Inhibition of Platelet Functions and Thrombosis through Selective or Nonselective Inhibition of the Platelet P2 Receptors with Increasing Doses of NF449 [4,4′,4″,4‴-(Carbonylbis(imino-5,1,3-benzentrietylblis-(carbonylimino))tetrakis-benzene-1,3-disulfonic Acid Octasodium Salt]  

Béatrice Hechler, Stéphanie Magnenat, Maddalena L. Zighetti, Matthias U. Kassack, Heiko Ullmann, Jean-Pierre Cazenave, Richard Evans, Marco Cattaneo, and Christian Gachet  

Institut National de la Santé et de la Recherche Médicale U.311, Etablissement Français du Sang-Alsace, Strasbourg, France (B.H., S.M., J.-P.C., C.G.); Centro Emofilia e Trombosi A. Bianchi Bonomi, Istituto di Ricovero e Cura a Carattere Scientifco Ospedale Maggiore, Università di Milano, Milan, Italy (M.L.Z.); Pharmazeutisches Institut, Universität Bonn, Bonn, Germany (B.H., S.M., J.-P.C., C.G.); Centro Emofilia e Trombosi, Ospedale San Paolo, Dipartimento di Medicina Chirurgia e Odontoiatria, Università di Milano, Milan, Italy (M.C.)  

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ABSTRACT  
Our aim was to determine whether the newly described P2X1 antagonist NF449 [4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzentrietylblis(carbonylimino))tetrakis-benzene-1,3-disulfonic acid octasodium salt] could selectively antagonize the platelet P2X1 receptor and how it affected platelet function. NF449 inhibited α,β-methyleneadenosine 5′-triphosphate-induced shape change (IC50 = 83 ± 13 nM; n = 3) and calcium influx (pA2 = 7.2 ± 0.1; n = 3) (pIC50 = 6.95) in washed human platelets treated with apyrase to prevent desensitization of the P2X1 receptor. NF449 also antagonized the calcium rise mediated by the P2X1 receptor, but with lower potency (IC50 = 5.8 ± 2.2 μM; n = 3). In contrast, it was a very weak antagonist of the P2Y1,2-mediated inhibition of adenylyl cyclase activity. Selective blockade of the P2X1 receptor with NF449 led to reduced collagen-induced aggregation, confirming a role of this receptor in platelet activation induced by collagen. Intravenous injection of 10 mg/kg NF449 into mice resulted in selective inhibition of the P2X1 receptor and decreased intravascular platelet aggregation in a model of systemic thromboembolism (35 ± 4 versus 51 ± 3%) (P = 0.0061; n = 10) but without prolongation of the bleeding time (106 ± 16 versus 78 ± 7 s; n = 10) (N.S.; P = 1.209). At a higher dose (50 mg/kg), NF449 inhibited the three platelet P2 receptors. This led to a further reduction in platelet consumption compared with mice injected with saline (13 ± 4 versus 42 ± 3%) (P = 0.0002; n = 5). NF449 also reduced dose-dependently the size of thrombi formed after laser-induced injury of mesenteric arterioles. Overall, our results indicate that NF449 constitutes a new tool to investigate the functions of the P2X1 receptor and could be a starting compound in the search for new antithrombotic drugs targeting the platelet P2 receptors.  

Among the three P2 receptor subtypes present on blood platelets, the G protein-coupled P2Y1 and P2Y12 subtypes are ADP receptors, whereas the P2X1 ion channel is activated by ATP. The P2Y1 and P2Y12 receptors are essential for normal aggregation in response to ADP: the Gq-coupled P2Y1 recep-
tor initiates platelet aggregation but is not sufficient for a full platelet response, whereas the Gi-coupled P2Y12 receptor is responsible for completion of the aggregation triggered by ADP and other aggregating agents and also potentiates platelet secretion (Gachet, 2001; Hechler et al., 2005). Due to its central role in the formation and stabilization of a thrombus, the P2Y12 receptor is a well established target of anti-thrombotic drugs such as clopidogrel (CAPRIE Steering Committee, 1996; Gachet, 2001; Savi et al., 2001; Savi and Herbert, 2005). Concerning the P2Y1 receptor, experimental thrombosis in P2Y1 knockout mice (Fabre et al., 1999; Léon et al., 1999) and mice treated with a selective P2Y1 antagonist (Baurand et al., 2001; Léon et al., 2001; Baurand and Gachet, 2003; Lenain et al., 2003a,b) has shown that this receptor could be a potential target for new anti-thrombotic drugs.

Study of the role of the P2X1 receptor in hemostasis and thrombosis has long been hindered by a lack of potent and selective P2X1 antagonists and by the rapid and long-lasting desensitization of the receptor (Vial et al., 1997; Jin et al., 1998; Takano et al., 1999). When P2X1 desensitization is prevented by addition of a high concentration of apyrase (ATP-diphosphohydrolase; EC 3.6.1.5.), this receptor triggers a transient platelet shape change in response to the poorly hydrolysable ATP analog αβ-methylene-ATP (αβMeATP) (Rolf et al., 2001). Despite the fact that activation of the P2X1 receptor alone cannot induce platelet aggregation, it contributes to aggregation in response to collagen (Oury et al., 2001; Hechler et al., 2003). The role of P2X1 in platelet functions seems to be particularly relevant under flow conditions characterized by high shear stress (Cattaneo et al., 2002; Hechler et al., 2003; Oury et al., 2004). A study of P2X1-deficient (P2X1−/−) mice has further indicated that this receptor contributes to the thrombosis of small arteries. P2X1−/− mice display resistance to the localized arterial thrombosis of mesenteric arteries triggered by laser-induced vessel wall injury and to the acute systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline (Hechler et al., 2003). Conversely, increased systemic thrombosis has been reported in mice overexpressing the human P2X1 receptor (Oury et al., 2003). Hence, this receptor could also constitute a potential target for new anti-thrombotic drugs. Whether combined inhibition of the platelet P2 receptors might lead to greater resistance to thrombosis has not yet been investigated.

The synthesis of a new potent P2X1 antagonist was recently reported: NF449 [4,4′,4′′-carbonyl(bis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid octasodium salt], an analog of suramin that displays good selectivity for the P2X1 receptor with respect to the P2X2 and P2X3 receptors (Hulsmann et al., 2003) and P2Y1, P2Y2, and P2Y11 receptor subtypes (Braun et al., 2001; Kassack et al., 2004). NF449 potently antagonizes contractions of rat vas deferens mediated by the native P2X1 receptor (pIC50 = 7.15 ± 0.03) or inward currents induced by ATP in follicle cell-free Xenopus laevis oocytes expressing a recombinant rat P2X1 receptor (pIC50 = 9.54 ± 0.01), whereas it behaves as a weak antagonist at P2Y1 receptors in guinea pig ileum (pIC50 = 4.85 ± 0.06). However, the effects of this molecule have not yet been tested on various P2 receptor subtypes coexpressed in the same cell. The aim of the present study was to determine first whether NF449 selectively antagonizes the platelet P2X1 receptor and second how it affects platelet functions in vitro and in vivo.

**Materials and Methods**

**Chemicals.** αβMeATP, ADP, insoluble bovine collagen type I, U46619, adrenaline, serotonin, MRS2179, TRAP-1 (SFLIRNP), prostaglandin E1 (PGE1), and essentially fatty acid-free human serum albumin were from Sigma (Saint Quentin-Fallavier, France). Equine collagen (Kollagenreagent Hamburg) was purchased from Hormon Chemie (Munich, Germany), and NF449 was provided by Teorics Cookson Inc. (Bristol, UK). Human fibrinogen was from Kabo (Stockholm, Sweden), fura-2/acetoxymethyl ester from Calbiochem (Meudon, France) and AR-C69931MX was from Astra Charnwood (Loughborough, UK). Apyrase (adenosine 5′-triphosphatase diphosphohydrolase; EC 3.6.1.5.) was purified from potatoes as described previously (Cazenave et al., 2004). RAM.2 rat monoclonal antibody against mouse GPIIbIIIa was kindly provided by F. Lanza (INSERM U.311, Strasbourg, France), and recombinant hirudin was provided by A. Pavirani (Transgene SA, Strasbourg, France). Human serum albumin was from the Etablissement Français du Sang-Alsace (Strasbourg, France). PPACK was from Calbiochem (San Diego, CA).

**Mouse Strains.** Wild-type (WT), P2X1 receptor-deficient mice (P2X1−/−) (Mulryan et al., 2000) and P2Y1 receptor-deficient (P2Y1−/−) mice (Léon et al., 1999) were of pure C57BL/6 genetic background. P2X1−/− mice (Le´on et al., 2001; Baurand and Gachet, 2003; Lenain et al., 2003a,b) has shown that this receptor contributes to the thrombosis of small arteries. P2X1−/− mice displayed resistance to the localized arterial thrombosis of mesenteric arteries triggered by laser-induced vessel wall injury and to the acute systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline (Hechler et al., 2003). Conversely, increased systemic thrombosis has been reported in mice overexpressing the human P2X1 receptor (Oury et al., 2003). Hence, this receptor could also constitute a potential target for new anti-thrombotic drugs. Whether combined inhibition of the platelet P2 receptors might lead to greater resistance to thrombosis has not yet been investigated.

**Aggregation of Washed Human Platelets.** Washed human platelets were prepared as described previously (Cazenave et al., 2004) and resuspended at a density of 3 × 10^11 platelets/l in Tyrode's buffer containing CaCl2, normally in the presence of 0.02 U/ml adenine nucleotides scavenger apyrase, a concentration sufficient to prevent desensitization of platelet responses to ADP (Baurand et al., 2000). This low concentration of apyrase was nevertheless not sufficient to block P2X1 desensitization. Therefore, in experiments where the platelet P2X1 receptor was required to remain functional, a higher concentration of apyrase (0.5 U/ml) was added at each step of the washing procedure and in the final resuspending buffer (Hechler et al., 2003). Platelets were kept at 37°C throughout all experiments.

Aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, ON, Canada). A 450-μl aliquot of platelet suspension was stirred at 1100 rpm and activated by addition of 50 μl of the appropriate agonist. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline and platelet shape change in response to P2X1 activation by measuring the maximum decrease in light transmission after addition of αβMeATP.

**Shear-Induced Platelet Aggregation in Human Platelet-Rich Plasma.** For the preparation of platelet-rich plasma (PRP), 9 volumes of blood were drawn into 1 volume of 763 μM PPACK in the presence of 0.5 U/ml apyrase; platelet count was adjusted to 3 × 10^11/l. Platelet aggregation and shape change were measured in an aggregometer (Chrono-log, Havertown, PA). NF449, at the indicated concentrations, was incubated with PRP at room temperature for 30 min before platelet stimulation with the agonists.

Shear-induced platelet aggregation was measured as described previously (Cattaneo et al., 1993). Briefly, duplicate PRP samples (350 μl) were exposed to controlled shear stress (100 dynes/cm²) in a stainless steel cone-and-plate viscometer (controlled stress rheometer; Carri-med, Dorking, UK) at 37°C for 1 min. The cone diameter was 6 cm and its angle was 0.21°. After being subjected to shear stress, 25 μl of PRP was added to 100 μl of 2.5% paraformaldehyde in phosphate-buffered saline, and the number of single platelets per microliter was counted in an automated cell counter (Micros 60; ABX, Milan, Italy). The appearance of platelet aggregates was accompanied by a decrease in the number of single platelets, which was
expressed as percentage of that present in the same PRP incubated in the cone-and-plate viscometer at 37°C for 1 min without shearing. 

**[Ca^{2+}]_i Measurements.** Loading of platelets with fura-2/ace-toxymethyl ester and intracellular calcium measurements were performed as described previously (Ohlmann et al., 2004).

**Measurement of Adenylyl Cyclase Activity.** A 450-μl aliquot of washed platelet suspension was stirred at 1100 rpm in an aggregometer cuvette, and the following reagents were added at 30-s intervals: PGE$_1$ (10 μM), NF449 and ADP or vehicle (Tyrode’s buffer). One minute later, the reaction was stopped by addition of 50 μl of ice-cold 6.6 N perchloric acid, and CAMP was extracted and quantified using a commercial radioimmunoassay (Amersham Biosciences Inc., Les Ulis, France) (Baurand et al., 2000).

**Scanning Electron Microscopy.** Washed human platelets were stimulated with 1 μM αβMeATP for 9 or 180 s, in the presence or absence of 0.3 μM NF449, in a final volume of 500 μl. The cells were then fixed by addition of an equal volume of fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose, 305 mMOSM/l, pH 7.3) previously warmed to 37°C. Samples were prepared for scanning electron microscopy as described previously (Ohlmann et al., 2000).

**Ex Vivo Platelet Aggregation.** Male mice weighing 25 to 30 g were anesthetized, the jugular vein was exposed surgically, and NF449 or physiological saline was injected within an infusion time frame of 3 to 4 s. At the times indicated, blood was drawn from the abdominal aorta into hirudin anticoagulant (100 U/ml) containing 0.5 U/ml apyrase. Platelet-rich plasma was prepared as described previously (Léon et al., 2001), the cell count was adjusted to 500,000 platelets/μl, and aggregation was measured as described above.

**Bleeding Time.** NF449 or physiological saline was injected into the jugular vein of 8-week-old mice, 1 min 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.** The model of acute systemic vascular thromboembolism induced by infusion of a mixture of collagen and adrenaline has been described previously (DiMinno and Silver, 1983; Léon et al., 1999). Briefly, male mice weighing 25 to 30 g were anesthetized and the jugular veins were exposed surgically. NF449 or physiological saline was injected into the left jugular vein, 30 s before injecting a mixture of 0.1 mg/kg collagen and 60 μg/kg adrenaline that was injected within an infusion time frame of 3 to 4 s into the right jugular vein. Two minutes later, blood was drawn from the abdominal aorta into 6 mM EDTA anticoagulant, and platelets were counted in an ACT Coulter DiffTM counter (Beckman-Coulter, Roissy, France).

The model of localized thrombosis in mesenteric arterioles triggered by laser-induced vessel wall injury has been described previously (Hechler et al., 2003). Mice weighing approximately 15 g and 3 to 5 weeks old were anesthetized, and their mesentery was exteriorized (Hechler et al., 2003). 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× objective of an inverted Leica DMIRB microscope with a Micropoint laser system (Photonics Instruments, St. Charles, IL) (Hechler et al., 2003). A superficial lesion was induced in arterioles with a diameter between 70 and 100 μm. The laser beam was focused on the luminal face of the vessel wall, and the firing duration was adjusted to cause endothelial desquamation in transmitted light. For each mouse, two to four arterioles were targeted over a period of 10 min with only one injury per vessel. To precisely define the contours of the thrombi and measure their surface area, 3,3'-dihexyloxycarbocyanine iodide (0.5 μg/g b.wt.) was injected into the jugular vein before vessel injury, and the fluorescence of platelets incorporated into the thrombi was visualized every 300 ms. Images were acquired sequentially, 7 s after the firing, with wide field and fluorescent light using a Cooke SensiCam charge-coupled device camera (Auburn Hill, MI) (2 × 2 binning) controlled by SlideBook software. The manipulator was unaware of the mouse treatment while performing these experiments.

**Results**

**Effect of NF449 on the Shape Change and Intracellular Calcium Rise Induced by αβMeATP in Washed Human Platelets.** In the presence of 0.5 U/ml apyrase ensuring functionality of the platelet P2X$_1$ receptor, increasing concentrations of αβMeATP induced a dose-dependent increase in platelet shape change (EC$_{50}$ = 880 ± 310 nM; n = 3) (Fig. 1A). Whereas NF449 by itself did not induce platelet shape change or aggregation even at a high concentration (100 μM) (data not shown), it dose-dependently inhibited the shape change triggered by 1 μM αβMeATP (IC$_{50}$ = 83 ± 13 nM; n = 3) (Fig. 1A). Figure 1B shows light transmission measurements and scanning electron micrographs illustrating the inhibitory effect of 0.3 μM NF449 on the morphological changes induced by 1 μM αβMeATP in human platelets.

At the intracellular level, αβMeATP (10 nM–100 μM) induced a dose-dependent rise in [Ca$^{2+}$]$_i$ (Fig. 1C). This response was abolished in the absence of external calcium (0.2 mM EGTA), indicating that it was entirely due to calcium entry from the external medium (data not shown). NF449 (0.03, 0.1, and 0.3 μM) induced a concentration-dependent parallel shift to the right of the concentration-response curve for [Ca$^{2+}$]$_i$ in the presence of αβMeATP (Fig. 1C), without significantly affecting the maximal response. A Schild plot analysis gave a mean pA$_2$ value for NF449 of 7.2 ± 0.1 (n = 3) and the pIC$_{50}$ was 6.95 at 0.3 μM αβMeATP (data not shown). Similarly, NF449 dose-dependently inhibited the [Ca$^{2+}$]$_i$ rise induced by αβMeATP in washed mouse platelets with a mean pA$_2$ value of 6.6 ± 0.1 (n = 3) (data not shown).

**Effect of NF449 on ADP-Induced Platelet Activation.** We next determined whether NF449 had a nonspecific inhibitory effect on the platelet P2Y$_1$ or P2Y$_{12}$ receptor. First, the effect of NF449 on ADP-induced aggregation of washed human platelets was examined in the presence of a lower concentration of apyrase (0.02 U/ml). Under these conditions, 10 μM aβMeATP did not trigger any detectable platelet shape change or [Ca$^{2+}$]$_i$ increase, confirming that the P2X$_1$ receptor was desensitized (data not shown). As seen in Fig. 2A, low concentrations of NF449 (0.3 or 1 μM) had no influence on ADP-induced platelet aggregation, whereas higher concentrations (30 or 100 μM) inhibited aggregation in response to ADP. This suggested that NF449 could interact with the P2Y$_1$ or P2Y$_{12}$ receptor. Similar experiments performed in washed mouse platelets also indicated that a high concentration of NF449 (100 μM) could inhibit ADP-induced aggregation (data not shown).

At the intracellular level, NF449 dose-dependently inhibited the [Ca$^{2+}$]$_i$ rise triggered by 0.3 μM ADP (IC$_{50}$ of 5.8 ± 2.2 μM; n = 3) (Fig. 2B). This suggested that NF449 antagonized the P2Y$_1$ receptor, which is in accordance with the inhibition of ADP-induced platelet aggregation and shape change observed in the presence of high concentrations of NF449. NF449 nevertheless displayed a lower potency for blockade of the P2Y$_1$ compared with the P2X$_1$ receptor. Similarly, NF449 dose-dependently inhibited the [Ca$^{2+}$]$_i$ rise...
induced by 0.3 μM ADP in washed mouse platelets (IC_{50} = 24 ± 2 μM; n = 3) (data not shown). In contrast, 10 μM NF449 had no effect on the [Ca^{2+}]_{i} increase in response to 1 μM serotonin in washed human platelets (data not shown).

In the P2Y_{12} receptor, NF449 (up to 1 mM) had no influence on basal levels of cAMP in washed human platelets or on the cAMP levels induced by 10 μM PGE_{1} (data not shown). NF449 (0.3 or 30 μM) had no significant effect on the inhibitory effect of NF449 on the platelet P2 receptors with NF449.
A

Fig. 2. Effect of NF449 on ADP-induced platelet activation. Experiments were performed in washed human platelets in the presence of a low concentration of apyrase (0.02 U/ml) to ensure desensitization of the P2X<sub>1</sub> receptor. A, effect of NF449 on platelet aggregation. Low concentrations of NF449 (0.3 or 1 μM) did not significantly modify ADP-induced aggregation [EC<sub>50</sub> values for ADP-induced platelet aggregation were 2.1 ± 0.4, 2.8 ± 0.6, and 4.2 ± 0.3 μM in the presence of 0, 0.3, and 1 μM NF449 (n = 3), respectively], whereas higher concentrations (30 or 100 μM) inhibited the dose-dependent increase in platelet aggregation induced by 0.05 to 500 μM ADP [EC<sub>50</sub> values for ADP-induced platelet aggregation were 8.6 ± 2.4 and 63 ± 21 μM in the presence of 30 and 100 μM NF449 (n = 3), respectively]. B, effect of NF449 on P2Y<sub>1</sub> receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub> mobilization. NF449 dose-dependently inhibited the [Ca<sup>2+</sup>]<sub>i</sub> rise triggered by 0.3 μM ADP [IC<sub>50</sub> = 5.8 ± 2.2 μM; n = 3]. C, effect of NF449 on P2Y<sub>12</sub> receptor-mediated inhibition of adenylyl cyclase activity. NF449 (0.3 or 30 μM) had no significant effect on the dose-dependent inhibition by ADP of the cAMP accumulation induced by 10 μM PGE<sub>1</sub>. In contrast, 100 μM or 1 mM NF449 shifted the dose-response curve for ADP-induced inhibition of adenylyl cyclase to the right. EC<sub>50</sub> values for ADP-induced inhibition of cAMP accumulation triggered by PGE<sub>1</sub> were 0.66 ± 0.27, 0.40 ± 0.10, 0.47 ± 0.02, and 1.44 ± 0.20 μM in the presence of 0, 0.3, 30, and 100 μM NF449 (n = 3), respectively. Data are mean values (±S.E.M.) from three separate experiments, each performed in duplicate.

bition by ADP (0.01–100 μM) of the cAMP accumulation triggered by 10 μM PGE<sub>1</sub> (Fig. 2C), whereas higher concentrations of NF449 (100 μM or 1 mM) shifted to the right the dose-response curve for ADP-induced inhibition of cAMP pro-
duction (Fig. 2C). Similar results were obtained in mouse platelets (data not shown). Conversely, the inhibition of PGE<sub>1</sub>-stimulated adenylyl cyclase activity induced by 1 μM adrenaline through activation of α<sub>2A</sub> adrenergic receptors
was not affected by NF449, even at a high concentration (1 mM) (data not shown).

Overall, NF449 antagonized the platelet P2X₁ receptor with good selectivity relative to the P2Y₁ receptor and displayed very low potency for inhibition of the P2Y₁₂ receptor. At an NF449 concentration of 0.3 μM, P2X₁ activation by 0.3 μM αβMeATP could be inhibited, leaving the P2Y₁ and P2Y₁₂ receptors fully functional. This concentration of NF449 was subsequently used to investigate the role of the P2X₁ receptor in aggregation of human platelets in response to various agents.

**Effect of Inhibition of the P2X₁ Receptor with NF449 on In Vitro Human Platelet Aggregation.** Previous studies in P2X₁⁻/⁻ mice (Hechler et al., 2003) or using selective desensitization of the P2X₁ receptor with αβMeATP (Oury et al., 2001) have established a role of this P2X₁ receptor in collagen-induced platelet activation. We therefore investigated whether similar results could be obtained through selective inhibition of the P2X₁ receptor with NF449. As shown in Fig. 3, the aggregation of washed human platelets induced by low concentrations of collagen (2.5, 2, or 1.5 μg/ml) was inhibited by 0.3 μM NF449 in the presence of 0.5 U/ml apyrase. Since NF449 had no effect on collagen-induced platelet aggregation under conditions where the P2X₁ receptor was desensitized (0.02 U/ml apyrase) (data not shown), the reduced aggregation must have been due to P2X₁ inhibition. In contrast, the aggregation induced by either the thromboxane A₂ analog U46619 (2.5 μM) or a low concentration of thrombin (0.04 U/ml), both of which trigger release of nucleotides from the platelet dense granules, was not modified by 0.3 μM NF449 (Fig. 3). This confirms previous results showing that the P2X₁ receptor is not essential for aggregation in response to these agonists (Hechler et al., 2003; Oury et al., 2003).

**Effects of NF449 on Shear-Induced Platelet Aggregation in Human PRP.** In general, the effects of NF449 on platelet function in human PPACK PRP containing 0.5 U/ml apyrase were very similar to those observed with washed platelets, although the inhibitory concentrations of the compound tended to be slightly higher, probably due to its binding to plasma proteins. NF449, at concentrations up to 10 μM, which completely inhibited 1 μM αβMeATP-induced platelet shape change, had no effects on platelet aggregation induced by 10 μM ADP, whereas at 100 μM, it caused approximately 40% inhibition of aggregation (data not shown). In mouse PRP, NF449 inhibited ADP-induced platelet aggregation at concentration higher than 100 μM (IC₅₀ = 580 μM). NF449, at concentrations up to 100 μM, had no effects on the inhibition by 10 μM ADP of PGE₁-induced increase in human platelet cAMP (data not shown). NF449 (1 and 10 μM) partially inhibited 2 μg/ml collagen-induced aggregation of human platelets, whereas it did not affect platelet aggregation induced by 1 μM U46619 or 20 μM TRAP-1 (data not shown). Similarly to the effects of NF449, P2X₁ desensitization obtained after incubation of PRP with 10 μM αβMeATP partially inhibited collagen-induced platelet aggregation, whereas it did not affect platelet aggregation induced by U46619 or TRAP-1 (data not shown), indicating that the observed effect of NF449 was simply mediated by P2X₁ receptor inhibition.

![Fig. 3. Effect of inhibition of the P2X₁ receptor with NF449 on platelet aggregation in response to other agonists.](image-url)

Experiments were performed in washed human platelets in the presence of a high concentration of apyrase (0.5 U/ml) to ensure functionality of the P2X₁ receptor. NF449 (0.3 μM) inhibited platelet aggregation in response to collagen (1.5, 2, or 2.5 μg/ml) but not in response to 2.5 μM U46619 or 0.04 U/ml thrombin. Data are from one experiment representative of three independent experiments giving identical results.
Since the role of P2X<sub>1</sub> in platelet function seems particularly relevant under flow conditions characterized by high shear stress, the effect of NF449 on shear-induced platelet aggregation in human PPACK-PRP was determined and compared with that of P2X<sub>1</sub> desensitization by αβMeATP. As shown previously (Cattaneo et al., 2002), the addition of 0.5 U/ml apyrase to PPACK-PRP significantly potentiated shear-induced platelet aggregation (Table 1). Preincubation of PPACK-PRP with 10 μM NF449 partially inhibited shear-induced platelet aggregation in the presence of apyrase, to a level that was similar to that observed in the same PRP to which apyrase had not been added. A short preincubation of PRP with 10 μM αβMeATP, which desensitized P2X<sub>1</sub>, caused a similar extent of inhibition of shear-induced platelet aggregation.

**Effect of Intravenous Administration of NF449 on ex Vivo Mouse Platelet Aggregation.** We next investigated whether NF449 had an impact on in vivo hemostasis and thrombosis in mice. First, to determine whether i.v. injection of NF449 resulted in inhibition of the ex vivo platelet aggregation induced by collagen or ADP, NF449 or saline was injected into anesthetized mice, and blood drawn 3 min later into 100 U/ml hirudin anticoagulant containing 0.5 U/ml apyrase was used to prepare PRP. At a dose of 10 mg/kg, NF449 inhibited the ex vivo aggregation triggered by 5 μg/ml collagen in WT mouse platelets without affecting that induced by 5 μM ADP (Fig. 4A, left), suggesting selective blockade of the P2X<sub>1</sub> receptor. In similar experiments performed on P2X<sub>1</sub>−/− mice, 10 mg/kg NF449 did not influence ex vivo aggregation in response to either collagen or ADP, confirming the presence of selective P2X<sub>1</sub> receptor inhibition (Fig. 4A, right).

At a higher dose (50 mg/kg), NF449 inhibited ex vivo platelet aggregation in response to not only 10 μg/ml collagen but also 5 μM ADP, indicating nonspecific inhibition of the P2Y<sub>1</sub> and/or P2Y<sub>12</sub> receptor (Fig. 4B). The P2Y<sub>1</sub>-mediated platelet shape change, visualized as the decrease in light transmission through the aggregometer cuvette (Fig. 4B), when aggregation was prevented by addition of a GPIIbIIIa blocking antibody (RAM.2), was indeed inhibited after treatment of mice with 50 mg/kg NF449 (Fig. 4B). The P2Y<sub>12</sub> receptor was also affected, since the P2Y<sub>12</sub>-mediated aggregation observed in P2Y<sub>1</sub>−/− mouse platelets at high agonist concentrations (Léon et al., 1999; Kauffenstien et al., 2001) was lost (Fig. 4B).

Plasma levels of NF449 after injection of 10 or 50 mg/kg NF449 into mice were determined by high-performance liquid chromatography as described previously (Kassack and Nickel, 1996). Injection of 10 mg/kg NF449 resulted 2 min 30 s later in a plasma concentration of 205 ± 1 μg/ml corresponding to 136 ± 1 μM, which progressively decreased to 142 ± 1 μg/ml (94 ± 1 μM) at 10 min and to 98 ± 2 μg/ml (65 ± 1 μM) at 30 min. Injection of 50 mg/kg NF449 resulted 2 min 30 s later in a plasma concentration of 627 ± 7 μg/ml, corresponding to 416 ± 4 μM, which also progressively decreased to 486 ± 1 μg/ml (323 ± 1 μM) at 10 min and to 321 ± 3 μg/ml (213 ± 2 μM) at 30 min.

These results indicate that NF449 selectively antagonized the P2X<sub>1</sub> receptor in vivo at a dose of 10 mg/kg, whereas at a higher dose (50 mg/kg), it nonselectively inhibited the three platelet P2 receptors. NF449 was used at both concentrations (10 and 50 mg/kg) to determine the impact of P2 receptor inhibition on hemostasis and thrombosis in mice.

**Bleeding Time.** To assess the impact of NF449 on hemostasis, the bleeding time was determined in mice after tail-tip amputation. The time to arrest of bleeding was similar in mice injected with 10 mg/kg NF449 (106 ± 16 s; n = 10) or saline (78 ± 7 s; n = 10) (P = 0.1209) (Fig. 5), consistent with our previous observation that P2X<sub>1</sub>−/− mice display an almost normal bleeding time (Hechler et al., 2003). Conversely, in mice receiving a higher dose of 50 mg/kg NF449, bleeding was markedly prolonged compared with those receiving saline and had to be stopped after 30 min by cauterization.

**Effect of i.v. Injection of NF449 on the Acute Thromboembolism Induced in Mice by Infusion of a Mixture of Collagen and Adrenaline.** P2X<sub>1</sub>−/− and P2Y<sub>12</sub>−/− mice and mice treated with clopidoogrel display resistance to thrombosis in a model of thromboembolism induced by infusion of a mixture of collagen and adrenaline (DiMinno and Silver, 1983; Léon et al., 1999; Hechler et al., 2003). In this model, measurement of the platelet count in whole blood before and after the challenge with collagen and adrenaline allows determination of the platelet consumption, an indicator of in vivo platelet aggregation.

As shown in Fig. 6A, mice injected with 10 mg/kg NF449 displayed reduced platelet consumption after intravenous infusion of 0.1 mg/kg collagen and 60 μg/kg adrenaline compared with mice injected with saline (35 ± 4 versus 51 ± 3%) (P = 0.0061; n = 10), reflecting decreased intravascular platelet aggregation. In P2X<sub>1</sub>−/− mice receiving 10 mg/kg NF449, platelet consumption was comparable with that in P2X<sub>1</sub>−/− mice receiving saline (35 ± 3 versus 40 ± 3%) (P = 0.1496; n = 10) (Fig. 6A), indicating that at a dose of 10 mg/kg, the inhibitory effect of NF449 was selective for the P2X<sub>1</sub> receptor.

After a higher dose of NF449 (50 mg/kg), WT mice exhibited a further reduction in platelet consumption after intravenous infusion of 0.1 mg/kg collagen and 60 μg/kg adrenaline compared with mice injected with saline (13 ± 4 versus 42 ± 3%) (P = 0.0002; n = 5) (Fig. 6B), reflecting a further decrease in intravascular platelet aggregation. These results indicate that mice injected with 10 mg/kg NF449 are resistant to the thromboembolism induced by collagen and adrenaline due to blockade of the P2X<sub>1</sub> receptor, whereas a higher dose of NF449 leads to greater protection against thrombosis.

Other experiments were performed in a model of localized thrombosis in mesenteric arterioles triggered by laser-induced vessel wall injury. Thrombus formation in mice receiv-

**TABLE 1**

Effect of NF449 and αβMeATP on shear-induced platelet aggregation in the presence of 0.5 U/ml apyrase

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Single Platelet Count % of basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tyrode</td>
<td>6</td>
<td>89.3 ± 4.6</td>
</tr>
<tr>
<td>B. 0.5 U/ml apyrase</td>
<td>6</td>
<td>60.8 ± 6.1</td>
</tr>
<tr>
<td>C. 0.5 U/ml apyrase</td>
<td>6</td>
<td>87.5 ± 4.0</td>
</tr>
<tr>
<td>+ 10 μM NF449</td>
<td>6</td>
<td>79.1 ± 4.7</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. P < 0.01, analysis of variance for repeated measures; B vs. A, P < 0.01; B vs. C, P < 0.01; B vs. D, P < 0.05; all other contrasts gave P values >0.05 (t-test for paired samples).
ing saline evolved biphasically with platelets quickly accumulating at the site of endothelial desquamation to form a parietal thrombus peaking at 38 s (Fig. 7). This was followed by progressive erosion, resulting in complete removal of the thrombus after 140 s. In mice receiving 10 mg/kg NF449, maximum thrombus surface area represented only 41% of that in mice receiving saline (Fig. 7), similarly to our previously published results on P2X$_{1-/-}$ mice (Hechler et al., Fig. 4. Effect of i.v. administration of NF449 on ex vivo mouse platelet aggregation. A, 10 mg/kg NF449 was injected into the jugular vein of anesthetized WT or P2X$_{1-/-}$ mice, and blood was drawn 3 min later from the abdominal aorta into 100 U/ml hirudin anticoagulant containing 0.5 U/ml apyrase. PRP was prepared and aggregation was induced with 5 μg/ml collagen, in the presence of 100 μM MRS2179 and 10 μM AR-C69931MX to prevent activation of the P2Y$_1$ and P2Y$_{12}$ receptors by secreted ADP, or with 5 μM ADP. Left, 10 mg/kg NF449 inhibited ex vivo aggregation of WT mouse platelets in response to collagen but not ADP; right, 10 mg/kg NF449 had no effect on collagen- or ADP-induced aggregation of P2X$_{1-/-}$ mouse platelets, indicating selective inhibition of the P2X$_1$ receptor. B, 50 mg/kg NF449 inhibited ex vivo aggregation of WT mouse platelets in response to either 10 μg/ml collagen or 5 μM ADP, suggesting inhibition of the P2Y ADP receptors. When 10 μg/ml RAM 2 against mouse GPIIbIIIa was added to prevent aggregation, NF449 did in fact inhibit the P2Y$_1$ receptor-mediated platelet shape change in response to 1 μM ADP, as visualized by the absence of a decrease in light transmission. Similarly, NF449 abolished the P2Y$_{12}$ receptor-mediated aggregation induced in P2X$_{1-/-}$ mouse platelets by a high concentration of ADP/βS (a nonhydrolysable analog of ADP). Data are from one experiment representative of three independent experiments giving identical results.
2003). Mice receiving 50 mg/kg NF449 exhibited a further reduction in maximum thrombus surface area, representing only 15% of that in mice receiving saline (Fig. 7). These results indicate that blockade of the P2X1 receptor with 10 mg/kg NF449 results in decreased localized arteriolar thrombosis after laser-induced vessel injury, whereas nonselective inhibition of the three platelet P2 receptors with a higher dose of NF449 (50 mg/kg) leads to greater protection against thrombosis.

Discussion

Progress in the field of P2 receptors has been impeded by the lack of appropriate ligands, especially P2 receptor subtype-selective antagonists. In particular, a clear and definitive elucidation of the role of the P2X1 receptor in platelet functions has been hindered by the lack of selective molecules. Several new antagonists at P2X receptors have been described over the past 4 years, but they are nevertheless limited in terms of their receptor affinity, subtype selectivity, and P2 receptor specificity (Lambrecht, 2000). The suspected ability of these molecules to act as substrates for ectonucleotidases or to inhibit these enzymes has also complicated their use. Thus, the availability of a new potent and selective antagonist of the P2X1 receptor (Braun et al., 2001; Hulsmann et al., 2003; Kassack et al., 2004) has provided a valuable tool to explore the role of this receptor in platelet functions.

NF449 is an analog of suramin with increased potency and selectivity for the P2X1 receptor compared with the previously synthesized structural analogs NF023 and NF279 (Lambrecht et al., 2002). We show here that NF449 is a strong competitive antagonist of the platelet P2X1 receptor,
displaying a pA₂ of 7.2 for inhibition of the calcium influx induced by αβMeATP, which is very similar to the reported pA₂ of NF449 (7.15 ± 0.03) at the native P2X₁ receptor of rat vas deferens (Braun et al., 2001). However, NF449 also weakly antagonizes the calcium mobilization mediated by the platelet P2Y₁ receptor (pIC₅₀ = 5.3). Since NF449 exhibited a pIC₅₀ of 6.95 at the platelet P2X₁ receptor using 0.3 μM αβMeATP (data not shown), it is 45-fold more potent at the P2X₁ than at the P2Y₁ receptor. Concerning the P2Y₁₂ receptor, NF449 only weakly inhibited ADP-induced inhibition of adenyl cyclase activity. As a result, the platelet P2 receptor selectivity profile of NF449 is P2X₁ > P2Y₁ > P2Y₁₂, which makes this molecule a valuable tool to investigate the functions of the platelet P2X₁ receptor in vitro. It has been reported that the antagonism of NF449 is specific for P2 receptors, since it had no significant effect on either the maximum response to or the potency of agonists activating α₁A₂-adrenoreceptors, H₁ histamine receptors, or muscarinic M₃ receptors (Braun et al., 2001). Moreover, the binding affinity of NF449 for β-adrenergic, adenosine A1, or angiotensin II type 1 receptors has been reported to be very low. We further showed that NF449 does not affect platelet activation in response to either serotonin or adrenaline, which provides additional evidence for its P2 selectivity and points to the interest of this compound for in vivo studies aimed at targeting P2 receptors.

At a dose of 10 mg/kg, NF449 selectively inhibited the mouse P2X₁ receptor in vivo but had no impact on the bleeding time, consistent with the almost normal bleeding time of P2X₁−/− mice (Hechler et al., 2003). NF449 (10 mg/kg) inhibited the acute thromboembolism induced by injection of a mixture of collagen and adrenaline and localized arterial thrombosis triggered by laser injury (Hechler et al., 2003). Hence, this molecule might constitute a starting compound in the search for antithrombotic drugs targeting the P2X₁ receptor and has the advantage of presenting an only minor hemorrhagic risk.

There already exists the rationale whereby each of the platelet ADP receptors is or might become a relevant clinical target for antithrombotic drugs (Gachet et al., 2005). This is in fact the case for the P2Y₁₂ receptor (Conley and Delaney, 2003). Thus, the thienopyridine compounds ticlopidine and clopidogrel, which are selective, irreversible P2Y₁₂ inhibitors, are now used clinically for antithrombotic treatment (Savi and Herbert, 2005). These compounds have been shown to be effective in numerous models of experimental thrombosis in animals as well as a great number of large multicentric clinical trials evaluating their effects on ischemic heart diseases, peripheral vascular disease, and stroke (Cazenave and Gachet, 2002; Gachet and Hechler, 2005). As an alternative to these irreversible agents, one might be able to use competitive P2Y₁₂ antagonists such as the oral, direct acting compound AZD6140, which is currently undergoing phase III clinical trials (Peters and Robbie, 2004). There also exists evidence suggesting that the P2Y₁ receptor might constitute a relevant target for new antithrombotic drugs. This receptor has been shown to play a role in thrombosis, since its absence or inhibition with the selective P2Y₁ antagonist MRS2179 reduces thrombus formation in an acute sys-
tomic model and in a model of localized thrombosis in mesenteric arterioles triggered by laser or ferric chloride injury (Lenain et al., 2003b). The anti-thrombotic protection was found to be equivalent to that in animals treated with clopidogrel and the combination of P2Y12 deficiency and clopidogrel treatment was better than either condition alone, opening up the possibility that combining P2Y1 and P2Y12 receptor antagonists could improve anti-thrombotic strategies (Lenain et al., 2003b).

The present work and recent studies in P2X1−/− mice (Hechler et al., 2003) indicate that the P2X1 receptor should also be considered as a potential anti-thrombotic target. Of particular interest is the observed inhibitory effect of NF449 on shear-induced platelet aggregation, which could be relevant to prevent arterial thrombosis at sites of vascular stenosis (Cattaneo et al., 2002; Hechler et al., 2003). Furthermore, it is possible that concomitant blockade of the P2X1, P2Y1, and P2Y12 receptors might lead to even greater anti-thrombotic activity. Here, we show that treatment with 50 mg/kg NF449 induced inhibition of the three platelet P2 receptors and led to a further inhibition of thrombosis in two different models compared with P2X1 inhibition alone, which supports this hypothesis. However, at this dose the bleeding time of mice was markedly prolonged, similarly as in P2Y12−/− mice or animals treated with clopidogrel (Foster et al., 2001). Although one cannot rule out the possibility that the prolongation of the bleeding time was due to binding of NF449 to targets other than P2 receptors in vivo, anti-thrombotic strategies based on combined inhibition of all three platelet P2 receptors might indeed lead to excessive derangement of hemostasis and deserves further investigation.

In conclusion, our results indicate that NF449 constitutes a valuable new tool to investigate the functions of the P2X1 receptor and might represent a starting compound in the search for new anti-thrombotic drugs targeting platelet P2 receptors.

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References


Address correspondence to: Dr. C. Gachet, INSERM U. 311, Etablissement Français du Sang-Alsace, 10, rue Spielmann, BP N° 36, 67065 Strasbourg Cédex, France. E-mail: christian.gachet@efs-alsace.fr