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Review

Adenosine diphosphate receptors on blood platelets — potential new targets for antiplatelet therapy

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Platelets play a key role not only in physiological haemostasis, but also under pathological conditions such as thrombosis. Platelet activation may be initiated by a variety of agonists including thrombin, collagen, thromboxane or adenosine diphosphate (ADP). Although ADP is regarded as a weak agonist of blood platelets, it remains an important mediator of platelet activation evoked by other agonists, which induce massive ADP release from dense granules, where it occurs in molar concentrations. Thus, ADP action underlies a positive feedback that facilitates further platelet aggregation and leads to platelet plug formation. Additionally, ADP acts synergistically to other, even weak, agonists such as serotonin, adrenaline or chemokines. Blood platelets express two types of P2Y ADP receptors: $P2Y_1$ and $P2Y_{12}$. ADP-dependent platelet aggregation is initiated by the $P2Y_1$ receptor, whereas $P2Y_{12}$ receptor augments the activating signal and promotes platelet release reaction. Stimulation of $P2Y_{12}$ is also essential for ADP-mediated complete activation of GPIIb-IIIa and GPIa-IIa, and further stabilization of platelet aggregates. The crucial role in blood platelet biology makes $P2Y_{12}$ an ideal candidate for pharmacological approaches for anti-platelet therapy.

Keywords: ADP receptors, platelet, $P2Y_{12}$, $P2Y_1$, antiplatelet therapy, ADP antagonists

BLOOD PLATELETS PLAY A ROLE IN BOTH HAEMOSTASIS AND THROMBOSIS

Blood platelets are small anuclear cells involved in blood coagulation. Upon blood vessel injury, the endothelial layer is damaged and platelets can interact with subendothelial adhesive proteins such as von Willebrand factor and collagen. This interaction first leads to the platelet adhesion and then to further platelet activation, release of the contents of intraplatelet granules and cell shape change. Altogether, an unique pathway of events leads to haemostatic plug formation and results in the arrest of bleeding. In addition to these types of early platelet response, altogether referred to as primary haemostasis, platelets are also crucial elements in promoting the activation of coagulation factors on surface plasma membranes (Hartwig, 2002; Gibbins, 2004).

The normal platelet response is required for the maintenance of haemostasis, but on the other hand, platelet hyperactivity leads to thrombotic phenotype (Ahmad *et al*., 2003). Arterial thrombi contain mainly platelets and are formed at sites of atherosclerotic vascular injury and disturbed blood flow. Therefore, the importance of antiplatelet therapy in the prevention of arterial thrombosis is unquestionable. For several decades, acetylsalicylic acid (aspirin) has been used as the most common antiplatelet agent. Despite its clinical effectiveness and relative safety, it is a rather weak antiplatelet agent having several limitations (Awtry & Loscalzo, 2000; Patrono, 2003; Schror, 1997; Wong *et al*., 2004). This prompted a search for other antiplatelet strategies including blocking of the interactions between one of the most common platelet activators – adenosine diphosphate – with its receptors on platelet surface (Gachet, 2001a; 2001b; Curtin *et al*., 2002; Cattaneo, 2003; Fox *et al*., 2004).

PLATELET ADENINE NUCLEOTIDE RECEPTORS

ADP was described about 40 years ago as a factor derived from red blood cells, affecting plate-

Abbreviations: ADP, adenosine diphosphate; A2P5P, adenosine-2′,5′-bisphosphate; A3P5P, adenosine-3′,5′-bisphosphate; A3P5PS, adenosine-3′-phosphate 5′-phosphosulfonate; cAMP, 3′,5′-cyclic adenosine monophosphate; Ap_4A, diadenosine tetraphosphate; GP, glycoprotein; LPA, lysophosphatidic acid; MRS 2179, methyl-2′-deoxyadenosine-3′,5′-bisphosphate; MRS 2279, (N)-methanocarba-N^6-methyl-2-chloro-2′-deoxyadenosine-3′,5′-bisphosphate; TxA, thromboxane A_2.

let adhesion and inducing platelet aggregation. It has been well documented that ADP is one of the most important mediators of both physiological haemostasis and thrombosis. Although ADP is regarded as a weak agonist of circulating blood platelets, it is an important mediator of platelet activation induced by other activators (thrombin, collagen), which promote ADP release from intraplatelet storage pools, like dense granules, where it is present in high concentrations. This results in a positive feedback that enhances platelet aggregation and proliferation of platelet plug. Additionally, ADP acts synergistically to all other platelet agonists, even the weak ones, such as serotonin, adrenaline or chemokines (Gachet & Cazenave, 2002; Andre *et al*., 2003). Currently, it is known that ADP initiates two signalling pathways in blood platelets: i) phospholipase C-mediated increase in cytosolic concentration of Ca^{2+}, and ii) inhibition of the formation of cyclic adenosine monophosphate (cAMP).

In general, adenine nucleotides act on cells *via* purinoreceptors (P2 receptors), which are present in various cell types: endothelial cells, smooth muscle cells, mastocytes, neuronal cells and blood cells. P2 receptors are divided into two major classes: the superfamily of G protein-coupled receptors (P2Y receptors), and the superfamily of ion channels-coupled receptors — P2X. Blood platelets express three types of P2 purinoreceptors: $P2X_1$, $P2Y_1$ and $P2Y_{12}$ (Ralevic & Burnstock, 1998; Gachet, 2001a; Gachet & Cazenave, 2002).

$P2X_1$ is a receptor for ATP but not ADP

Human $P2X_1$ is a protein composed of 399 amino acids and consists of two transmembrane domains and a large extracellular domain with 10 cysteine residues. The N- and C-terminal regions are located inside the cell. Several years ago it was believed that $P2X_1$ is mainly a receptor for adenosine triphosphate and its potential role in platelet response to ADP could be minor (Gachet, 2001a). Interestingly, however, some authors reported that $P2X_1$ was responsible for a rapid (about 10 ms) ADP-induced entry of calcium ions into platelet cytoplasm (MacKenzie *et al*., 1996). Recently, it was pointed out that commercially available ADP preparations are contaminated by ATP. Using HPLC and hexokinase in order to remove the ATP contamination, Mahaut-Smith *et al*. (2000) found that such a treatment abolishes $P2X_1$ activation. Notably, Greco *et al*. (2001) have found on megakaryocytic cell lines an ADP-sensitive and α,β-methylene ATP-insensitive form of $P2X_1$ receptor ($P2X_{1del}$), differing from the wild type $P2X_1$ ($P2X_{1WT}$) by a deletion of a 17 amino-acid extracellular sequence. Other studies, however, demonstrated that $P2X_{1del}$ form of the receptor is below the detection limit in blood platelets. Altogether, most data indicate that $P2X_1$ is a receptor for ATP but not for ADP (Oury *et al*., 2002; Vial *et al*., 2002).

$P2Y_1$ receptor

$P2Y_1$ receptor is composed of 373 amino acids and has a structure typical for G protein-coupled receptors. $P2Y_1$ displays rather low tissue specificity being found in heart, blood vessels, smooth muscle cells, connective and neural tissues, testis, prostate, ovary and blood platelets (Ralevic & Burnstock, 1998). Initially, on the basis of pharmacological studies, it was proposed that in platelets $P2Y_1$ (earlier termed the $P2_T$ receptor) is responsible for ADP-induced aggregation, Ca^{2+} mobilisation and inhibition of adenylate cyclase (Mills, 1996; Hourani, 2000). Currently, it is known that this receptor plays a key role in platelet shape change, as well as in the initiation of platelet response to ADP. It mediates the first, reversible phase of platelet aggregation (Gachet, 2001a; Jin *et al*., 2002), whilst the amplification of platelet aggregation and the enhancement of platelet secretion is caused by the other receptor for ADP-$P2Y_{12}$, coupled to and responsible for adenylate cyclase inhibition (Gachet, 2001a; Jin *et al*., 2002). The importance of the role of $P2Y_1$ receptor in primary haemostasis was proved in experiments on $P2Y_1$ receptor-null mice (Leon *et al*., 1999). It was found that these animals had prolonged bleeding time and an unchanged platelet count after bolus injection of ADP, compared to normal bleeding time and the platelet count decreased by more than 50% in wild-type mice. Furthermore, $P2Y_1$ receptor-null mice were characterised by two-fold lower mortality in response to a bolus injection of a mixture of collagen and adrenaline (Leon *et al*., 1999).

Inhibitors of $P2Y_1$

The action of ADP on $P2Y_1$ receptor can be blocked by specific antagonists, including adenosine-2′,5′-bisphosphate (A2P5P), adenosine-3′,5′-bisphosphate (A3P5P) and adenosine-3′-phosphate,5′-phosphosulphate (A3P5PS). They competitively block the receptor, however, only at concentrations as high as micromolar (Boyer *et al*., 1996). Another group of efficient agents includes N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2179) (Boyer *et al*., 1998) and (N)-methanocarba-N^6-methyl-2-chloro-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2279) (Nandanan *et al*., 2000). Both groups of $P2Y_1$ inhibitors are known to block ADP-induced platelet aggregation and shape change (Hechler *et al*., 1998; Haseruck *et al*., 2004). Also, ADP-induced $[Ca^{2+}]_i$ increase was found to be inhibited in human platelets by either A2P5P or A3P5P, while these nucleotides had no

antagonistic effect on the $[Ca^{2+}]_i$ increases induced by either thrombin or thromboxane A_2 analogue (Hechler *et al*., 1998). Recently, studies have been undertaken to verify whether an inhibition of $P2Y_1$ can effectively and safely block thrombus formation *in vivo* (Cattaneo, 2003). Using mouse model, it has been demonstrated that MRS 2179 administration results in decreased, localized thrombus formation and lowered thrombin generation (Leon *et al*., 2001; Lenain *et al*., 2003).

$P2Y_{12}$ receptor

$P2Y_{12}$ receptor, the existence of which had been predicted several years ago and which had been known in the literature under the synonyms $P2T_{AC}$, $P2Y_{cyc}$ or P_{2T}, was cloned by Hollopeter *et al*. in 2001. The receptor acts *via* heterotrimeric G_{i2} protein and inhibits adenylate cyclase, thus leading to a drop in platelet cAMP concentration. According to the present knowledge, ADP-dependent platelet aggregation is initiated by the $P2Y_1$ receptor, whereas $P2Y_{12}$ receptor enhances the activating signal. Stimulation of $P2Y_{12}$ is also essential for complete activation of glycoprotein IIb-IIIa by ADP or the stabilization of platelet aggregates. In blood platelets it is mainly $P2Y_{12}$ that underlies the ADP-induced generation of thromboxane A_2 (TxA_2) (Kunapuli *et al*., 2003), and it also promotes a release reaction from intraplatelet granules (Gachet, 2001a; Storey, 2001; Andre *et al*., 2003). Overall, $P2Y_{12}$ plays a crucial role in activation of circulating platelets and their recruitment to the site of vascular injury, as well as in the enhancement of platelet activation evoked by other platelet agonists (Dorsam & Kunapuli, 2004).

It has been demonstrated that $P2Y_{12}$, unlike the other purinoreceptors described above, is present mainly on blood platelets and, to a much lesser extent, on neuronal cells in the brain, and it does not occur in other tissues. It seems apparent, therefore, that $P2Y_{12}$ is an ideal candidate for pharmacological approaches aimed at anti-platelet effects (Gachet & Cazenave, 2002).

Inhibitors of $P2Y_{12}$

The first $P2Y_{12}$ antagonists were thienopyridine derivatives. These compounds gain activity after being metabolised in the liver due to the action of cytochrome P-450. They irreversibly block $P2Y_{12}$ as a result of a covalent modification of cysteine residues located in the extracellular portion of the receptor (Conley & Delaney, 2003; Ding *et al*., 2003). Actually, thienopyridines were developed and used in the clinics many years before $P2Y_{12}$ was cloned and its role in platelet activation understood. Numerous reports have demonstrated that two thienopyridines: Ticlopidine and Clopidogrel have more beneficial effects in a broad range of patients, compared to the current standard, aspirin (Arjomand *et al*., 2003; Bhatt *et al*., 2002; Curtin *et al*., 2002), even though, their use in clinical practice has also some limitations (Bennett *et al*., 2000).

Recently, extensive studies have been performed aimed at designing of new competitive inhibitors of $P2Y_{12}$ derived of the chemical structure of ATP, a natural weak and non-selective antagonist of $P2Y_{12}$ receptor. The blocker AR-C69931MX, designed by AstraZeneca, is an analogue of ATP that was found to be a potent and selective antagonist of $P2Y_{12}$. In spite of the positive and promising outcomes of the second phase of clinical trials, the compound has not been demonstrated to be active when administered orally (Storey, 2001; Cattaneo, 2003). This prompted a search for novel efficient blockers. Some literature data point that under *in vitro* conditions diadenosine tetraphosphate (Ap_4A) is a competitive inhibitor of ADP-induced platelet aggregation (Chan *et al*., 1997). Recently, a novel group of stable analogues of Ap_4A in which polyphosphate chain was substituted by either a polyalcoholic chain or a fragment of bis(hydroxymethyl)phosphinic acid was designed, and the adenylated derivatives of bis (hydroxymethyl)phosphinic acid have been shown to be potent inhibitors of platelet aggregation. The efficiency of these compounds was confirmed not only by classical aggregometry under static conditions (Walkowiak *et al*., 2002), but also in a dynamic model mimicking blood flow in arterial vessels (Watala *et al*., 2003).

Recent studies have shown that $P2Y_1$ and $P2Y_{12}$ contribute to platelet aggregation induced by lysophosphatidic acid (LPA). Since LPA is present in oxidized low-density lipoprotein (ox-LDL), the receptors could play a role in platelet hyperreactivity and atherosclerosis. Haseruck *et al.* (2004) found that both $P2Y_1$ antagonists, such as A3P5P and MRS2179, and $P2Y_{12}$ antagonists completely inhibited platelet aggregation induced by LPA in whole blood, platelet-rich plasma and washed platelets. Moreover, a combination of $P2Y_1$ and $P2Y_{12}$ antagonists efficiently inhibited formation of LPA-induced platelet-monocyte aggregates. Notably, there was no effect of aspirin on LPA-induced aggregation.

Genetic polymorphisms of $P2Y_{12}$ receptor

Platelet response is characterised by significant individual variability, underlined among others by genetic factors (Lasne *et al*., 1997; Feng *et al*., 1999). In the light of recent publications it seems that the phenomenon of genetically determined intra-donor variability concerns also platelet sensitivity to inhibitors (Michelson *et al*., 2000; Szczeklik *et al*., 2000; Rozalski & Watala, 2002; Luzak *et al*., 2003). Since the $P2Y_{12}$ receptor has been cloned quite recently (Hol-

lopeter *et al*., 2001), little is known about genetic variation of the receptor. In their recent paper, Fontana and co-workers (2003a) reported five genetic polymorphisms of the $P2Y_{12}$ receptor. It was found that four of these polymorphisms are in complete linkage disequilibrium giving rise to two haplotypes: H1 and H2. It was demonstrated that the maximal aggregation evoked by low concentrations of ADP was elevated in the carriers of H2 haplotype; the highest values were observed in individuals with the H2/H2 genotype. Furthermore, the decrease in cAMP level in platelet cytoplasm caused by stimulation with ADP was augmented in carriers of the H2 haplotype (Fontana *et al*., 2003a). Another paper (*a case-control study*) provided evidence that the H2 haplotype was a risk factor of peripheral arteries disease (Fontana *et al*., 2003b).

It remains to be elucidated whether the H2 haplotype of $P2Y_{12}$ affects platelet sensitivity to $P2Y_{12}$ blockers.

CONCLUSIONS

In general, the use of inhibitors blocking the interaction of ADP with the $P2Y_1$ and $P2Y_{12}$ receptors seems promising as antiplatelet agents. The outcomes of large clinical trials and smaller studies on the effects of purinoreceptor antagonists indicate that new generation drugs antagonizing ADP receptors (Clopidogrel) may be more effective than the most commonly used antiplatelet drug, aspirin, in reducing the combined risk of myocardial infarction, ischaemic stroke, vascular disease or cardiovascular fatal events. Also, the overall clinical safety profile of thienopyridine-derived blockers, at least as good as that of medium-dose aspirin, seems encouraging. Despite these advantageous characteristics, the cost: effectiveness ratio is much worse for thienopyridines, the only widely used purinoreceptor blockers in today's clinics, than for aspirin. Therefore, the use of currently available purinoreceptor antagonists is considered rather in a combination therapy with other antiplatelet agents than as a single agent treatment in long-term antiplatelet therapy. Hence, the invention of low-cost new generation blockers of ADP receptors, preferably suitable for oral administration, is challenging. The fact that ADP is a rather small and relatively simple molecule makes designing and testing novel competitive blockers with a desirable pharmacokinetic profile fairly straightforward.

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