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MRS2500 [2-Iodo-*N*⁶-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate], a Potent, Selective, and Stable Antagonist of the Platelet P2Y₁ Receptor with Strong Antithrombotic Activity in Mice

Béatrice Hechler, Christelle Nonne, Eun Joo Roh, Marco Cattaneo, Jean-Pierre Cazenave, François Lanza, Kenneth A. Jacobson, and Christian Gachet

Institut National de la Santé et de la Recherche Médicale, U311, Strasbourg, France; Etablissement Français du Sang-Alsace, Strasbourg, France (B.H., C.N., J.-P.C., F.L., C.G.); University of Strasbourg, Strasbourg, France (B.H., J.-P.C., F.L., C.G.); Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (E.J.R., K.A.J.); and Unità di Ematologia e Trombosi, Ospedale San Paolo, Dipartimento di Medicina Chirurgia e Odontoiatria, Università di Milano, Milano, Italy (M.C.)

Abstract

The platelet P2Y₁ ADP receptor is an attractive target for new antiplatelet drugs. However, because of the lack of strong and stable antagonists, only a few studies have suggested that pharmacological inhibition of the P2Y₁ receptor could efficiently inhibit experimental thrombosis *in vivo*. Our aim was to determine whether the newly described potent and selective P2Y₁ receptor antagonist MRS2500 [2-iodo-*N*⁶-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate] could inhibit platelet function *ex vivo* and experimental thrombosis in mice *in vivo*. MRS2500 was injected intravenously into mice, and its effect on *ex vivo* platelet aggregation and in several models of thrombosis *in vivo* was determined. MRS2500 displayed high potency and stable and selective P2Y₁ receptor inhibition *ex vivo*. Although MRS2500 injection resulted in only moderate prolongation of the bleeding time, it provided strong protection in systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline. MRS2500 also potently inhibited localized arterial thrombosis in a model of laser-induced vessel wall injury with two degrees of severity. Moreover, combination of MRS2500 with clopidogrel, the irreversible inhibitor of the platelet P2Y₁₂ receptor for ADP, led to increased antithrombotic efficacy compared with each alone. These results add further evidence for a role of the P2Y₁ receptor in thrombosis and validate the concept that targeting the P2Y₁ receptor could be a relevant alternative or complement to current antiplatelet strategies.

Adenine nucleotides play a central role in hemostasis and in thrombosis. Their effects are mediated by three separate P2 receptors on blood platelets: the ATP-gated P2X₁ cation channel and the G protein-coupled ADP receptors, P2Y₁ and P2Y₁₂. These receptors play selective roles in platelet activation and thrombus formation, which imply that they are attractive targets for antiplatelet drugs (Gachet and Hechler, 2005). Among these, the P2Y₁₂ receptor is the molecular target of the blockbuster antithrombotic drug clopidogrel (Savi and Herbert, 2005), and extensive work is in progress in many laboratories and companies to provide new compounds acting at the P2Y₁₂ receptor (Niitsu et al., 2005; van Giezen and Humphries,

2005). However, pharmacological studies as well as studies with P2X₁ and P2Y₁-deficient (–/–) mice indicate that these receptors could also be relevant targets for new antiplatelet compounds (Gachet and Hechler, 2005).

Concerning the P2Y₁ receptor, studies with P2Y₁^{–/–} mice indicated defective aggregation in response to ADP and low concentrations of collagen (Fabre et al., 1999; Léon et al., 1999). These animals displayed resistance to systemic thromboembolism induced by infusion of either a mixture of collagen and adrenaline (Fabre et al., 1999; Léon et al., 1999) or tissue factor (Léon et al., 2001), pointing to the essential role of this receptor in thrombin-dependent and -independent models of thrombosis. Moreover, in a model of localized arterial thrombosis of mesenteric arterioles triggered by ferric chloride injury, P2Y₁^{–/–} mice also displayed reduced and delayed thrombus formation compared with the wild type (Lenain et al., 2003). In this model, the extent of inhibition was found to be equivalent to that of clopidogrel-treated animals at the maximal effective dose (Lenain et al., 2003). In addition, the combination of P2Y₁ deficiency and clopidogrel treatment was found to be more efficient than each alone, opening the possibility that a combination of P2 receptor antagonists could improve antithrombotic strategies (Lenain et al., 2003). Altogether, these results suggested the P2Y₁ receptor to be a potential target for new antiplatelet compounds.

These results could be reproduced by intravenous administration of the selective P2Y₁ antagonist MRS2179 (Léon et al., 2001), one of the first high-affinity ($K_d = 109$ nM), potent, and selective P2Y₁ receptor antagonists (Boyer et al., 1998; Baurand et al., 2001). MRS2179 inhibits aggregation of human platelets induced by ADP with an IC₅₀ value in the submicromolar range (Boyer et al., 1998; Baurand et al., 2001). However, the inhibitory effect of MRS2179 on platelet function *ex vivo* is very transient (less than 5 min) and requires high doses (50 mg/kg), thus limiting animal studies (Baurand et al., 2001; Baurand and Gachet, 2003). Recently, synthesis of the most potent and selective P2Y₁ receptor antagonist MRS2500 was reported (Kim et al., 2003). This compound displays 100-fold higher affinity ($K_i = 0.78$ nM) compared with MRS2179 (Waldo et al., 2002; Kim et al., 2003) at recombinant human P2Y₁ receptor and inhibits platelet aggregation to ADP with an IC₅₀ in the nanomolar range (Cattaneo et al., 2004). Its chemical structure makes it a good lead candidate, because in addition to its potency, it is expected to display more *in vivo* stability than ribose-containing nucleotides toward nucleotidases due to the (*N*)-methanocarbaring system (Ravi et al., 2002). The aim of the present study was to evaluate the potential of MRS2500 as a prototypical antithrombotic agent *ex vivo* and *in vivo*.

Materials and Methods

Chemicals

ADP, insoluble bovine collagen type I, U46619 and adrenaline were from Sigma Chemical (Poole, Dorset, UK). The anesthetic drugs xylazine (Rompun) and ketamine (Imalgene 1000) were from Bayer AG (Wuppertal, Germany) and Merial (Lyon, France), respectively. MRS2500 was synthesized as described previously (Kim et al., 2003). MRS2179 was provided by Tocris Cookson Inc. (Bristol, UK), and DIOC₆ was from Invitrogen (Carlsbad, CA). Equine collagen (Kollagenreagent Horm) was purchased from Hormon Chemie (Munich, Germany). Recombinant hirudin was kindly provided by A. Pavirani (Transgene SA, Strasbourg, France). Clopidogrel was a generous gift from Sanofi-Aventis (Bridgewater, NJ).

Mouse Strains

Wild-type (WT) and P2Y₁^{–/–} mice (Léon et al., 1999) were of pure (nine generation back-cross) C57BL/6 genetic background and were maintained in the animal facilities of the Etablissement Français du Sang-Alsace (Alsace, France).

Ex Vivo Platelet Aggregation

Male mice weighing 20 to 25 g were anesthetized by injection of 150 μ l i.p. of 0.2% xylazine base and 1% ketamine. The jugular vein was exposed surgically, and MRS2500 or saline was injected at the indicated dose within an infusion time frame of 3 to 4 s. At the time indicated, blood was drawn from the abdominal aorta into citrate (3.15%) as an anticoagulant and platelet-rich plasma (PRP) was prepared (Léon et al., 2001). Platelet count was adjusted to 500×10^3 platelets/ μ l. Aggregation was measured at 37°C by a turbidimetric method in a dualchannel aggregometer (Payton Associates, Scarborough, Canada). The extent of aggregation was estimated quantitatively by measuring the maximal curve height above baseline.

Bleeding Time

MRS2500 or physiological saline was injected into the jugular vein of 8-week-old anesthetized male mice, one minute before severing 3 mm from the distal end of the tail. The tail was immediately immersed in normal saline (37°C), and the time from severing to cessation of bleeding was recorded as the bleeding time. If the blood flow did not cease after 30 min, the tail was cauterized and the bleeding time was recorded as >1800 s.

In Vivo Models of Thrombosis

A model of acute systemic vascular thromboembolism induced by infusion of a mixture of collagen and adrenaline was performed as described previously (DiMinno and Silver, 1983; Léon et al., 1999). Male mice (20–25 g) were anesthetized, and the jugular veins were exposed surgically. MRS2500 or physiological saline was injected into the left jugular vein 30 s before injecting a mixture of collagen (0.15 mg/kg) and adrenaline (60 μ g/kg), which was injected into the right jugular vein. Two minutes later, blood was drawn from the abdominal aorta into EDTA anticoagulant (6 mM) and platelets were counted in an ACT Coulter Diff counter (Beckman Coulter, Fullerton, CA). For mortality experiments, mice were injected with a higher dose of collagen (0.21 mg/kg) and adrenaline (60 μ g/kg). Mice were observed for 30 min, and the mice that recovered were killed thereafter.

The model of localized thrombosis in mesenteric arterioles triggered by laser-induced vessel wall injury has been described previously (Hechler et al., 2003; Nonne et al., 2005). Mice weighing approximately 15 g and 3 to 5 weeks old were anesthetized, and their mesentery was exteriorized. Localized injury of the luminal surface of a mesenteric arteriole was induced with a pulsed nitrogen dye laser (440 nm) applied through the microscope 40 $\times$ objective of an inverted Leica DMIRB microscope with a Micropoint laser system (Photonics Instruments, St Charles, IL). Reproducible superficial or severe injuries were induced by adjusting the laser intensity and number of pulses, as previously described (Nonne et al., 2005). For each type of lesion, three to seven arterioles were targeted over a period of 45 min with only one injury per vessel. To precisely define the contours of the thrombi and measure their surface area, DIOC₆ (0.5 nmol/g body weight) was injected into the jugular vein and images were acquired sequentially with wide field and fluorescent light using a Cooke SensiCam CCD camera (Auburn Hill, MI) controlled by SlideBook software (Intelligent Imaging Innovations, Denver, CO). The manipulator was unaware of the mouse genotype and treatment while performing these experiments.

Results

MRS2500 Selectively Inhibits Ex Vivo Mouse Platelet Aggregation Induced by ADP

Because of its potency and expectation of enhanced stability (Ravi et al., 2002), we started by injection of 2 mg/kg MRS2500 into mice and checked for the inhibition of platelet aggregation ex vivo in platelet-rich plasma at several time points (1, 5, 15, 30, and 60 min). MRS2500

inhibited the ex vivo platelet aggregation and shape change triggered by ADP (1 μ M) for at least 60 min (Fig. 1A). At a higher concentration of ADP (5 μ M), aggregation and shape change were inhibited at 5 min but reappeared at 15 min (Fig. 1A). For comparison, MRS2179 (50 mg/kg) inhibited aggregation induced by ADP (5 or 1 μ M) for 1 min only (Fig. 1A). A higher dose of MRS2500 (4 mg/kg) further inhibited platelet aggregation induced by ADP (5 μ M) for at least 30 min (Fig. 1B). Lower doses of MRS2500 (0.01 or 0.5 mg/kg) also inhibited ADP (1 μ M)-induced platelet aggregation for at least 5 min (data not shown). Overall, these results indicate increased potency and stability of MRS2500 compared with MRS2179 in vivo in mice.

To check for the selectivity of MRS2500 toward the P2Y₁ receptor, the compound (4 mg/kg) or saline was injected into anesthetized P2Y₁^{-/-} mice. PRP was prepared, and platelet aggregation was induced with ADP (100 μ M), collagen (2 μ g/ml), or U46619 (5 μ M). As shown in Fig. 2, MRS2500 (4 mg/kg) had no effect on the P2Y₁₂-mediated aggregation observed in P2Y₁^{-/-} mouse platelets at the high concentration of ADP (Léon et al., 1999; Kauffenstein et al., 2001), indicating that MRS2500 did not inhibit the P2Y₁₂ receptor. Likewise, aggregation induced by collagen or U46619, the stable analog of thromboxane A₂, was not inhibited by MRS2500 in P2Y₁^{-/-} mouse platelets (Fig. 2). Overall, these results indicate that MRS2500 displays high selectivity, potency, and stability in vivo.

Bleeding Time

The bleeding time was measured in mice after tail-tip amputation. Mice injected with MRS2500 (0.01 or 0.5 mg/kg) displayed a normal bleeding time (122 $\pm$ 33 s, *n* = 10; N.S., *P* = 0.0806 and 116 $\pm$ 25 s, *n* = 10; N.S., *P* = 0.0649) compared with mice receiving saline (65 $\pm$ 7 s, *n* = 10). Bleeding time was similarly not substantially longer in five of 10 WT mice injected with MRS2500 (2 mg/kg) (82 $\pm$ 10 s, *n* = 5) compared with mice injected with saline (65 $\pm$ 7 s, *n* = 10) (N.S., *P* = 0.1138) (Fig. 3), whereas among the other five mice, one displayed a bleeding time of 915 s and the remaining four displayed a bleeding time longer than 1800 s, which is consistent with our previous observations in P2Y₁^{-/-} mice (Léon et al., 1999).

Decreased Acute Systemic Thromboembolism through Selective Inhibition of the P2Y₁ Receptor with MRS2500

We used the model of acute vascular occlusion induced by intravenous injection of a mixture of collagen (0.15 mg/kg) and adrenaline (60 μ g/kg) to evaluate the effect of MRS2500 on intravascular platelet aggregation in vivo. To evaluate the selectivity of MRS2500 in this model, the experiment was also performed on P2Y₁^{-/-} mice. WT and P2Y₁^{-/-} mice were injected with MRS2500 (2 mg/kg) or saline 30 s before the thrombogenic mixture, and platelet consumption was determined (Léon et al., 1999). WT mice injected with MRS2500 displayed reduced platelet consumption compared with mice injected with saline (50 $\pm$ 3 versus 71 $\pm$ 4%) (***, *P* = 0.0004, *n* = 10), reflecting decreased intravascular platelet aggregation. In P2Y₁^{-/-} mice, platelet consumption was not further decreased by MRS2500 (50 $\pm$ 3 versus 53 $\pm$ 4%) (N.S., *P* = 0.5152, *n* = 10) (Fig. 4A), demonstrating the selectivity of MRS2500 toward the P2Y₁ receptor. In addition, in P2Y₁^{-/-} mice receiving saline, platelet consumption was comparable with that in WT mice receiving MRS2500 (53 $\pm$ 4 versus 50 $\pm$ 3%) (N.S., *P* = 0.5450, *n* = 10) (Fig. 4A). Histological analysis of the lungs of WT mice injected with MRS2500 showed that only a few vessels were obstructed and that the thrombi were rarely occlusive compared with WT mice receiving saline (Fig. 4A).

A higher dose of collagen (0.21 mg/kg) was injected in WT mice to induce mortality by extensive obstruction of the lung microcirculation (DiMinno and Silver, 1983; Léon et al., 1999). All 10 mice receiving saline died within 15 min (Fig. 4B), whereas among 10 mice injected with MRS2500 (2 mg/kg), five died and the five others survived the challenge for the 30-min observation period (Fig. 4B). The effect of MRS2500 treatment on survival was

statistically significant by log-rank test (***, $P < 0.0001$, $n = 10$), and the percentage of survivors among mice receiving MRS2500 was significantly greater than that among mice receiving saline (**, $P = 0.0098$, $n = 10$, χ^2 test). With a lower dose of MRS2500 (0.5 mg/kg), five of 10 mice also survived the challenge for the 30-min observation period (***, $P = 0.0004$, $n = 10$, log-rank test), whereas with a dose of 0.01 mg/kg, six mice died and the four others survived the challenge (**, $P = 0.0074$, $n = 10$, log-rank test). It can be noticed that the time to death increased as the dose of MRS2500 administered into mice increased (Fig. 4B). Overall, these results indicate that MRS2500 efficiently inhibits acute systemic intravascular thrombosis in mice.

MRS2500 Strongly Inhibits Localized Arterial Thrombosis

MRS2500 was also tested in a previously described model of localized thrombosis in mesenteric arterioles triggered by laser-induced vessel wall injury (Hechler et al., 2003; Nonne et al., 2005). Reproducible superficial and severe injuries were induced by adjusting the laser intensity and number of pulses as described under Materials and Methods. Thrombi that form after the two types of injury are inhibited by a GPIIb-IIIa blocker and the P2Y₁₂ inhibitor clopidogrel (Nonne et al., 2005). However, thrombi that form after deeper injury only are inhibited by hirudin, suggesting that they are dependent on thrombin formation (Nonne et al., 2005).

In superficial injuries, thrombus formation classically evolved in two phases with platelets quickly accumulating at the site of endothelial desquamation to form a parietal thrombus peaking at 48 s followed by progressive erosion resulting in a 84% decrease in thrombus surface area after 160 s (Fig. 5A, black curve). A dose-dependent inhibition of thrombus formation was observed with increasing doses of MRS2500. Maximum thrombus surface area with 0.01 mg/kg MRS2500 represented only 63% of that in mice receiving saline and 30.5 to 33% with 0.1, 0.25, 0.5, and 1 mg/kg MRS2500 (Fig. 5A). At the highest dose of 2 mg/kg, thrombus formation was further decreased (86.5% reduction in size) (Fig. 5A). In the severe injury model, a parietal thrombus formed progressively during the first 90 s and reached a 14-fold larger size than in the superficial lesion model, leading to near occlusion of the vessel lumen (Fig. 5B), and the thrombus size did not significantly decrease during the following 3 min. Higher doses of MRS2500 were required to inhibit thrombosis. MRS2500 (1 and 2 mg/kg) reduced the maximum thrombus surface area by 32% (Fig. 5B). With MRS2500 (1 mg/kg), a stepwise drop in thrombus size followed by thrombus reformation was observed, indicating thrombus instability. With MRS2500 (2 mg/kg), thrombus size rapidly decreased (Fig. 5B). Overall, these results indicate that MRS2500 is a potent inhibitor of arterial thrombus formation after mild or severe lesion of the vessel wall.

This effect of MRS2500 was selective because P2Y₁^{-/-} mice were insensitive to MRS2500 treatment in both superficial (Fig. 5C) and severe injuries (Fig. 5D). In P2Y₁^{-/-} mice, thrombus size was reduced compared with WT mice but to a lower extent, as compared with that in WT mice receiving MRS2500 (Fig. 5, C and D). Again, stepwise drops were noticed in the descending portion of the curve, indicating a tendency to embolization of platelet aggregates (Fig. 5, C and D).

Association of MRS2500 with Clopidogrel Improves Protection against Thrombosis

We investigated whether combined addition of the P2Y₁ receptor antagonist with the antiplatelet drug clopidogrel, which targets the P2Y₁₂ ADP receptor, could improve protection against thrombosis in the severe injury model. Clopidogrel was used at 50 mg/kg (administered orally twice into mice: the day before and two hours before the experiment), a dose leading to a complete irreversible inhibition of the P2Y₁₂ receptor (Gachet et al., 1995). In agreement with our previous report (Nonne et al., 2005), thrombus formation was greatly reduced by

clopidogrel (75% reduction in size at 2 min) and the combination of MRS2500 (2 mg/kg) with clopidogrel resulted in almost complete inhibition of thrombus formation (Fig. 6). Overall, these results indicate that inhibition of the P2Y₁ receptor together with P2Y₁₂ receptor blockade leads to potent antithrombotic efficacy.

Discussion

The aim of the present study was to evaluate the antithrombotic properties of a potent and stable competitive P2Y₁ receptor antagonist MRS2500 to both obtain insight in the performance of the compound and to further validate the concept that targeting the P2Y₁ receptor could be a relevant alternative or complement to other antiplatelet strategies. Previous studies with MRS2179 already indicated ex vivo inhibition of ADP-induced platelet aggregation and in vivo inhibition of systemic thromboembolism (Baurand et al., 2001; Léon et al., 2001; Baurand and Gachet, 2003). However, the lack of stability in vivo precluded the possibility to further evaluate this compound in animals. Therefore, MRS2500 was designed for optimized antagonism of the P2Y₁ receptor (Jacobson et al., 2005). The presence of the rigid methanocarba (a bicyclo[3.1.0]hexane) ring constrains the nucleotide in a North conformation that is preferred by the P2Y₁ receptor (Nandanan et al., 2000; Kim et al., 2003; Ohno et al., 2004). In addition to enhancing affinity as a P2Y₁ receptor antagonist, this moiety as a ribose substitute also prolongs stability toward ectonucleotidases. Indeed, a 5'-monophosphate derivative in this chemical series was shown to be nearly resistant to the action of 5'-nucleotidase (Ravi et al., 2002). The high potency and selectivity of MRS2500 for P2Y₁ receptor inhibition was demonstrated in vitro both in 1321N1 human astrocytoma cells expressing the recombinant human P2Y₁ receptor and in human platelets (Kim et al., 2003; Cattaneo et al., 2004).

Here, we show that MRS2500 also displays high potency and stable and selective P2Y₁ receptor inhibition in vivo. Injection of MRS2500 (2 mg/kg) into mice did not prolong the bleeding time in half of the mice. In the other half, one had mild prolongation and the others displayed bleeding time longer than 1800 s. The same was already observed and reported concerning the P2Y₁^{-/-} mice and is thought to be related to thrombus instability (Léon et al., 1999). One has to keep in mind that, in contrast, compounds targeting GPIIb-IIIa or P2Y₁₂ induce a dramatic prolongation of the bleeding time in all of the animals tested (data not shown) (for review see Hodivala-Dilke et al., 1999; Foster et al., 2001).

MRS2500 provided strong antithrombotic activity in systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline, which is in agreement with the resistance of P2Y₁^{-/-} mice to thrombosis in this model (Fig. 4) (Léon et al., 1999). This compound was also found to be effective in a model of laser-induced vessel wall injury with two degrees of severity, the more severe injury being dependent on thrombin formation (Nonne et al., 2005). The P2Y₁ receptor has already been shown to contribute to acute systemic thrombin-dependent thrombosis induced by infusion of tissue factor (Léon et al., 2001). This is consistent with the fact that the P2Y₁ receptor is involved in the procoagulant activity of platelets, indirectly through platelet P-selectin exposure and formation of platelet-leukocyte conjugates, leading to leukocyte-tissue factor exposure (Léon et al., 2003).

The residual thrombus formed after laser injury in P2Y₁-deficient mice or in mice receiving MRS2500 displayed decreased stability and a tendency to embolization (Fig. 5). van Gestel et al. (2003) have shown previously that P2Y₁ blockade with A3P5P, a first-generation selective P2Y₁ receptor antagonist, resulted in decreased thrombus stability in a model of thromboembolism induced by mechanical vessel wall puncture of rabbit mesenteric arterioles. Overall, these results suggest a role of the P2Y₁ receptor in thrombus stability at the vessel wall, as already suggested in the case of bleeding time measurements.

Interestingly, MRS2500 treatment could result in greater inhibition of thrombus formation compared with P2Y₁ deficiency. We have extensively checked for a possible nonselective effect of the compound, which could be excluded. This observation suggests that compensatory processes occurred in the knockout mice, as already described in other mouse strains (Gingrich and Hen, 2000). This difference was not observed in the model of acute systemic thromboembolism, which would rule out the possibility of compensatory processes within the platelet and would suggest that the compensatory process involves the much more complex pathogenesis of thrombi on injured vessels. However, further investigation would be required to address this point

As reported previously, current antiplatelet drugs, such as clopidogrel, a GPIIb-IIIa blocker, and hirudin, were also effective in localized thrombosis after laser-induced severe injury (Nonne et al., 2005). Because a combination of antiplatelet drugs that inhibit multiple pathways of platelet activation is more efficient than each drug alone, as has been demonstrated for clopidogrel and aspirin in large clinical trials (Bhatt and Topol, 2003), we investigated whether the combined addition of the P2Y₁ receptor antagonist with clopidogrel could improve protection against thrombosis, which indeed was the case (Fig. 6). Similar results were previously reported in a model of localized arterial thrombosis of mesenteric arterioles triggered by ferric chloride injury (Lenain et al., 2003). Thus, this combination could be promising in view of future drug association. The P2Y₁ receptor is widely distributed in many tissues, including heart, blood vessels, smooth muscle cells, neural tissue, testis, prostate, and ovary (Ralevic and Burnstock, 1998), which could be a disadvantage compared with the P2Y₁₂ receptor, the expression of which is restricted to the platelet lineage (Foster et al., 2001). However, there is a long list of drugs that act on multiple sites in a beneficial or even synergistic manner. The most prominent example of such a drug among antiplatelet agents is aspirin, which ubiquitously targets cyclo-oxygenase 1 in the body (Patrino, 2001). With regard to the platelet P2 receptors, one can speculate that a molecule with a dual selectivity for P2Y₁ and P2Y₁₂ receptors would combine the advantages of platelet selectivity (P2Y₁₂) with higher efficacy (both P2Y₁ and P2Y₁₂). Possible synergism between P2Y₁ and P2Y₁₂ receptors could reduce the dose of the drug required or allow the use of antagonists with lower potency. It is to be noted that, because inhibition of the P2Y₁ receptor results in only moderate prolongation of the bleeding time compared with P2Y₁₂ inhibition, combined receptor inhibition might diminish hemorrhagic risk.

The concept of multivalent P2 receptor antagonists has been put forward recently by the use of the suramin analog NF449 (Kassack et al., 2004; Hechler et al., 2005). Indeed, a low dose of NF449 selectively inhibits the P2X₁ receptor and leads to decreased thrombosis. At a higher dose (50 mg/kg), NF449 inhibits specifically the three platelet P2 receptors and leads to a strong reduction in thrombosis (Hechler et al., 2005), adding further evidence that targeting multiple P2 receptors might be a promising antithrombotic strategy.

Overall, our results indicate that the P2Y₁ receptor may become a relevant clinical target alone or in combination with current antithrombotic strategies. These promising results warrant the use of this compound in established experimental thrombosis models in higher animals, such as the pig or dog, to test them alone or in combination with current antiplatelet drugs.

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ABBREVIATIONS

P2X₁ and P2Y₁	platelet P2X ₁ and platelet P2Y ₁ , respectively
WT	wild type

PRP	platelet-rich plasma
U46619	9,11-dideoxy-11 α ,9 α -epoxy-methanoprostaglandin F _{2α}
DIOC₆	3,3'-dihexyloxacarbocyanine iodide
MRS2179	2'-deoxy- <i>N</i> ⁶ -methyl adenosine 3',5'-diphosphate
MRS2500	2-iodo- <i>N</i> ⁶ -methyl-(<i>N</i>)-methanocarpa-2'-deoxyadenosine-3',5'-bisphosphate
NF449	4,4',4'',4' ''-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid octasodium salt
A3P5P	adenosine-3'-phosphate-5'-phosphate

Fig. 1.

Increased potency and stability of MR2500 compared with MRS2179 in vivo in mice. A, MRS2500 (2 mg/kg), MRS2179 (50 mg/kg), or saline was injected into the jugular vein of anesthetized WT mice, and blood was drawn at various time (1, 5, 15, 30, and 60 min) from the abdominal aorta into citrate (3.15%) anticoagulant. PRP was prepared, and aggregation was induced with ADP (5 μ M) (left) or ADP (1 μ M) (right). B, similar experiments were performed, as detailed in A, using MRS2500 (4 mg/kg) and MRS2179 (50 mg/kg), and blood was drawn at 15 and 30 min. Aggregation of citrated PRP was induced with ADP (5 μ M). Data are mean values ($\pm$ S.E.M.) from three separate experiments.

Fig. 2.

MRS2500 does not inhibit aggregation induced by collagen, U46619, or ADP in P2Y₁^{-/-} platelets. MRS2500 (4 mg/kg) was injected into the jugular vein of anesthetized P2Y₁^{-/-} mice, and blood was drawn 10 min later from the abdominal aorta into citrate (3.15%) anticoagulant. PRP was prepared, and aggregation was induced with ADP (100 μM), collagen (2 μg/ml), or U46619 (5 μM), a stable analog of thromboxane A₂. MRS2500 (4 mg/kg) had no inhibitory effect on aggregation induced by ADP, collagen, or U46619, overall indicating selective inhibition of the P2Y₁ receptor.

Fig. 3.

Tail-bleeding time. The bleeding time was measured in mice after tail-tip amputation. Mice injected with MRS2500 (0.01 or 0.5 mg/kg) displayed a normal bleeding time (122 ± 33 s, $n = 10$; N.S., $P = 0.0806$ and 116 ± 25 s, $n = 10$; N.S., $P = 0.0649$) compared with mice receiving saline (65 ± 7 s, $n = 10$). Bleeding time was similarly not substantially longer in five of 10 WT mice injected with MRS2500 (2 mg/kg) (82 ± 10 s, $n = 5$) compared with mice injected with saline (65 ± 7 s, $n = 10$; N.S., $P = 0.1138$), whereas among the other five mice, one displayed a bleeding time of 915 s and the remaining four displayed a bleeding time longer than 1800 s.

Fig. 4.

Resistance of mice injected with MRS2500 (2 mg/kg) to acute systemic intravascular thromboembolism. A (top), representative histological sections of lungs of WT mice receiving saline or MRS2500 (2 mg/kg) and challenged with collagen (0.15 mg/kg) and adrenaline (60 μ g/kg). Bar = 100 μ m. Note the frequent occurrence of occlusive intravascular thrombi in lungs of WT mice injected with saline (arrows) (inset, bar = 10 μ m) compared with unobstructed vessels in lungs of WT mice receiving MRS2500 (2 mg/kg) (arrows and inset). A (bottom), WT mice injected with MRS2500 (2 mg/kg) displayed reduced platelet consumption after intravenous infusion of the mixture of collagen (0.15 mg/kg) and adrenaline (60 μ g/kg) compared with mice injected with saline (50 ± 3 versus $71 \pm 4\%$) (***, $P = 0.0004$,

$n = 10$), reflecting decreased intravascular platelet aggregation. Platelet consumption was not further decreased compared with $P2Y_1^{-/-}$ mice receiving saline (50 ± 3 versus $53 \pm 4\%$) (N.S., $P = 0.5152$; $n = 10$). In addition, in $P2Y_1^{-/-}$ mice receiving saline, platelet consumption was comparable with that in WT mice receiving MRS2500 (2 mg/kg) (53 ± 4 versus $50 \pm 3\%$) (N.S., $P = 0.5450$; $n = 10$). B, time from collagen (0.21 mg/kg) and adrenaline ($60 \mu\text{g/kg}$) injection to death of mice. Results are expressed as the percentage of mice alive as a function of time. All 10 mice receiving saline died within 15 min, although among the 10 mice injected with MRS2500 (2 mg/kg), five died and the five others survived the challenge for the 30-min observation period. The effect of MRS2500 treatment on survival was statistically significant by log-rank test (***, $P < 0.0001$, $n = 10$), and the percentage of survivors among mice receiving MRS2500 was significantly greater than that among mice receiving saline (**, $P = 0.0098$, $n = 10$, χ^2 test). With a lower dose of MRS2500 (0.5 mg/kg), five out of 10 mice also survived the challenge for the 30-min observation period (***, $P = 0.0004$; $n = 10$, log-rank test), although with a dose of 0.01 mg/kg, six mice died and the four others survived the challenge (**, $P = 0.0074$, $n = 10$, log-rank test).

Fig. 5.

Resistance of mice injected with MRS2500 to localized thrombosis of mesenteric arterioles triggered by laserinduced injury. Superficial (A and C) and severe lesions (B and D) were generated in WT and $\text{P2Y}_1^{-/-}$ mice receiving saline or MRS2500 at the indicated dose. Top panels are representative photographs of the thrombus at maximal size after superficial (A) or severe (B) lesions; $\rightarrow$ represents blood flow direction. Bar = $30\ \mu\text{m}$. Bottom panels are mean thrombus surface area ($\pm$ S.E.M.) at each time point (0.3 s intervals) in WT receiving saline (black curve) and in WT mice receiving increasing doses of MRS2500 (0.01, 0.1, 0.25, 0.5, 1, or 2 mg/kg). A, in the presence of increasing doses of MRS2500, thrombus size progressively decreased compared with mice receiving saline ($n = 33$ vessels in 10 mice) in the superficial lesions (MRS2500 0.01 mg/kg, $n = 7$ vessels in two mice; N.S., $P = 0.1392$) (MRS2500 0.1 mg/kg, $n = 20$ vessels in six mice; N.S., $P = 0.0752$) (MRS2500 0.25 mg/kg, $n = 14$ vessels in three mice, *, $P = 0.0174$) (MRS2500 0.5 mg/kg, $n = 15$ vessels in five mice, *, $P = 0.0119$) (MRS2500 1 mg/kg, $n = 12$ vessels in three mice, **, $P = 0.0056$) (MRS2500 2 mg/kg, $n = 16$ vessels in four mice, ***, $P = 0.0001$). B, in the severe injury, protection against thrombosis was also found in WT mice injected with increasing doses of MRS2500 compared with WT animals ($n = 31$ vessels in 15 mice) and the thrombus showed a tendency to embolize

(MRS2500 0.1 mg/kg, $n = 5$ vessels in three mice; N.S., $P = 0.7837$) (MRS2500 0.25 mg/kg, $n = 3$ vessels in two mice; N.S., $P = 0.5042$) (MRS2500 0.5 mg/kg, $n = 7$ vessels in four mice; N.S., $P = 0.1525$) (MRS2500 1 mg/kg, $n = 3$ vessels in three mice, *, $P = 0.0336$) (MRS2500 2 mg/kg, $n = 28$ vessels in 13 mice, ***, $P < 0.0001$). C, thrombus surface area after superficial injury in $P2Y_1^{-/-}$ mice was not further decreased by MRS2500 (2 mg/kg), indicating selective $P2Y_1$ receptor inhibition ($n = 7$ vessels in three $P2Y_1^{-/-}$ mice treated with saline or MRS2500; N.S., $P = 0.8125$). In $P2Y_1^{-/-}$ mice receiving saline, thrombus size was reduced compared with WT mice receiving saline ($n = 11$ vessels in four mice) but to a lower extent compared with that in WT mice receiving MRS2500 (2 mg/kg) ($n = 7$ vessels in two mice) (WT versus $P2Y_1^{-/-}$; N.S., $P = 0.2772$, MRS2500 versus $P2Y_1^{-/-}$ *, $P = 0.0424$). D, after severe injury in $P2Y_1^{-/-}$ mice, thrombus surface area was not further decreased by MRS2500 (2 mg/kg) ($n = 7$ vessels in four $P2Y_1^{-/-}$ mice treated with saline or MRS2500; N.S., $P = 0.3125$), again indicating selective $P2Y_1$ receptor inhibition.

Fig. 6.

Association of MRS2500 with clopidogrel improves protection against thrombosis. Severe lesions were generated in WT mice receiving saline, MRS2500 (2 mg/kg), clopidogrel (50 mg/kg) (administered orally twice into mice: the day before and 2 h before the experiment), or a combination of MRS2500 (2 mg/kg) and clopidogrel (50 mg/kg). In the presence of clopidogrel (50 mg/kg) or MRS2500 (2 mg/kg), thrombus size was significantly less than in mice receiving saline ($n = 7$ to 9 vessels in three mice) (80 and 32% reduction in size for clopidogrel and MRS2500, respectively, at 3 min) and combination of MRS2500 (2 mg/kg) with clopidogrel treatment resulted in almost complete inhibition of thrombus formation (97.5% reduction in size at 3 min) ($n = 7$ vessels in three mice; ***, $P = 0.006$).