

Theme Issue Article

P2 receptors, platelet function and pharmacological implications

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Summary

ADP and ATP play a crucial role in platelet activation and their receptors are potential targets for antithrombotic drugs. The ATP-gated cation channel P2X₁ and the two G protein-coupled ADP receptors, P2Y₁ and P2Y₁₂, selectively contribute to platelet aggregation and formation of a thrombus. Owing to its central role in the growth and stabilization of a thrombus, the P2Y₁₂ receptor is an established target of antithrombotic drugs like the thienopyridines clopidogrel or prasugrel, or competitive antag-

onists such as cangrelor or AZD6140. The optimal inhibition of this receptor to reach clinical efficacy while preserving patients from unacceptable bleeding is a matter of debate. On the other hand, studies in P2Y₁ and P2X₁ knockout mice and using selective P2Y₁ and P2X₁ antagonists have shown that these receptors are also attractive targets for new antithrombotic compounds. Finally, the regulation by the P2 receptors of the platelet involvement in inflammatory processes is also briefly discussed.

Keywords

Haemostasis, thrombosis, ADP, P2Y₁, P2Y₁₂, P2X₁, antiplatelet drugs, thienopyridine, clopidogrel, prasugrel, AZD6140, cangrelor

Thromb Haemost 2008; 99: 466–472

Introduction

Extracellular nucleotides and their receptors are important components of the cardiovascular system and are involved in functions like platelet activation and the control of vascular tone (1). Adenosine diphosphate (ADP) plays crucial roles in the physiological process of haemostasis and in the development and extension of arterial thrombosis (2). By itself ADP is a weak agonist of platelet aggregation inducing only reversible responses as compared to strong agonists such as thrombin or collagen. However, due to its presence in large amounts in the platelet dense granules and its release upon activation at sites of vascular injury, ADP is an important so-called secondary agonist which amplifies most of the platelet responses and contributes to the stabilization of the thrombus. The receptors for extracellular nucleotides belong to the P2 family which consists of two classes of membrane receptors: P2X ligand-gated cation channels (P2X_{1–7}) and G protein-coupled P2Y receptors (P2Y_{1,2,4,6,11,12,13,14}) (3). Starting from the concept of a unique P2T receptor (T for thrombocyte) originally postulated on the basis of pharmacological data (4), a model of three platelet P2 receptors progressively

emerged (5, 6). These are the P2X₁ cation channel which is activated by ATP and two G protein-coupled receptors, P2Y₁ and P2Y₁₂, both activated by ADP. Each of these receptors has a specific function during platelet activation and aggregation, which naturally has implications for their involvement in thrombosis.

The respective roles of the three platelet P2 receptors during activation (Fig. 1)

The P2Y₁ receptor is widely distributed in many tissues including heart, blood vessels, smooth muscle cells, neural tissue, testis, prostate and ovary (3). About 150 P2Y₁ receptor binding sites are expressed per platelet (7), which is very low as compared for instance to the TP receptors or the thrombin receptor PAR-1 (1000 to 2000 sites/platelet). As it is coupled to G α_q , the P2Y₁ receptor triggers the mobilization of calcium from internal stores, which results in platelet shape change and weak, transient aggregation in response to ADP (8–10). The P2Y₁ receptor is absolutely required for ADP-induced platelet aggregation. Its pharmacological inhibition or genetic deficiency results in complete

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Received November 12, 2007
Accepted after minor revision January 10, 2008
Prepublished online February 14, 2008
doi:10.1160/TH07-11-0673

absence of platelet aggregation and shape change in response to ADP. As a consequence, at the intracellular level, the calcium signal is abolished while the ability of ADP to inhibit cAMP formation is preserved (11, 12). The P2Y₁ receptor also participates in the aggregation response to collagen and plays a key role in collagen-induced shape change when TXA₂ formation is prevented (12, 13). Overall, the P2Y₁ receptor mediates weak responses to ADP but is nevertheless a crucial factor in the initiation of the platelet activation induced by ADP or collagen.

The P2Y₁₂ receptor, despite its being well known and characterized on the basis of both pharmacological and genetic evidence, was the last to be cloned (14). This receptor is deficient in patients with selective defects of ADP-induced platelet aggregation (15) and is also the molecular target of the antiplatelet drugs clopidogrel and prasugrel, which are thienopyridine compounds, and AZD6140 and cangrelor which are competitive antagonists of the receptor (5). Its tissue distribution is very limited, although not entirely restricted to platelets as it is also present in brain (14), glial cells (16) and possibly smooth muscle cells (17). The P2Y₁₂ receptor is coupled to G α_{i2} and is responsible for completion of the platelet aggregation response to ADP initiated by P2Y₁. It plays a central role in amplification of the aggregation induced by all known platelet agonists whatever their signalling pathway, including collagen, thrombin, immune complexes, TXA₂, adrenaline and serotonin (6, 18). The P2Y₁₂ receptor is also involved in the potentiation of platelet secretion (19). All these features make this receptor a pivotal factor in sustaining platelet aggregation and hence in promoting thrombus growth and stabilization. Its genetic deficiency or pharmacological inhibition results in strong inhibition of platelet aggregation induced by low to medium concentrations of any platelet agonist. The bleeding time is markedly prolonged in P2Y₁₂-deficient mice (14, 20) as it is in patients with severe P2Y₁₂ deficiency (15) as well as in animals treated with high doses of clopidogrel or other P2Y₁₂ antagonists (see below). The intracellular pathways through which P2Y₁₂ amplifies platelet responses include inhibition of cyclic AMP production, vasodilator-stimulated phosphoprotein (VASP) dephosphorylation (21), phosphoinositide 3-kinase (PI 3-K) (22, 23) and small GTPase Rap1B (24, 25) activation.

Co-activation of the P2Y₁ and P2Y₁₂ receptors is necessary for normal ADP-induced platelet aggregation since separate inhibition of either of them with selective antagonists results in a dramatic decrease in aggregation (9, 10, 26). However, the two receptors have different functions since, except in the case of collagen-induced activation, P2Y₁ plays a minor role when platelet aggregation is induced by other agonists whereas P2Y₁₂ supports amplification of these responses. For long, one intriguing question was: why is ADP a *weak* agonist as compared for example to TXA₂ or thrombin, inducing only reversible aggregation and unable by itself to induce platelet secretion (provided mM calcium concentration present in the medium)? In fact, these features result from the very low level of expression of the P2Y₁ receptor at the platelet membrane as compared to other G-protein coupled receptors (around 150/platelet versus >1500/platelet, as already mentioned). Indeed, over-expressing the P2Y₁ receptor resulted in full secretion and irreversible aggregation of washed mouse platelets (27). On the other hand, in human and wild-type mouse

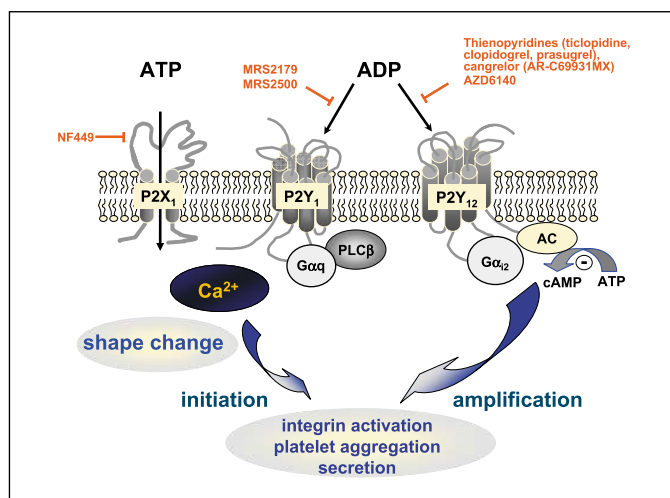


Figure 1: Current model of three platelet P2 receptors. The P2X₁ receptor is responsible for rapid calcium influx and platelet shape change in response to ATP and contributes to the platelet activation induced by low concentrations of collagen. The P2Y₁ and P2Y₁₂ receptors are essential for normal aggregation in response to ADP: the G_q-coupled P2Y₁ receptor is responsible for intracellular calcium mobilization, shape change and initiation of aggregation and the G_i-coupled P2Y₁₂ receptor for completion of the ADP-induced aggregation response and for potentiation of the aggregation and secretion induced by other agents through various intracellular pathways. Selective antagonists allow discrimination of the roles of the three receptors. P2Y₁₂ is the target of the antithrombotic drugs ticlopidine, clopidogrel and prasugrel which are thienopyridine compounds and of direct antagonists such as AZD6140 or cangrelor. P2Y₁ and P2X₁ are potential targets for new antiplatelet compounds.

platelets, high concentration of ADP (100 μ M) is able to trigger weak secretion (28). Altogether, these data tell us that the low level of expression of the P2Y₁ receptor limits the platelet responses when ADP is the sole agonist. Such a situation may happen in the circulation when red blood cells release ADP.

The P2Y₁ and P2Y₁₂ receptors are also differentially involved in the procoagulant activity of platelets. While both receptors are indirectly involved through their role in platelet P-selectin exposure and in the formation of platelet-leukocyte conjugates leading to leukocyte tissue factor exposure (29, 30), the P2Y₁₂ receptor is also directly implicated in the exposure of phosphatidylserine at the surface of platelets (29, 31, 32).

The third component of the platelet P2 receptors is P2X₁, a ligand-gated cation channel responsible for a fast calcium entry induced by ATP (33). Although unable to trigger platelet aggregation by itself, the P2X₁ receptor induces transient shape change (34) and participates in collagen- and shear-induced aggregation. One hallmark of this receptor is that it requires high shear conditions to fully play its role in the formation of a thrombus (35–37).

Desensitization

An important phenomenon in controlling thrombus growth is the regulation of platelet reactivity after stimulation and receptor desensitization is one general mechanism used by cells to adapt

their responsiveness. Once initially activated by ADP, platelets become transiently unresponsive to a second stimulation with the same agonist. Full ADP-dependent responses recover within 15 to 30 minutes. The phenomenon has been shown to be caused by selective desensitization of the P2Y₁ receptor with a resultant loss of shape change and aggregation (38). Conversely, the P2Y₁₂ receptor remains functional and conserves its ability to amplify the platelet aggregation induced by other agonists (39), suggesting that the two receptors are differentially regulated upon activation. What could the physiological relevance of this be? Again, one would suggest that platelets must have a system to regulate their responses when ADP, released from red blood cells, is the sole agonist in the medium, a situation where P2Y₁ plays a major role. On the other hand, even in platelets refractory to stimulation by ADP, the P2Y₁₂ receptor would still be able to ensure their reactivity at sites of injury where additional agonists might be present, thus preventing loss of haemostatic function. In view of the impact of P2Y₁₂ deficiency/inhibition in terms of bleeding, such an hypothesis makes sense. The matter will nevertheless require further study since others have reported results in contradiction with ours concerning desensitization of the P2Y₁₂ receptor (40).

Finally, the P2X₁ receptor is also desensitized and this occurs very quickly and requires lower concentrations of nucleotides than for the metabotropic receptor P2Y₁. The physiological implications of P2X₁ desensitization are still not well understood but might be related to the need to confine thrombus growth to the site of a lesion and prevent uncontrolled extension of the platelet aggregates.

Polymorphisms of the P2Y receptors

P2Y₁ and P2Y₁₂ have been shown to display gene sequence variations which have been proposed to be associated with variable platelet responsiveness to ADP. In P2Y₁₂, the polymorphisms are in the intronic part of the gene and have no obvious impact on the coding sequence. Two haplotypes have been described, H1 and H2, the latter being proposed to be linked to an increased reactivity to ADP (41). The H2 haplotype was reported to be associated with peripheral arterial disease in a case-control study (42) and, more recently, with coronary artery disease (43).

In P2Y₁, a silent polymorphism was identified at position 1622 (A/G), which led to increased platelet aggregation in response to a low concentration of ADP (0.1 μM) in subjects carrying the G allele (44). These authors also reported the previously described polymorphisms of the P2Y₁₂ gene, but did not confirm the increased platelet response associated with the H2 haplotype. Later, in a study aimed at evaluating the impact of the gain of function P2Y₁₂ receptor H2 haplotype on individual response to clopidogrel treatment, Bura et al. did not retrieve their original finding (45). It thus appears that polymorphisms of the non-coding region of the P2Y₁₂ receptor gene do not have any impact on the receptor function, nor on the individual responsiveness to clopidogrel (see below). Whether polymorphism of the P2Y₁ receptor has an impact on the platelet physiology or in clinical pharmacology probably requires further studies.

The platelet P2 receptors as molecular targets for antithrombotic drugs

The P2Y₁₂ receptor

Long before its molecular cloning, the pharmacological importance of this receptor in haemostasis and thrombosis was well recognized. This was due to the fact that the potent antithrombotic thienopyridine compounds ticlopidine and clopidogrel, of which an active liver metabolite selectively and irreversibly targets the P2Y₁₂ receptor, were used as molecular tools to characterize platelet responses to ADP and the role of the latter in thrombosis (46). The thienopyridine compounds are prodrugs which have to be metabolized by the liver in order to generate active metabolites. Clopidogrel treatment leads to a dose-dependent inhibition of platelet aggregation in response to ADP with conserved shape change and transient weak aggregation driven by P2Y₁. At the intracellular level, P2Y₁₂ blockade results in the inhibition of the ability of ADP to inhibit cyclic AMP production while calcium signalling is preserved. Platelet aggregation in response to other agents is also affected through the effect on released ADP, which normally amplifies their responses and stabilizes the aggregates (see above). The active metabolite of clopidogrel (47) covalently binds cysteine residues of the P2Y₁₂ receptor, thus precluding the binding of ADP (48–50). Moreover, it has been recently reported that clopidogrel's active metabolite disrupts homopolymers of the P2Y₁₂ receptor expressed in lipid rafts and partitions them from the lipid rafts (51). Further studies are required to confirm these important findings.

Comprehensive reviews have been published emphasizing the clinical relevance of the P2Y₁₂ receptor as a target for antiplatelet drugs (18, 52) and surveying P2Y₁₂ targeting compounds (5, 46, 53–55). Large-scale clinical trials have demonstrated the beneficial effects of thienopyridines in the prevention of major cardiac events after coronary artery stent insertion and in the secondary prevention of major vascular events in patients with a history of cerebrovascular, coronary or peripheral artery disease.

Interindividual variability in the response to clopidogrel

Despite appropriate protocols and improved procedures, an important inter-individual variability has been observed in the response to clopidogrel (56) and a concept of “clopidogrel resistance” has been put forward on the basis of the observation that 5 to 10% of patients under treatment experienced acute or subacute thrombosis after a coronary event or implantation of a coronary stent (57–61) while an average of 30% of patients under clopidogrel treatment display biological parameters which do not differ from those of untreated subjects (57, 59, 61–63). Many clinical studies now strongly suggest that these patients are at risk for recurrent ischemic events (64, 65) and should benefit from adaptation of their treatment.

The mechanisms responsible for the inter-individual variability and the so-called “resistance” to clopidogrel have not yet been entirely defined. Poor compliance to the treatment, variable metabolism of the prodrug in the liver (66), drug-drug interactions (67), genetic polymorphisms of the platelet P2Y₁₂ receptor (41), a greater extent of P2Y₁-dependent platelet aggregation (68) or upregulation of other pathways are the hypotheses gen-

erally put forward, which nevertheless remain to be demonstrated (60, 69, 70). Recent studies discussed the impact of polymorphisms in the CYP2C19 (71, 72) while CYP3A4, and CYP3A5 and CYP2C9 have also been reported to be involved in clopidogrel metabolism.

New P2Y₁₂ receptor targeting drugs

Prasugrel (CS-747, LY640315) is a new thienopyridine compound which has higher efficacy, faster onset of action and less variability than clopidogrel. This is due to a slightly different metabolic pathway and better rate of active metabolite generation as compared to clopidogrel (53, 73–76). A large scale clinical trial, TRITON-TIMI 38, including 13,609 patients planned for percutaneous coronary intervention (PCI) upon assessment of coronary anatomy, 3/4 with unstable angina, 1/4 with myocardial infarction, has demonstrated the overall superiority of prasugrel (60-mg loading dose followed by 10-mg maintenance dose) in comparison to clopidogrel (300-mg loading dose, 75-mg maintenance dose) with a total of 19% reduction of ischemic events with, particularly, 52% decreased stent thrombosis (77). This study definitely demonstrated that inhibiting more P2Y₁₂-dependent platelet activation results in improved clinical outcome. However, this has a price which is a 32% increase of major bleeding, including fatal bleeding. Although not really surprising, these results are very important and will most probably modify the overall procedures and use of current and future antiplatelet drugs (78). Particularly, one question is whether increasing both loading and maintenance doses of clopidogrel could reach the same order of efficacy as prasugrel. The PRINCIPLE-TIMI 44 study compared 600-mg loading dose and 150-mg/day maintenance dose clopidogrel to 60-mg loading dose and 10-mg/day maintenance dose prasugrel in terms of inhibition of platelet aggregation (IPA) and platelet reactivity index (PRI) as calculated from the VASP phosphorylation assay in patients undergoing PCI. The conclusions were that clopidogrel did not reach the same inhibition as prasugrel (79). However, this is a small sized trial (201 patients) with no clinical endpoint. The ongoing CURRENT study, which should be completed this year, is a clinical endpoint study in 14,000 patients with unstable angina and non-ST elevation myocardial infarction undergoing PCI. Its objective is to determine whether a high-dose regimen of clopidogrel (600-mg loading dose and 150-mg/day maintenance dose) is superior to a standard regimen of clopidogrel and whether high dose of aspirin is superior to low dose of aspirin in preventing cardiovascular death, myocardial infarction or stroke (80).

Again, adapting the antiplatelet drug regimen would probably improve both clinical efficacy and safety provided appropriate laboratory tests are employed to monitor and adjust the treatment in individual patients (81, 82).

Competitive P2Y₁₂ antagonists

“P2T receptor antagonists” were discovered in the mid-1990s and turned out to be competitive P2Y₁₂ antagonists. Cangrelor and the more recent compound AZD6140 are under clinical evaluation, the latter being orally active while cangrelor requires intravenous administration (54, 83–88).

Theoretically, use of such molecules would have an advantage mainly in acute situations like myocardial infarction, where fast blockade of the ADP receptor should be beneficial as compared to the delayed action of thienopyridine compounds. The rapid cessation of activity would also be beneficial in terms of safety. A second theoretical advantage of using competitive P2Y₁₂ antagonists could be if there is less inter-individual variability in the response to the treatment. The CHAMPION-PCI and CHAMPION-PLATFORM studies are currently assessing whether cangrelor is superior to clopidogrel or placebo, respectively, in patients undergoing PCI. The PLATO study is currently assessing whether this agent has clinical efficacy superior to clopidogrel in the management of ACS (88).

The P2Y₁ receptor as a target for new antiplatelet compounds

A consideration of the role of P2Y₁ in platelet aggregation and experimental thrombosis provides the rationale for suggesting this receptor to be a relevant target for new antiplatelet compounds. Thus, P2Y₁-knockout mice and animals treated with selective P2Y₁ antagonists display resistance to the systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline (12, 89) or when thromboembolism was induced by infusion of tissue factor (32). A role of the P2Y₁ receptor has also been demonstrated in localized thrombosis, using intravital microscopy after ferric chloride or laser induced injury of mouse mesenteric arteries (90, 91).

The above results clearly indicate that the P2Y₁ receptor should be regarded as an attractive target for antiplatelet compounds. Moreover, a combination of P2Y₁ deficiency or inhibition and clopidogrel treatment has been found to confer better thromboresistance than either condition alone, suggesting that a combination of P2 receptor antagonists could improve antithrombotic strategies (90, 91). It is worthy of note that inhibition of the P2Y₁ receptor results in only moderate prolongation of the bleeding time, which could be advantageous in terms of safety.

The P2X₁ receptor as a target for new antiplatelet compounds

Since the P2X₁ receptor plays an important role in thrombus formation only under high-shear conditions, it might represent the ideal target for an antithrombotic drug. P2X₁-deficient mice have in fact no prolongation of their bleeding time as compared to the wild type, indicating that they conserve normal haemostasis. In contrast, they display resistance to the systemic thromboembolism induced by injection of a mixture of collagen and adrenaline and to localized laser-induced injury of the vessel wall of mesenteric arteries (35). Conversely, increased systemic thrombosis has been reported in mice overexpressing the human P2X₁ receptor (92). Moreover, the P2X₁ antagonist NF449 (93) has an inhibitory effect on platelet activation *ex vivo* and thrombosis *in vivo* (94). These results clearly indicate that the P2X₁ receptor should also be considered as a potential target for antiplatelet strategies, with the interesting feature that P2X₁ antagonists should be effective only at sites of severe stenosis where shear forces are very high, without having a deleterious effect on normal haemostasis.

The platelet P2 receptors in inflammation, atherosclerosis and angiogenesis

Inflammation plays a major role in the progression of atherothrombosis and angiogenesis. Hence, inflammatory markers are elevated in patients with stable or unstable ischemic diseases (95–97). Among all blood cell types, monocytes, T lymphocytes and platelets are the key agents, and the contribution of blood platelets to the development of atherosclerosis has been established in many studies. Since the purinergic ligand-receptor system exists in all cell types and tissues involved in inflammation and atherosclerosis and has such importance in platelet physiology, these ligands and receptors should also be regarded as important partners in atherosclerosis (98). In addition to their short-term effects on vascular tone and platelet activation, nucleotides and P2 receptors are involved in long-term trophic effects on cell growth, proliferation and death which have important implications for both atherosclerosis and angiogenesis (1, 98). What do we know about the contributions of the individual platelet P2 receptors to these processes? Once again, most of our present knowledge comes from the observed P2Y₁₂ antagonistic effects of thienopyridines *in vitro*, in animal models or in patients. Decreased exposure of P-selectin, diminished formation of platelet-leukocyte aggregates and less subsequent tissue factor exposure have been documented (29, 84). The inhibition of CD40L exposure and release (99) and the reduction of circulating levels of C-reactive protein (97) in response to these molecules clearly indicate a prominent role of the P2Y₁₂ receptor. Thus, in addition to their anti-aggregatory activity, the efficacy of these drugs might be related to blockade of the contribution of platelets to inflammation (46, 97, 100). The role of the P2Y₁₂ receptor not only in platelet aggregation but also in the activation of multiple inflammatory and trophic processes may be expected to result in its direct involvement in the progression of atherosclerosis, which has been reported recently (101).

Very much less is known about the involvement of the P2Y₁ and P2X₁ receptors. The P2Y₁ receptor plays a role in P-selectin exposure, the formation of platelet-leukocyte aggregates and tissue factor exposure when platelets are stimulated with ADP, collagen or low concentrations of thrombin receptor agonist peptides, as has been shown *in vitro* (29, 31). At present, no *ex-vivo* data for markers of inflammation are available from animal mod-

els. Using P2Y₁-knockout mice crossed with apolipoprotein E (ApoE)-knockout mice, we observed a reduction of the size of atherosclerotic plaques as compared to controls (102).

The haemostatic system and platelets are known to play a key role in angiogenesis (103). On the other hand, it is known that ATP and ADP act as mitogenic/apoptotic factors for vascular cells, and the involvement of their receptors is now subject to increasing study (1). Concerning more specifically the platelet P2 receptors, early work showed a beneficial effect of ticlopidine in the treatment of diabetic retinal angiopathy but at the non proliferative stage (104). Although the precise mechanism is not yet fully understood, part of the *in-vivo* effect of the drug is probably due to its antiplatelet properties, i.e. to inhibition of the P2Y₁₂ receptor. Goepfert et al. have shown that angiogenesis is impaired in an *in-vivo* model (matrigel™ invasion) in CD39-deficient mice, which could tentatively be attributed to desensitization of P2Y₁ or P2Y₂ receptors (105). Whereas inhibition of macrophage migration was proposed to explain the results, the role of blood platelets, known to display reduced reactivity after desensitization of P2Y₁, could not be ruled out. All these studies are partial and somewhat preliminary but further work should rapidly enable us to assess the importance of this system and its relevance as a pharmacological target to modulate vascular remodelling and angiogenesis in an inflammatory context.

Conclusions

Owing to the central role of ADP and ATP in haemostasis and thrombosis, there is no doubt that their receptors are relevant targets for antiplatelet drugs and research is very active in this field. The interest of P2Y₁₂ antagonists, either irreversible like the thienopyridine compounds or competitive like cangrelor or AZD6140, requires no additional proof. P2Y₁ and P2X₁ would also appear to be promising targets. The fact that a combination of clopidogrel and aspirin is much more efficient than either drug alone indicates that inhibition of several pathways of platelet activation will be critical to achieve effective antithrombotic strategies. Whether combined inhibition of the P2 receptors would also be beneficial requires further investigation. The tools now exist to allow progress to be made in preclinical studies including in better defined animal models and using new antagonists. When P2Y₁ or P2X₁ antagonists or mixed compounds will be tried in humans remains an open question.

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