IKKβ phosphorylation, IκBα degradation, and p65 phosphorylation, as potential targets of antiplatelet and antithrombotic treatments (Chen et al., 2016; Fuentes, Rojas, & Palomo, 2016). There have been reports that protein acetylation is involved in the regulation of adhesion, aggregation, and granule release of platelet, which may also function as a novel direction in antiplatelet therapy (Latorre & Moscardo, 2016).

Reactive oxygen species (ROS) may participate in the regulation of platelet activation; therefore, factors involved in redox regulation can be tested for their antiplatelet effects (Ferroni et al., 2012). Examples include the oxygenase platelet-type 12-(S)-lipoxygenase (12-LOX) which has been shown to potentiate platelet activation and the selective 12-LOX inhibitor, ML355, which decreases thrombosis without prolonging hemostasis (Luci et al., 2010). More clinical studies are, however, required to determine their safety and effectiveness (Mangin, Tourdot, & Holinstat, 2017)

5. Targeting the pathways involved in platelet activation/aggregation: Glycoprotein IIb/IIIa (integrin αIIbβ3)

Glycoprotein (GP) IIb/IIIa is the most abundant receptor expressed on the membranes of platelets and megakaryocytes. Upon activation, the "inside-out" signaling causes conformational changes in the receptor to acquire an affinity to its ligands; fibrinogen, vWF, fibronectin, and vitronectin. Of these ligands, fibrinogen results in a GPIIb/IIIa cross-link between platelets, resulting in platelet aggregation (Shattil & Newman, 2004). Therefore, GPIIb/IIIa inhibition is described as a very effective approach in antiplatelet treatment.

The development of GPIIb/IIIa inhibitors was mostly performed from 1990 to 2000. Several pilot studies (Harrington et al., 1995; Nicholson et al., 1991; Peerlinck et al., 1993; Theroux et al., 1996) explored the pharmacokinetics, pharmacodynamics, as well as the antiplatelet aggregation effects of several of these inhibitors. These included the antibody fragment agents such as c7E3 (Abciximab) and hC4G1, peptide glycoprotein/mimic integrilin, and SC47,643, as well as the non-peptide antagonists, MK-383 (tirofiban), and lamifiban. These early studies demonstrated that GPIIb/IIIa inhibitors effectively inhibited platelet aggregation without any significant risk of bleeding.

The clinical trials during this period led to the approval of three GPIIb/IIIa inhibitors by the FDA: Abciximab in 1997, and Eptifibatide and Tirofiban in 1998. These agents were administered to patients via the intravenous route. Abciximab is an antigen-binding fragment (Fab) of the humanized monoclonal antibody, 7E3. Abciximab binds to GP IIb/IIIa in an irreversible manner, blocking the binding of other ligands (Tam, Sassoli, Jordan, & Nakada, 1998). Since the agent is not cleared via the renal pathway, dose adjustment in patients with renal dysfunction is not required. Another inhibitor, eptifibatide, was derived from a protein found in the venom of *Sistrurus miliarius barbouri*. Eptifibatide is a heptapeptide containing the KGD sequence (Tcheng & O'Shea, 1999), and unlike abciximab, has a reversible binding affinity to the GPIIb/IIIa receptor. Tirofiban, another approved GP IIb/IIIa inhibitor, is a nonpeptide mimic that act as an arginine-glycine-aspartic acid (RGD). Like eptifibatide, tirofiban binds reversibly to the GPIIb/IIIa receptor, with high specificity.

The first major clinical trial on GPIIb/IIIa inhibitors was the EPIC trial in 1994 (Popma & Satler, 1994). Together with the later expanded EPILOG trial (van de Werf, 1996), abciximab was demonstrated to reduce the risk of composite ischemic outcomes in the patients with elective and non-elective PCI. The results of the EPIC and EPILOG trials supported abciximab as a standard of care in PTCA without stenting. Later, the EPISTENT trial ("Randomised placebo-controlled and balloon-angioplasty-controlled trial to assess safety of coronary stenting with use of platelet glycoprotein-IIb/IIIa blockade," 1998) showed that abciximab had benefits in PCI with stenting, and was associated with an increased longevity of event-free survival.

PRISM and PRISM-PLUS ("A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina," 1998; "Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction,"1998) were the critical studies that led to the approval of tirofiban. PRISM-PLUS demonstrated that adding tirofiban significantly lowered the mortality and MI rates more than only heparin administration did. After the trial, tirofiban became the most widely used small molecule GPIIb/IIa inhibitor to treat ACS from 1998 to 2000. However, the later results from the TARGET trial (Doggrell, 2001) demonstrated that tirofiban was less efficient in IPA than abciximab. Several other studies have shown a lower efficiency when tirofiban is administered, compared to the other GPIIb/IIIa inhibitors; this led to a decrease in its usage. A high dose bolus regimen of tirofiban was approved for PCI in Europe, in 2010. The TENACITY trial in 2011 demonstrated that this regimen achieved greater inhibition of platelet aggregation (Moliterno, 2011). Additionally, in the PRISM-PLUS trial (Theroux et al., 2000; Zhao, Theroux, Snapinn, & Sax, 1999) aimed at evaluating the efficacy of empiric GPIIb/IIIa inhibition in patients with NSTEMI, the results showed that the addition of eptifibatide to aspirin, and unfractionated heparin in NSTE-ACS, improved the ischemic outcomes. These results, combined with efforts to evaluate the usage of clopidogrel, led to its application in a dual antiplatelet therapy (aspirin plus clopidogrel, eptifibatide, or tirofiban).

The shortage of GPIIb/IIIa inhibitors was indicated in some of the later studies. The GUSTO IV-ACS trial (Ottervanger et al., 2003) in 2001 showed that abciximab treatment did not confer any benefit in terms of reducing the mortality or MI in patients with UA/NSTEMI; administering the drug may have also been harmful to patients. The ISAR REACT trial (Gratsianskii, 2004) and the later ISAR REACT 2 trial (Kastrati et al., 2006), demonstrated that the benefits of abciximab in PCI after clopidogrel administration were limited in certain patients with STEMI and high-risk NSTE-ACS. Furthermore, the ACUITY trial (Stone et al., 2006) in 2006 which aimed to evaluate the direct thrombin inhibitor, bivalirudin, showed that it was as effective as combining UFH or low molecular weight heparin (LMWH) with a GPIIb/IIIa inhibitor. This trial resulted in a recommendation by the ACC/AHA guideline to omit GPIIb/IIIa inhibitors in NSTEMI patients receiving bivalirudin. Another study, the HORIZONS-AMI trial (Mehran et al., 2009), further confirmed the efficacy of bivalirudin, and in 2009, the EARLY-ACS trial (Giugliano et al., 2009) showed that triple antiplatelet treatment (eptifibatide along with aspirin and clopidogrel) in UA/NSTEMI patients resulted in higher bleeding rates, without reducing ischemic endpoints.

More trials were also performed to compare the GPIIb/IIIa inhibitors. The TARGET trial (Mukherjee et al., 2005) was the first in this category, and the results showed that abciximab was superior to tirofiban's original dosing regimen. The MULTISTRATEGY study (Iavelov, 2008), which compared high-dose bolus tirofiban to abciximab in patients with STEMI, showed similar efficacies, bleeding profiles, and overall mortality between the drugs; however, abciximab was associated with a more significant moderate to severe thrombocytopenia. Additionally, a meta-analysis of 31 trials and real-world registry data showed a similar efficacy between tirofiban and abciximab (Valgimigli et al., 2010).

In recent years, the use of GPIIb/IIIa inhibitors has been reduced for several reasons. Besides the superiority of the thrombin inhibitor (bivalirudin) over GPIIb/IIIa inhibitors as demonstrated in the HORIZONS-AMI trial, newer P2Y12 inhibitors such as prasugrel and ticagrelor (from studies such as the FABOLOUS PRO Trial (Valgimigli et al., 2010)) have exhibited a faster and more pronounced antiplatelet effect; therefore, replacing the GPIIb/IIIa inhibitors. The approval of cangrelor further decreased the application of the GPIIb/IIIa inhibitors.

Some of the later studies attempted to identify other GPIIb/IIIa receptor inhibitors, such as roxifiban (DMP754) and elarofiban. (Murphy et al., 2003). Antiplatelet agents such as NQ304, which target the downstream intracellular pathway of GPIIb/IIIa receptor (Zhang et al., 2001) and monoclonal antibodies (mAbs) against the integrin PSI domain (with endogenous thiol isomerase function) (Zhu et al., 2017), have been suggested as novel targets for antiplatelet therapy. Recently, novel agents targeting GPIIb rather than GPIIIa, such as RUC-1 and RUC-4, have been suggested for use as a prehospital therapy for MI (Blue et al., 2009; Li et al., 2014).

GPIIb/IIIa inhibitors are effective antiplatelet agents for ACS patients. The major clinical concern however, is its heterogeneous pharmacodynamics. Evaluating the factors that influence the pharmacokinetics, as well as conducting a timely platelet response assessment, may serve as the focus of future studies geared to developing antiplatelet agents that target this pathway. In addition, factors that interact with GPIIb/IIIa, such as the CD40 ligand (CD40L) (Andre et al., 2002), may be considered as future targets in the development of antiplatelet agents.

6. Targeting platelet aggregation/interaction with coagulation

6.1. Targeting PAR

PARs are cell surface receptors activated via proteolytic cleavage of the extracellular domain. Four PAR members have been identified; PAR-1, PAR-3 and PAR-4 are activated by thrombin, while PAR-2 is activated by trypsin (Nieman, 2016). Human platelets express PAR-1 and PAR-4, and PAR-1 is suggested to be the key mediator of the thrombin-induced signaling pathway in platelets. Thrombin cleavage of the extracellular loop results in a new amino acid terminus that binds intra-molecularly to the proximal part of the receptor, activating intracellular signaling (Cunningham, McIntosh, Bushell, Sloan, & Plevin, 2016). A PAR-1 antagonist is an attractive target for antiplatelet and antithrombotic therapy as it inhibits thrombin-mediated platelet activation, without affecting thrombin's role in the coagulation cascade; a limited impact on bleeding also results. A strong interaction between the receptor and antagonist is required to effectively inhibit the intramolecular binding of a tethered ligand. Only two on-going pipelines of the PAR-1 antagonists, atopaxar (E-5555) and vorapaxar (SCH 530348) which are under investigation, are currently being used despite the great potential of other drugs in this category.

After early testing of the safety and exploratory efficacy of SCH530348 (Goto et al., 2010), the pharmacokinetics and pharmacodynamics of vorapaxar in healthy subjects demonstrated rapid and sustained inhibition of platelet aggregation, without an effect on bleeding or clotting times (Kosoglou et al., 2012). In addition, end-stage renal disease (Kosoglou et al., 2012) and hepatic impairment (Statkevich et al., 2012) did not clinically affect the pharmacokinetics and pharmacodynamics of this drug. A phase III trial TRACER study performed in high-risk patients with NSTE ACS to evaluate the efficacy and safety of SCH530348 (Held et al., 2014), failed to establish the efficacy of vorapaxar on the primary outcome (a 2-year rate of composite cardiovascular events). However, the exploratory analysis indicated that vorapaxar was associated with a reduction in MI. Another study, the TRA 2 P-TIMI 50 trial, was performed to evaluate the effect of vorapaxar on the rate of coronary stent thrombosis in stable patients with a history of coronary stenting, prior MI, peripheral arterial disease, or stroke. Although the risk of cardiovascular death, MI, or stroke in patients with PAD was not significantly reduced by vorapaxar treatment, a significant reduction in acute limb ischemia and peripheral revascularization was observed. Treatment with vorapaxar is also associated with an increased risk of bleeding (Bonaca et al., 2013; Bonaca et al., 2014; Morrow et al., 2009). In 2014, vorapaxar became the first PAR-1 antagonist approved for use in the US, by the FDA, to function as an antiplatelet agent in patients with cardiovascular diseases.

The efficacy and safety of another oral PAR-1 antagonist agent, atopaxar, has been evaluated in Japanese patients with ACS or high-risk coronary artery disease; this drug also reached Phase II in the clinical trial (Goto, Ogawa, Takeuchi, Flather, & Bhatt, 2010). The results showed that atopaxar resulted in significant platelet inhibition, without increasing the clinically-significant bleeding. A significant dose-dependent increase in liver function abnormalities was, however, observed, and development of the compound was discontinued in 2012. Other agents of PAR-1 antagonists, such as KC- A0Y (Lee, 2011) in the pilot stage, has also been reported.

The affinity of PAR-4 for thrombin is much lower than that of PAR-1. It is suggested that PAR-4 may be a rescue pathway that is only engaged in when there is a high concentration of thrombin such as in occlusive thrombosis. Therefore, antiplatelet agents against PAR-4 may be more selective in inhibiting thrombus, with no effect exhibited on normal hemostasis. PAR-4 antagonist development is still, however, in the early stages (Dumas et al., 2012). A phase II clinical trial of the oral PAR-4 antagonist, BMS-986120, was also reported (Wilson et al., 2018).

Currently, most PAR-1-targeting agents, except vorapaxar, are still in early clinical stages. More importantly, the application of these agents may be limited by several factors including: excessive bleeding which has been observed in clinical trials; agents more prone to being used as an adjunct in PAD and post-MI settings; unfamiliarity of physicians to these agents; and cost. For further clinical development and the future search for new agents, more clinical trials and a wider application base are required to address these issues.

6.2. Targeting P-selectin

Platelet activation induces the expression of P-selectin on its surface. By interacting with its ligand PSGL-1, P-selectin can trigger an "out–inside" signaling pathway, and induce the formation of platelet–monocyte aggregates and vascular inflammation to facilitate the formation of thrombus. Therefore, blocking P-selectin/PSGL-1 interaction is a potential direction in antiplatelet therapy (Furie & Furie, 2004). The inhibitory effect of PSI-697, a novel P-selectin antagonist, on platelet–monocyte aggregate formation failed in an early stage trial. Therefore, the clinical efficacy of PSI-697 requires establishment (Japp et al., 2013). Recently, crizanlizumab, an antibody against P-selectin, was developed to prevent Pain Crises in Sickle Cell Disease instead of for use as an antiplatelet therapy. The result of the Phase II SUSTAIN clinical trial with crizanlizumab has been published (Ataga et al., 2017). Currently, the study of antiplatelet agents targeting P-selectin is rare; however, the previous study has suggested that its use is a new direction in the development of novel antiplatelet agents.

7. Anti-coagulant

Platelet aggregation and subsequenent blood coagulation cascades together result in thrombosis. Anti-coagulant agents targeting thrombin inhibitor such as non-vitamin K antagonist oral anticoagulants, and factor Xa inhibitors including Rivaroxaban, Apixaban, otmixaban, betrixaban, etc., are also important part for anti-thrombotic agent development. Since this review is focusing on antiplatelet molecular mechanism, we did not include the agents targeting molecules outside platelet in the current summary. The development and clinical effects of these agents have been reviewed in some previous studies (Becattini, Lignani, & Agnelli, 2012; Khadse, Sharma, Murumkar, Rajput, & Yadav, 2018).

8. Discussion and future directions

Although antiplatelet agents are commonly prescribed to effectively prevent cardiovascular and cerebrovascular events, two major adverse events often occur: 1) intersubject variability in the antithrombotic effects, which is largely due to their suboptimal ADME properties; and 2) the risk of internal bleeding when the physiological homeostasis of pro- vs. anti-thrombotic functions is compromised. Therefore, the discovery and development of the next generation of antiplatelet drugs should focus on two aspects: 1) Reducing or eliminating interpatient variability of drug efficacy, by improving pharmacological properties such as rapid onset of action, minimum drug-drug interactions and lower intersubject pharmacokinetic variations; and 2) Identifying new targets or mechanisms that enable selective inhibition of the pathological process of thrombosis while exerting minimum effects on the physiological thrombotic hemostasis, to significantly lower the drug-induced risk of bleeding and enhance drug tolerability and safety. In addition, the antiplatelet agents with a differential manner of action, such as reversible inhibition with a short target resident time, could confer a wider therapeutic window. Combining different agents, with a reduced dose of each, may also function as a strategy to mitigate the side effect of bleeding, while maintaining sufficient efficacy.

Multiple progresses in addressing the liabilities associated with antiplatelet drugs have been achieved by generating novel antagonists for the P2Y family of receptors. Compared to classical P2Y12 receptor antagonists, a new generation of P2Y12 receptor antagonists, such as ticagrelor and cangrelor, have exhibited favorable pharmacokinetic profiles, with less PK and efficacy variations among patients. Interestingly,

these new P2Y12 inhibitors bind both rapidly and reversibly to the target, resulting in a fast and complete inhibition of platelet function and a lower risk of bleeding. ACT-246475, another novel reversible antagonist of P2Y12 (currently under clinical development), has not been observed to affect vascular muscle relaxation or contraction, but has demonstrated a wider therapeutic window than ticagrelor in a rat model (Juif, Boehler, Dobrow, Ufer, & Dingemanse, 2019; Rey et al., 2017). Finally, it was reported that the P2Y1 receptor antagonists can significantly reduce the formation of thrombus with less BT. Therefore, combining the P2Y12 and P2Y1 antagonists may overcome the disadvantages of P2Y12 antagonist monotherapy (Benimana, Zhao, Kong, Li, & Xie, 2017). Antagonists of the P2Y family of receptors, with complimentary activities, may be the future direction of antiplatelet therapy owing to their increased efficacy and lower side effects.

A recent study on a previously underappreciated platelet receptor, PAR-4, has shed light on a distinct platelet regulatory mechanism. In contrast to PAR-1, PAR-4 is a low-affinity thrombin receptor that mediates a later stage of platelet activation which can only be triggered by a high concentration of thrombin; an event critical for pathological thrombosis. This suggests that PAR-4 does not only function as a "backup" thrombin receptor to PAR-1, but that it regulates platelet function at a distinct stage, which may be preferentially required for pathological thrombosis. The results from an ex vivo study of the PAR-4 inhibitor, BMS-986120, supported the hypothesis that PAR-4 inhibition only affects thrombus formation at high shear conditions (mimic stenosis, diseased arteries), with no similar results at low shear condition (mimic epicardial arteries and some veins) (Wilson et al., 2018). Thus, inhibiting PAR-4 might provide a wider therapeutic window than that of existing antiplatelet agents. Considering the unique mechanism of the PAR-4 antagonists, future combination with different antiplatelet agents (aspirin, P2Y12 antagonists or vorapaxar) should be explored to evaluate the antithrombotic efficacy and safety profiles.

Recent progress in the development of new P2Y12 antagonists has lowered the clinical usage of GPIIb/IIIa receptor antagonists. This type of antagonists blocks the final common pathway of platelet aggregation and often results in a higher risk of bleeding and thrombocytopenia. These risks may however be reduced with novel agents, such as RUC-1, which bind to the GPIIb/IIIa receptor in a different mode, without inducing conformation changes in the receptor.

As previously discussed, the ultimate challenge in the development of the next generation of antiplatelet therapy is being able to separate the agent's antithrombotic efficacy from the side effect of bleeding. Therefore, it is worth exploring the novel targets such as the TP receptor, serotonin receptor, EP3 receptor, GPIb-IX-V receptor, GPVI receptor, P-selectin, and PI3Kβ, which are all based on a new mechanism of action and participate in different pathways or stages of platelet adhesion, activation or aggregation. Optimizing, repurposing, and combining currently available drugs may also function as another cost-effective ways to expand the therapeutic window of antiplatelet therapy.

Understanding the mechanisms that targeted molecules are involved in, as well as the mechanisms used by agents to interact with these molecules, are critical to the development process of antiplatelet agents. In addition, the pharmacokinetics, efficacy, and safety issues encountered during the different stages of preclinical and clinical studies are usually underlined by these mechanisms. The current review summarize the entire mechanism of platelet involvement in thrombosis, and the development of antiplatelet agents targeting surface receptors or downstream intracellular signaling pathways. For future development of antiplatelet agents, we propose that the correlation between the functional mechanism of targeted molecules and the potential effectiveness of agents under development, be considered.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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