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Prevention of platelet glycoprotein IIb/IIIa activation by a novel tyrosine kinase inhibitor 3,4-Methylenedioxy-β-nitrostyrene

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Running title: MNS inhibits platelet GPIIb/IIIa activation

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Abbreviations: Fluo-3/AM, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxyl]-2-[2-amino-5-methylphenoxy]ethane-N,N,N',N'-tetraacetic acid/ acetoxymethyl ester; MARCKS, myristoylated alanine-rich C kinase substrate; MLCK, myosin light chain kinase; MNS, 3,4-methylenedioxy-β-nitrostyrene; PDBu, phorbol 12,13-dibutyrate; U46619, 9, 11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$.

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ABSTRACT

Binding fibrinogen to activated glycoprotein (GP) IIb/IIIa is the final common pathway of platelet aggregation, and has become a successful target for antiplatelet therapy. In the present study, we found that a small chemical compound, 3,4-methylenedioxy-β-nitrostyrene (MNS), exhibited potent and broad-spectrum inhibitory effects on human platelet aggregation caused by various stimulators. Moreover, addition of MNS to human platelets that had been aggregated by ADP caused a rapid disaggregation. We demonstrated that the anti-aggregatory activity of MNS is due to inhibition of GPIIb/IIIa activation by measuring the binding amount of PAC-1 in platelets. On the other hand, MNS is not a direct antagonist of GPIIb/IIIa, since MNS did not affect fibrinogen binding to fixed ADP-stimulated platelets. Investigating how MNS inhibits GPIIb/IIIa activation, we found that MNS potently inhibited the activity of tyrosine kinases (Src and Syk), prevented protein tyrosine phosphorylation, and cytoskeletal association of GPIIb/IIIa and talin, but had no direct effects on protein kinase C, Ca^{2+} mobilization, Ca^{2+}-dependent enzymes (myosin light chain kinase and calpain) and arachidonic acid metabolism, and did not affect the cellular levels of cyclic nucleotides. Therefore, MNS represents a new class of tyrosine kinase inhibitor that potently prevents GPIIb/IIIa activation and platelet aggregation without directly affecting other signaling pathways required for platelet activation. Because MNS inhibits GPIIb/IIIa functions in a manner different from GPIIb/IIIa antagonists, this feature may provide a new strategy for treatment of platelet-dependent thrombosis.

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INTRODUCTION

Platelets play a central role in the process of arterial thrombosis which leads to myocardial infarction and ischemic stroke, the two leading causes of death in the industrialized world (Packham, 1994; Ruggeri, 2002). Following vessel injury, platelets rapidly adhere to the newly exposed sub-endothelial matrix, such as collagen and von Willebrand factor. Platelet adhesion is followed by shape change, spreading, and release of both ADP and thromboxane A_2, which act as secondary agonists to recruit more circulating platelets to the site of injury (Jackson et al., 2003). Activated platelets also facilitate thrombin generation by providing a catalytic surface on which coagulation activation occurs (Lundblad and White, 2005). Thrombin is not only responsible for the formation of fibrin but also an extreme platelet activator. Upon platelet stimulation, the major platelet integrin glycoprotein (GP) IIb/IIIa undergoes a conformational change that allows it to bind plasma fibrinogen with high affinity. The mechanism of GPIIb/IIIa activation is termed inside-out signaling and it results in the formation of intercellular bridges between platelets and finally in platelet aggregation (Shattil et al., 1998; Levy-Toledano, 1999).

The cyclooxygenase inhibitor aspirin and the ADP receptor antagonists, such as clopidogrel, are widely used antiplatelet drugs and have been shown to be beneficial in the treatment of arterial thrombotic diseases (Antiplatelet Trialists' Collaboration,

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1994a and b; Antithrombotic Trialists' Collaboration; 2002). However, the efficacy of aspirin and ADP receptor antagonists is limited because there are other pathways of platelet aggregation. Several GPIIb/IIIa antagonists have been developed based on the fact that the binding of fibrinogen to GPIIb/IIIa is the final common pathway for platelet aggregation. Although intravenous GPIIb/IIIa antagonists have had a significant impact on outcomes of percutaneous coronary intervention by reducing the rate of post-procedure ischemic complications, their efficacy in the treatment of acute coronary syndromes is restricted to high-risk patients (Boersma et al., 2002). Moreover, the oral GPIIb/IIIa antagonists have been discontinued because of a lack of efficacy and increased mortality (Leclerc, 2002; Quinn et al., 2003). It has been suggested that the deleterious effects of GPIIb/IIIa antagonists may be due to paradoxical GPIIb/IIIa activation and increased fibrinogen binding to blood platelets (Peter et al., 1998; Honda et al., 1998). Therefore, there remains an unmet need for the development of more effective and safe antiplatelet agents.

In our continuing search for new antiplatelet agents, more than one thousand synthetic compounds, natural products, and plant extracts were randomly screened for their ability to inhibit platelet aggregation. Among them, 3,4-methylenedioxy-β-nitrostyrene (MNS), a synthetic small-molecule compound, was found to possess potent inhibitory effect on platelet aggregation caused by

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various stimulators. In this study, we demonstrate that MNS inhibits platelet aggregation by preventing GPIIb/IIIa activation, and that this effect is due to the inhibition of platelet tyrosine kinases.

MATERIALS AND METHODS

Materials. 3,4-Methylenedioxy-β-nitrostyrene (MNS, Fig. 1A), bovine α-thrombin, U46619 (9, 11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$), collagen (type I, bovine Achilles tendon), phorbol 12,13-dibutyrate (PDBu), fluo-3/AM (1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxyl]-2-[2-amino-5-methylphenoxy]ethane-N,N,N',N'-tetraacetic acid/ acetoxymethyl ester) were obtained from Sigma Chem. Co. U.S.A. The thromboxane B_2 immunoassay kit and the cAMP/cGMP immunoassay kit were purchased from Amersham Co. U.S.A. FITC-labeled PAC-1 was from BD Biosciences, U.S.A. FITC-conjugated anti-CD62P (P-selectin) was from Serotec, U.K. Monoclonal anti-talin antibody was from Sigma Chem. Co. U.S.A. Anti-integrin β_3, anti-phospho-myosin light chain, anti-phosphotyrosine monoclonal antibody PY20, phospho-MARCKS- specific polyclonal antibody, anti-Syk, and anti-PLCγ2 were purchased from Santa Cruz Biotechnologies, U.S.A. Anti-phospho-Src (Tyr416) antibody was obtained from Cell Signaling Technology, U.S.A. Baculovirus produced

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human Src, Syk and JAK2 were purchased from Upstate Biotech. Inc. U.S.A. Recombinant human FAK was purchased from Biosource International Inc. U.S.A. The tyrosine kinase activity assay kit was from Chemicon International Inc. U.S.A. All other chemicals were purchased from Sigma Chem. Co. U.S.A.

Preparation of washed human platelets. Human blood anticoagulated with acid citrate dextrose (ACD) was obtained from healthy human volunteers who had not taken any drugs within the last two weeks. The platelet suspension was then prepared according to the washing procedure described previously (Wu et al., 2003). Platelets were finally suspended in Tyrode's solution containing Ca^{2+} (2 mM), glucose (11.1 mM) and bovine serum albumin (3.5 mg/ml) at a concentration of 3×10^8 platelets/ml.

Measurement of platelet aggregation. Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., U.S.A.). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or MNS at 37 ^{o}C for 3 min under a stirring condition (1200 rpm) prior to the addition of the platelet activators. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of inducers.

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Measurement of platelet cyclic nucleotide contents. The contents of cyclic nucleotides in platelets were measured by the method described previously (Wu et al., 2004). In brief, the platelet suspension was incubated with MNS at 37 °C for 3 min under a stirring condition (1200 rpm) in a light-transmission aggregometer. The reaction was stopped by adding EDTA (10 mM) followed immediately by boiling for 2 min. Upon cooling to 4 °C, cell debris was removed by centrifugation at 13,000 × g for 5 min. The supernatant was then used to assay for cAMP and cGMP using enzyme immunoassay kits.

Measurement of the formation of thromboxane B_2. Because thromboxane A_2 is very unstable and rapidly converted to more stable metabolite thromboxane B_2, we thus measured the latter instead of thromboxane A_2. After challenge of platelets with the aggregation inducer for 5 min, EDTA (2 mM) and indomethacin (50 μM) were added. The platelet suspensions were centrifuged for 3 min at 13,000 rpm, the contents of thromboxane B_2 in the supernatants were assayed using an enzyme immunoassay kit according to the procedure described by the manufacturer.

Measurement of P-selectin expression and PAC-1 binding by flow cytometry.

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Washed human platelets (3 x 10^7 platelets/ml) were pre-incubated with DMSO or test compounds for 5 min, and then treated with or without thrombin (0.2 U/ml) in the presence of excessive amounts of FITC-conjugated monoclonal antibody (anti-CD62P or PAC-1) for 15 min at room temperature. The samples were then fixed at 4 °C with 1 % paraformaldehyde. Flow cytometric analysis was performed on a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. Platelets were identified by logarithmic signal amplification for forward and side scatter. The levels of P-selectin expression and PAC-1 binding were expressed as the percentages of cells positive for anti-CD62P and PAC-1, respectively. The negative cut-off for each antibody was set using resting platelets that gave less 4 % of positive results.

Measurement of intracellular Ca^{2+} mobilization. Platelets pelleted from platelet-rich plasma were resuspended in Ca^{2+}-free Tyrode’s solution, and then incubated with fluo-3/AM (2 μM) at 37 °C for 30 min. In order to prevent leakage of dye, probenecid (2.5 mM) was added to the buffers throughout the experiments (Merritt et al., 1990). After washing twice, the fluo-3-loaded platelets were finally suspended in Ca^{2+}-free Tyrode's solution at a concentration of 5×10^7 platelets/ml. The fluo-3-loaded platelets were preincubated with MNS in the presence of 1 mM extracellular calcium at 37 °C for 3 min prior to the addition of the platelet activators.

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Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo, Japan).

Platelet lysis and immunoprecipitation. To prepare whole platelet lysates, the reaction was terminated at the indicated time points by adding an equal volume of 2 × SDS sample buffer (1×, 2 % SDS, 62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol). The samples were boiled for 5 min and subjected to immunoblotting analysis. For preparation of immunoprecipitated proteins, platelet suspensions (containing 1×10^9 cells) were lysed by adding a half-volume of ice-cold 3 × radioimmunoprecipitation assay (RIPA) buffer (1×, 1% Triton X-100, 75 mM NaCl, 50 mM Tris-HCl, pH 7.4, 4 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM sodium orthovanadate). Lysates were incubated for 30 min on ice, clarified by centrifugation at 12,000 × *g* for 30 min, precleared with protein A-Sepharose beads, and incubated for 2 h at 4 °C with anti-Syk or anti-PLCγ2. Immune complexes were precipitated with protein A-Sepharose beads for an additional 3 h, sedimented by brief centrifugation, and washed three times in ice-cold 1 × RIPA buffer. The anti-Syk and anti-PLCγ2 immunoprecipitates were subjected to immunoblotting analysis.

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Fractionation of Platelets.

After the indicated time of activation, platelets were lysed with an equal volume of 2 × ice-cold lysis buffer (1×, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM sodium orthovanadate) and kept on ice for 30 min. The lysates were centrifuged at 15,000 *g* for 5 min at 4 °C. The resulting pellet (the Triton-insoluble fraction) was washed with 1 × lysis buffer, solubilized in 1 × SDS sample buffer, and subjected to immunoprecipitation analysis as described above.

Immunoblotting. Whole platelet lysates, subcellular fractions, and immunoprecipitates from the same cell equivalents were electrophoresed on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Trans-blot, Bio-Rad). The membranes were blocked overnight in 5 % non-fat dry milk in TBST (Tris-buffered saline supplemented with 0.1% Tween 20), washed three times in TBST, and incubated for 1 h in the antibody solution of interest in TBST with 1% bovine serum albumin. The membrane was immunoblotted with primary antibodies against phosphotyrosine, Syk, phospho-Src, PLCγ2, integrin β3, talin, phospho-MARCKS or phospho-myosin light chain. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr. After washing

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with TBST, protein bands on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (Western Lightning®, Perkin Elmer, USA).

Tyrosine kinase assay. For immune complex kinase assay, platelets treated with MNS, PP1, or piceatannol were lysed and immunoprecipitated with either anti-Src or anti-Syk antibody. In some experiments, recombinant human Src, Syk, FAK, or JAK2 was used and treated with MNS or tyrosine kinase inhibitors for 5 min. The activity of the immunoprecipitated kinases or the recombinant enzymes was determined by using a tyrosine kinase assay kit (Chemicon International, Inc., Temecula, CA). Samples were incubated with biotinylated poly [Glu:Tyr] (4:1), an exogenous substrate, in kinase assay buffer (20 mM HEPES, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM sodium orthovanadate, 1 mM DTT) for 37 °C for 30-45 minutes. After stopping the enzyme reaction with EDTA (final concentration of 20 mM), both the phosphorylated and dephosphorylated substrates were immobilized by binding to the streptavidin-coated plate. The fraction of phosphorylated substrate is determined using a phosphotyrosine monoclonal antibody conjugated to HRP and an ensuing chromagenic substrate reaction.

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Fibrin clot retraction. Washed human platelets (3×10^8 platelets/ml) were suspended in Tyrode's solution containing fibrinogen (250 μg/ml) and incubated with DMSO or MNS for 3 min without stirring in a glass tube. Fibrin clot formation was induced by adding thrombin (1 U/ml). Clot retraction was allowed to develop at 37 °C for 4h, and pictures were taken at 0.5, 1, 2, 3 and 4h using a digital camera. The images were analyzed, and the areas occupied by clots were calculated using Scion Image software. Data were expressed as follows: percentage of clot retraction = [1 - (area t/area t_0)] x 100.

Statistics. Results are expressed as the mean ± standard error of the mean (S.E.M.) and comparisons were made using Student's t test. A probability of 0.05 or less was considered significant.

RESULTS

Effect of MNS on the aggregation of washed human platelets. In washed human platelets, MNS completely inhibited U46619 (2 μM, a thromboxane A_2 mimic)-, ADP (5 μM)-, arachidonic acid (AA, 100 μM)-, collagen (10 μg/ml)-, and thrombin (0.1

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U/ml)-induced platelet aggregation in a concentration-dependent manner with IC_{50} values of 2.1 ± 0.5, 4.1 ± 0.5, 5.8 ± 0.9, 7.0 ± 0.4, and 12.7 ± 1.1 μM, respectively (Fig. 1B). In addition, platelet aggregation caused by either the calcium ionophore A23187 (1 μM) or the protein kinase C (PKC) activator PDBu (200 nM) was also inhibited by MNS with IC_{50} values of 25.9 ± 3.1 and 4.8 ± 0.6 μM, respectively.

Moreover, the addition of MNS (5- 20 μM) to human platelets that had been aggregated by ADP caused rapid disaggregations in a concentration-dependent manner (Fig. 1C). This ability of MNS to induce platelet disaggregation was even more potent than that of prostaglandin E_1 (PGE_1).

Effect of MNS on P-selectin expression and thromboxane formation in platelets. We next examined whether MNS could inhibit the processes of platelet activation, such as α-granule secretion and thromboxane formation. In the present study, the α-granule secretion was monitored by the surface expression of P-selectin, a major platelet α-granule protein that is crucial in promoting platelet-leukocyte interactions and fibrin accumulation (Shebuski and Kilgore, 2002; Andre, 2004). As shown in Fig. 2, thrombin-induced P-selectin expression on platelets was decreased by MNS to levels comparable to those observed in PGE_1-treated platelets.

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To investigate whether MNS interferes with AA metabolism and thromboxane formation in human platelets, AA (200 μM) was added to platelet suspension to elicit thromboxane formation. Even at the highest concentration (20 μM) used, MNS did not affect the thromboxane formation caused by AA in human platelets (ng/3 x 10^8 platelets: 241.9 ± 47.2 vs. control 231.8 ± 29.4, n=4).

Effect of MNS on platelet GPIIb/IIIa. Platelet GPIIb/IIIa activation was monitored by the binding of FITC-labeled PAC-1, since this monoclonal antibody only binds to the activated form of GPIIb/IIIa. Figure 3A shows that MNS (1-20 μM) inhibited thrombin-induced PAC-1 binding to platelets in a concentration range similar to that observed with the inhibition of platelet aggregation.

To determine whether MNS directly interferes with fibrinogen binding to platelet GPIIb/IIIa, the effect of MNS on fibrinogen-induced aggregation in ADP-treated fixed platelets was tested. Similar to PGE_1, neither pretreatment nor post-treatment of platelets with MNS affected fibrinogen-induced platelet aggregation. In contrast, RGDS (100 μM) not only prevented fibrinogen-induced platelet aggregation but also elicited disaggregation of platelets that had been aggregated by fibrinogen (Figure 3B).

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Effect of MNS on cyclic nucleotide levels in platelets. To assess whether the action of MNS was due to elevation of intracellular levels of cAMP and/or cGMP, which are two major inhibitory messengers in regulating platelet aggregation, the effect of MNS on cyclic nucleotide levels in platelets was examined. In washed human platelets, we found that MNS affected neither cAMP levels (pmol/8 x 10^8 platelets: 4.77 ± 0.18 vs. basal 4.61 ± 0.98, n=4) nor cGMP levels (pmol/8 x 10^8 platelets: 0.65 ± 0.09 vs. basal 0.88 ± 0.14, n=4). These results indicate that the antiplatelet effect of MNS is not related to cyclic nucleotides.

Effect of MNS on the intracellular free calcium of platelets. As shown in Figure 4, in the presence of 1 mM of extracellular calcium, pretreatment of fluo-3-loaded platelets with MNS (20 μM) did not inhibit the calcium signal elicited by thrombin, ADP, U46619, or AA. In contrast, collagen-induced intracellular calcium mobilization was almost abolished by MNS treatment.

Effect of MNS on myosin light chain kinase and calpain. Because MNS did not inhibit the intracellular calcium increase caused by various agonists other than collagen, we next examined whether MNS interfered with myosin light chain kinase (MLCK) and calpain, which are downstream of intracellular calcium and may play

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important roles in platelet activation (Croce et al., 1999; Azam et al., 2001).

As shown in Figure 5A, MNS had no apparent inhibitory effect on MLC phosphorylation induced by either thrombin or A23187. In contrast, the MLC kinase inhibitor ML-7 (50 μM) markedly inhibited MLC phosphorylation elicited by these two inducers.

To investigate the effect of MNS on calpain activation, the cleavage of talin, a major substrate of activated calpain in platelets, was examined. Figure 5B shows, MNS did not affect talin cleavage in A23187-treated platelets. Although MNS inhibited thrombin-induced talin cleavage, this effect may have resulted from the inhibition of platelet aggregation, since thrombin cannot induce talin cleavage in the absence of platelet aggregation (in an un-stirred condition). In contrast, the calpain inhibitor calpeptin (100 μM) completely prevented both A23187- and thrombin-induced talin cleavages. However, calpeptin had no inhibitory effect on platelet aggregation (data not shown). These results suggest that MNS blocked GPIIb/IIIa activation without inhibiting the function of calpain.

Effect of MNS on protein kinase C activation. In addition to calcium signaling, agonist-induced protein kinase C (PKC) activation may also contribute to the regulation of GPIIb/IIIa activation (van Willigen et al., 1996; Hers et al., 1998). We

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thus examined the effect of MNS on PKC activation by measuring the phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), which is a major substrate of PKC (Elzagallaai et al., 2000). As shown in Fig. 6, MNS (20 μM) markedly inhibited thrombin- but not PDBu-induced MARCKS phosphorylation. In contrast, the PKC inhibitor GF109203X (10 μM) completely abolished MARCKS phosphorylation caused by these two inducers. Based on the fact that PDBu-induced platelet aggregation is completely prevented by MNS, these results indicate that MNS inhibits GPIIb/IIIa activation without directly inhibiting PKC.

Effect of MNS on protein tyrosine phosphorylation in platelets. Fig. 7A shows that thrombin stimulation of platelets leads to time-dependent tyrosine phosphorylation of a number of proteins with molecular weights of 130, 110, 90, 80, 75, and 60 KD. A similar profile was observed following collagen-induced platelet activation. Pretreatment of platelets with MNS (20 μM) markedly inhibited protein tyrosine phosphorylation at either 0.5 or 3 min after thrombin or collagen stimulation. The concentration of MNS required to inhibit protein tyrosine phosphorylation was comparable to that required to inhibit platelet aggregation.

The tyrosine kinase inhibitor genistein at 20 μM inhibited collagen-induced platelet aggregation by 81 %, and markedly prevented protein tyrosine

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phosphorylation (Fig. 7A). However, a 30-fold higher concentration (600 μM) of genistein was required to significantly prevent thrombin-induced protein tyrosine phosphorylation. At 600 μM, genistein partially inhibited thrombin-induced platelet aggregation by 56 %.

We also examined the effect of MNS on tyrosine phosphorylated proteins in the cytoskeleton. Upon thrombin stimulation, there was a time-dependent increase in the levels of phosphotyrosine of multiple proteins in the cytoskeletal fraction (Fig. 7B). Pretreatment of platelets with MNS (20 μM) almost completely reduced the quantity of tyrosine-phosphorylated proteins in the cytoskeleton at either 0.5 or 3 min after thrombin stimulation. Genistein (600 μM) also decreased tyrosine-phosphorylated proteins in the cytoskeleton, but to a lesser extent than that observed with MNS (Fig. 7B).

Effect of MNS on thrombin-induced cytoskeletal association of GPIIb/IIIa and talin. Upon stimulation of platelet with thrombin, GPIIb/IIIa and talin were detectable in Triton X-100-insoluble cytoskeletal fraction within 0.5 min and became more pronounced at 3 min (Fig. 8A). Both MNS (20 μM) and genistein (600 μM) markedly inhibited talin and GPIIb/IIIa redistribution to cytoskeletal fractions.

The ability of platelets to retract fibrin clots is dependent on the anchorage of

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GPIIb/IIIa to the cytoskeleton (Tuszynski et al., 1984). Therefore, we next examined the effect of MNS on platelet-mediated fibrin clot retraction. As shown in Fig. 8B, a delay in the kinetics of clot retraction was observed in MNS-treated platelets compared with control platelets. In the control group, clot retraction was significant at 30 min and reached a maximum by 1 h, while platelets treated with MNS (20 μM) started retracting by 2 h and were almost completely retracted by the end of 3 h.

Effect of MNS on activation of Src, Syk, and PLCγ2. In order to investigate the mechanism of inhibition of agonist-induced protein tyrosine phosphorylation by MNS, the activation of two major tyrosine kinases Src and Syk in platelets was examined. We assessed the autophosphorylation of Src in thrombin- or collagen-stimulated platelets by Western blotting using a specific anti-phospho-Src (Y-416) antibody, which specifically detects Src phosphorylated at Tyr-416, a process indicative of Src activation (Osusky et al., 1995). Fig. 9A shows that stimulation of platelets with thrombin or collagen induced Tyr-416 phosphorylation of Src within 1 min. MNS pretreatment of platelets led to marked inhibition of Src activation by both these two agonists. In order to further determine the effect of MNS on endogenous Src activity, the immune complex kinase assay was performed. As shown in Fig. 9B, thrombin treatment of human platelets led to a marked increase in tyrosine phosphorylation and

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kinase activity of Src. As expected, both MNS and the Src inhibitor PP1 significantly inhibited thrombin-induced Src phosphorylation and kinase activity.

Similar to Src, either thrombin- or collagen-induced tyrosine phosphorylation of Syk was also decreased by MNS (Fig. 9C). The immune complex kinase assay also demonstrated that thrombin-induced increase in Syk activity was inhibited by either MNS or the Syk inhibitor piceatannol.

In human platelets, PLCγ2 is downstream of Syk and is essential for both collagen-elicited IP_3 formation and intracellular Ca^{2+} mobilization. As shown in Fig. 9D, both thrombin and collagen induced tyrosine phosphorylation of PLCγ2 in human platelets. Treatment of platelets with MNS resulted in attenuation of PLCγ2 phosphorylation induced by these two agonists.

Direct effect of MNS on purified tyrosine kinases. We then examined the direct inhibition of MNS on tyrosine kinase activity by using recombinant human Src and Syk. The results in Table 1 demonstrate that MNS at 20 μM profoundly inhibited Syk activity by more than 90% with an IC_{50} value of 2.5 μM, which was about 15-fold more potent than piceatannol, a well-known inhibitor of Syk. At higher concentrations, MNS also inhibited Src activity, but the potency was less pronounced (IC_{50} = 29.3 μM). To further examine the selectivity of MNS for other tyrosine kinases,

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recombinant human FAK and JAK2 were used. Table 1 shows that even at the highest concentration (50 μM) tested, MNS only inhibited FAK and JAK2 by 36.6 ± 17.7 % and 5.2 ± 2.2 %, respectively. These data indicate that MNS selectively inhibits the kinase activity of Syk and, to a lesser extent, Src, but not FAK and JAK2. In contrast, genistein inhibited Src, Syk, and FAK with similar IC_{50} values indicating it is a non-selective inhibitor of tyrosine kinases.

DISCUSSION

In the present study, we show that MNS, a β-nitrostyrene derivative, exhibited potent and broad-spectrum inhibitory effects on human platelet aggregation caused by various receptor agonists and chemical reagents (a PKC activator and a Ca^{2+} ionophore) that bypass receptor-mediated processes. In addition to preventing platelet aggregation, MNS also induced rapid disaggregation of aggregated platelets. These results imply that MNS may act on GPIIb/IIIa, the final common pathway of platelet aggregation. In the following experiment, we have demonstrated that the anti-aggregatory effect of MNS is due to inhibition of GPIIb/IIIa activation by measuring the binding amount of PAC-1 in platelets. On the other hand, MNS is apparently not a direct antagonist of GPIIb/IIIa, since MNS did not affect fibrinogen binding to fixed ADP-stimulated platelets.

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The activation of GPIIb/IIIa is controlled in a complex manner by positive and negative signaling elements in blood platelets. Elevation of intracellular cyclic nucleotide levels, either by stimulation of cyclases or by inhibition of phosphodiesterases, is the most potent inhibitory mechanism of GP IIb/IIIa activation (Haslam et al., 1999; Schwarz et al., 2001). However, the failure of MNS to increase cAMP and cGMP levels in platelets excludes the possibility that cyclic nucleotides are involved in the action of MNS. Several positive signaling molecules have been implicated in the regulation of GPIIb/IIIa function; among them, phosphatidylinositide-specific phospholipase C (PLC) plays a central role (Nozawa et al., 1993). Two major forms of PLC, β and γ, have been identified in human platelets. The β forms are regulated by $G_{\alpha q}$ protein-coupled receptors for soluble agonists (thrombin, ADP, and thromboxane A_2), whereas the γ forms (predominantly PLCγ2) are activated by tyrosine phosphorylation in response to adhesion receptors for subendothelial matrix (collagen, vWF) (Levy-Toledano, 1999; Gibbins, 2004). Once activated, PLC hydrolyzed phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol-1,4,5-triphosphate which promote activation of PKC and the increase of cytosolic Ca^{2+}, respectively. We show here that collagen-induced tyrosine phosphorylation of PLCγ2 and Ca^{2+} signaling were both abolished by MNS, indicating that the activity of PLCγ2 had been inhibited. In contrast, MNS did not

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inhibit the increase in intracellular Ca^{2+} caused by soluble agonists, indicating that MNS has no inhibitory effect on PLCβ activation. Furthermore, calcium-induced activation of both MLCK and calpain were not affected by MNS, even though platelet aggregation was inhibited. These results suggest that MNS is able to prevent GPIIb/IIIa activation and platelet aggregation by acting at sites distal to the PLC/Ca^{2+} pathway. Interestingly, we found that the calpain inhibitor calpeptin did not inhibit either thrombin- or A23187-induced platelet aggregation, even though calpain activation was completely prevented. This result is inconsistent with that of Croce et al. (1999), and the reason for this discrepancy is not clear yet. To examine the effect of MNS on the PKC pathway, we found that although MNS was able to prevent thrombin-induced MCRCKS phosphorylation, it did not inhibit PDBu-induced MCRCKS phosphorylation despite the fact that platelet aggregation was completely abolished. The indirect inhibition of thrombin-induced PKC activation by MNS might result from its interfering with tyrosine kinases, since PKCα and PKCδ are known to be functionally regulated by tyrosine phosphorylation (Pula et al., 2005; Murugappan et al., 2005). Moreover, we also found that the Src inhibitor PP1 decreased thrombin-induced MARCKS phosphorylation in platelets (data not shown).

Protein tyrosine phosphorylation is another important stimulation pathway involved in the regulation of GPIIb/IIIa activation and platelet aggregation (Jackson et

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al., 1996; Shattil et al., 1998). Platelets contain high amounts of different non-receptor tyrosine kinases, including Src family kinases (Src, Fyn, Lyn, Hck, Yes), Syk, FAK family kinases (FAK, PYK), and JAK family kinases (JAK, TYK). Upon stimulation of platelets with agonists, there is a dramatic increase in the levels of phosphotyrosine of multiple platelet proteins. A role for protein tyrosine phosphorylation in inside-out GPIIb/IIIa signaling is suggested by the observation that tyrosine kinase inhibitors decrease GPIIb/IIIa activation and platelet aggregation (Asahi et al., 1992; Rendu et al., 1992; Furman et al., 1994). Furthermore, Syk null murine platelets have been shown to cause a reduction in fibrinogen binding in response to ADP and epinephrine (Law et al., 1999). Protein tyrosine phosphorylation also plays a critical role in outside-in GPIIb/IIIa signaling events, such as cytoskeletal recruitment of various proteins (talin, GPIIb/IIIa, PLCγ), cytoskeleton reorganization, and clot retraction (Banno et al., 1996; Santos et al., 2000; Schoenwaelder et al., 1994). In our experiments, MNS effects on agonist-induced total protein tyrosine phosphorylation in platelets and activation of Src and Syk paralleled a reduction in the activation of GPIIb/IIIa and platelet aggregation, indicating that the inhibition of inside-out GPIIb/IIIa signaling by MNS is due to the inhibition of tyrosine kinases and protein tyrosine phosphorylation. Furthermore, platelets treated with MNS resulted in a complete absence of thrombin-induced tyrosine-phosphorylated proteins in the

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cytoskeletal fraction, and largely prevented the cytoskeletal association of talin and β_3 integrin, suggesting that MNS disrupted cytoskeletal reorganization and the interaction between GPIIb/IIIa and cytoskeleton. This would contribute to the significant inhibition of clot retraction, which is dependent on the anchorage of GPIIb/IIIa to the contractile cytoskeleton (Tuszynski et al., 1984).

Several tyrosine kinase inhibitors have been used to investigate the role of protein tyrosine phosphorylation in platelet functions; however, these compounds usually suffer limitations, such as lack of potency, specificity and/or cell permeability. For example, it has been demonstrated that besides inhibiting tyrosine kinases, genistein also antagonizes the thromboxane A_2 receptor on platelets (Nakashima et al., 1991; McNicol 1993). Additionally, erbstatin was found to directly inhibit protein kinase C besides tyrosine kinases (Bishop et al., 1990). In this study, we show that MNS is more potent than genistein in inhibiting agonist-induced protein tyrosine phosphorylation in platelets. In the pure enzyme system, MNS is also more potent than genistein and piceatannol in inhibiting the activities of tyrosine kinases, especially that of Syk. Moreover, MNS does not inhibit PLCβ/Ca^{2+} signaling and arachidonic acid metabolism and has no direct effect on PKC, MLCK and calpain, suggesting its high specificity for tyrosine kinases. In addition, MNS can readily penetrate the plasma membrane since it effectively inhibits tyrosine kinases both *in*

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vitro as well as in intact cells.

As noted in previous reports (Ozaki et al., 1993; Rendu et al., 1992) and in this study, although tyrosine kinase inhibitors, including genistein, PP1 and piceatannol, are effective in inhibiting collagen-induced platelet aggregation (IC_{50}: 13.7 ± 0.6 μM, 2.1 ± 0.4 μM, and 17.2 ± 2.3 μM, respectively, n=3) they are much less effective in inhibiting platelet aggregation caused by thrombin (IC_{50} >200 μM). In contrast, though MNS had distinct effect on Ca^{2+} signaling triggered by thrombin and collagen, it inhibited platelet aggregations by these two stimulators in a similar range of concentration. These data indicate that tyrosine kinases sensitive to MNS may be involved in the final common steps leading to the activation of GPIIb/IIIa. The discrepancy between MNS and other tyrosine kinase inhibitors in inhibition of thrombin-induced platelet aggregation can probably be attributed to their distinct specificity and efficacy toward diverse tyrosine kinases, which is supported by the observation that treatment with MNS caused a different pattern of inhibition of protein tyrosine phosphorylation in agonist-stimulated platelets compared with genistein. Further investigation of the effect of MNS for different tyrosine kinases in platelets may help to elucidate the precise mechanism for how and which tyrosine kinases are involved in the final steps of inside-out GPIIb/IIIa signaling pathway.

In conclusion, MNS represents a new class of tyrosine kinase inhibitor that

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potently prevents GPIIb/IIIa activation and platelet aggregation caused by various stimulators without directly affecting other signaling pathways required for platelet activation. In addition, MNS not only inhibits platelet aggregation but also promotes platelet disaggregation, indicating that MNS has the ability to reverse the processes of GPIIb/IIIa and thereby has the potential to accelerate thrombolysis. Since MNS inhibits GPIIb/IIIa functions in a manner different from GPIIb/IIIa antagonists, it might avoid adverse effects of the latter, such as paradoxical GPIIb/IIIa activation. These features of MNS may provide a new strategy for treatment of platelet-dependent thrombosis.

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Figure legends

Figure 1. (A) Chemical structure of MNS. (B) Concentration-inhibition curves for MNS on human platelet aggregation. Washed human platelets were incubated with DMSO (vehicle control) or MNS at 37 ^{o}C for 3 min, then arachidonic acid (AA, 100 μM), collagen (10 μg/ml), thrombin (0.1 U/ml), U46619 (2 μM), or ADP (5 μM) was added to trigger platelet aggregation. Values are presented as means ± S.E.M. (n = 4). (C) Disaggregation of ADP-aggregated platelets by MNS and PGE_1. Washed platelets were challenged by ADP (10 μM) to induce platelet aggregation. DMSO (control), PGE_1 (10 μM) or MNS (5, 10 and 20 μM) was added to aggregated platelets at times indicated by downward arrowheads. Representative tracings of three independent experiments are shown.

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Figure 2. Effect of MNS on P-selectin expression. Washed human platelets were pre-incubated with DMSO or test compounds for 5 min, and then treated with or without thrombin (0.2 U/ml) in the presence of FITC-conjugated anti-CD62P for 15 min at room temperature. The samples were then fixed at 4 °C with 1% paraformaldehyde. P-selectin expression was measured by flow cytometry as described in Materials and Methods. Results are presented as mean ±S.E.M. (n =3).

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#*P* < 0.001 as compared with the resting value. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 as compared with the control.

Figure 3. (A) Effect of MNS on GPIIb/IIIa activation. Washed human platelets were pre-incubated with DMSO or test compounds for 5 min, and then treated with or without thrombin (0.2 U/ml) in the presence of FITC-conjugated PAC-1 for 15 min at room temperature. The samples were then fixed at 4 °C with 1% paraformaldehyde. The percentage of PAC-1 positive platelets was analyzed by flow cytometry as described in Materials and Methods. Results are presented as mean ±S.E.M. (n =3). #*P* < 0.001 as compared with the resting value. *** *P* < 0.001 as compared with the control. (B) Effects of MNS, PGE_1 and RGDS on fibrinogen-induced aggregation in ADP-stimulated fixed platelets. Washed human platelets were treated with ADP (20 µM) and then fixed with 0.8 % paraformadehyde for 30 min at room temperature. The fixed platelets were washed and resuspended in Tyrode's solution. The fixed platelets were treated with DMSO (control), MNS (20 µM), PGE_1 (10 µM) or RGDS (100 µM) before (left panels) or after (right panels, at times indicated by downward arrowheads) addition of fibrinogen (200 µg/ml). Representative tracings of three independent experiments are shown.

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Figure 4. Effect of MNS on intracellular calcium mobilization in human platelets. Fluo-3-loaded human platelets were incubated with DMSO or MNS (20 μM) at 37 ^{o}C for 3 min in the presence of 1 mM extracellular Ca^{2+}, arachidonic acid (AA, 100 μM), collagen (10 μg/ml), thrombin (0.1 U/ml), ADP (5 μM), or U46619 (2 μM) was then added to trigger the increase of $[Ca^{2+}]_i$ (fluorescence, F). Similar results were obtained in three separate experiments.

Figure 5. Effects of MNS on thrombin- or A23187-induced myosin light chain (MLC) phosphorylation (A) and talin proteolysis (B). Human platelets were pre-incubated with MNS (20 μM), ML-7 (50 μM) or calpeptin (100 μM) at 37 ^{o}C for 3 min, and then stimulated with either A23187 (1 μM) or thrombin (0.1 U/ml) for another 30 seconds. After stimulation, platelets were lysed and electrophorezed as described in Materials and Methods. Western blot analysis was performed on platelet lysates using anti-phospho-MLC antibody and anti-talin antibody, respectively. Similar results were obtained in three separate experiments.

Figure 6. Effect of MNS on thrombin- or PDBu-induced MARCKS phosphorylation. Washed platelets were pre-incubated with MNS (20 μM) or GF109203X (10 μM) for 3 min at 37°C, and then stimulated with either thrombin (0.1 U/ml) or PDBu (200 nM)

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for another 30 seconds. Western blot analysis was performed on platelet lysates using anti-phospho-MARCKS antibody. Similar results were obtained in three separate experiments.

Figure 7. Effects of MNS and genistein on thrombin- and collagen-induced protein tyrosine phosphorylation in human platelets. (A) Washed platelets were pre-incubated with DMSO, MNS (20 μM) or genistein (600 μM for thrombin treatment, but 20 μM for collagen treatment) at 37°C for 3 min. And then, platelets were treated with or without thrombin (0.1 U/ml) or collagen (10 μg/ml) for the indicated periods. Western blot analysis was performed on whole platelet lysates using a monoclonal anti-phosphotyrosine antibody (PY20). (B) Washed platelets were pre-incubated with DMSO, MNS (20 μM) or genistein (600 μM) at 37°C for 3 min. After treatment with or without thrombin (0.1 U/ml), platelets were lysed with Triton X-100 lysis buffer. The Triton X-100-insoluble (cytoskeletal) fraction was separated with centrifugation. Western blot analysis was performed on cytoskeletal fractions using PY20. Similar results were obtained in three separate experiments.

Figure 8. (A) Effect of MNS on thrombin-induced cytoskeletal association of talin and GPIIb/IIIa. Washed platelets were pre-incubated with DMSO, MNS (20 μM) or

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genistein (600 μM) at 37°C for 3 min. After treatment with or without thrombin (0.1 U/ml), platelets were lysed with Triton X-100 lysis buffer and the cytoskeletal fraction was isolated. Western blot analysis was performed on cytoskeletal fractions using anti-talin antibody and anti-β3 integrin antibody, respectively. Similar results were obtained in three separate experiments. (B) Effect of MNS on fibrin clot retraction. Washed platelets were suspended in Tyrode's solution containing fibrinogen (250 μg/ml) and incubated with DMSO or MNS for 3 min without stirring in a glass tube. Fibrin clot formation was induced by adding thrombin (1U/ml). Clot retraction was allowed to develop at 37 °C for 4h. Upper panel, time frames of a retracting clot at the indicated time points. Lower panel, kinetic curves of retraction in the presence of different concentrations of MNS were generated by plotting clot areas versus time. Results represent the means from two independent experiments.

Figure 9. (A) Effect of MNS on the activation of Src. Washed platelets were pre-incubated with DMSO, MNS (20 μM) or PP1 (20 μM) at 37°C for 3 min, and then stimulated with thrombin (0.1 U/ml) or collagen (30 μg/ml) for the indicated periods. Western blot analysis was performed on platelet lysates using a specific anti-phospho-Src antibody (Y-416). (B) Platelet lysates were immunoprecipitated with anti-Src antibody, and the immunoprecipitates were subjected to Western blot analysis

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for phosphotyrosine (PY20) or in vitro kinase assay. Results of the in vitro kinase assay are presented as mean ±S.E.M. (n =3). (C) Effect of MNS on Syk phosphorylation and kinase activity. Washed platelets were pre-incubated with DMSO, MNS (20 μM) or piceatannol (100 μM) at 37°C for 3 min, and then stimulated with thrombin (0.1 U/ml) or collagen (30 μg/ml) for the indicated periods. Platelet lysates were immunoprecipitated with anti-Syk antibody, and the immunoprecipitates were subjected to Western blot analysis for phosphotyrosine (PY20) or in vitro kinase assay. Results of the in vitro kinase assay are presented as mean ±S.E.M. (n =3). (D) Effect of MNS on PLCγ2 phosphorylation. PLCγ2 was immunoprecipitated from lysates made from platelets stimulated with thrombin (0.1 U/ml) or collagen (30 μg/ml) in the presence of DMSO or MNS (20 μM). The immunoprecipitates were subjected to Western blot analysis for phosphotyrosine (PY20).

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Table 1. Effects of MNS and Src/Syk inhibitors on the enzyme activities of recombinant human Src, Syk, FAK, and JAK2. The enzyme activities of these tyrosine kinases were measured by phosphorylation of biotinylated poly [Glu:Tyr] 4:1 substrate in the presence of various concentration of MNS, PP1, piceatannol or genistein as described in Materials and Methods. Results are represented as percent inhibition of control enzyme activity and indicated as means ± S.E.M. (n = 3).

		% Inhibition of control enzyme activity			
		Src	Syk	FAK	JAK2
MNS	50 μM	88.5 ± 6.1	-	11.6 ± 9.5	5.2± 2.2
	20 μM	22.4 ± 6.7	94.4 ± 2.3	-	-
	10 μM	10.9 ± 4.4	82.0 ± 4.2	-	-
	5 μM	0.8 ± 0.5	67.0 ± 8.3	-	-
	2 μM	-	44.2 ± 4.1	-	-
	1 μM	-	16.4 ± 4.5	-	-
PP1	1 μM	99.1 ± 0.7	8.3 ± 2.5	-	-
Piceatannol	100 μM	42.8 ± 1.4	87.5 ± 8.3	-	-
	20 μM	7.1 ± 4.7	37.2 ± 3.0	-	-
Genistein	100 μM	74.3 ±9.9	73.6 ± 2.8	78.2 ± 9.0	18.8 ± 3.4
	50 μM	27.8 ± 10.7	58.4 ± 3.5	30.5 ± 15.8	-

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Fig. 1

A

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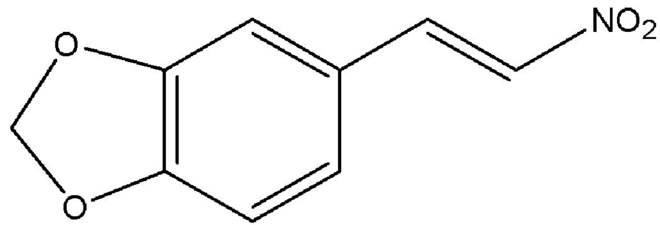

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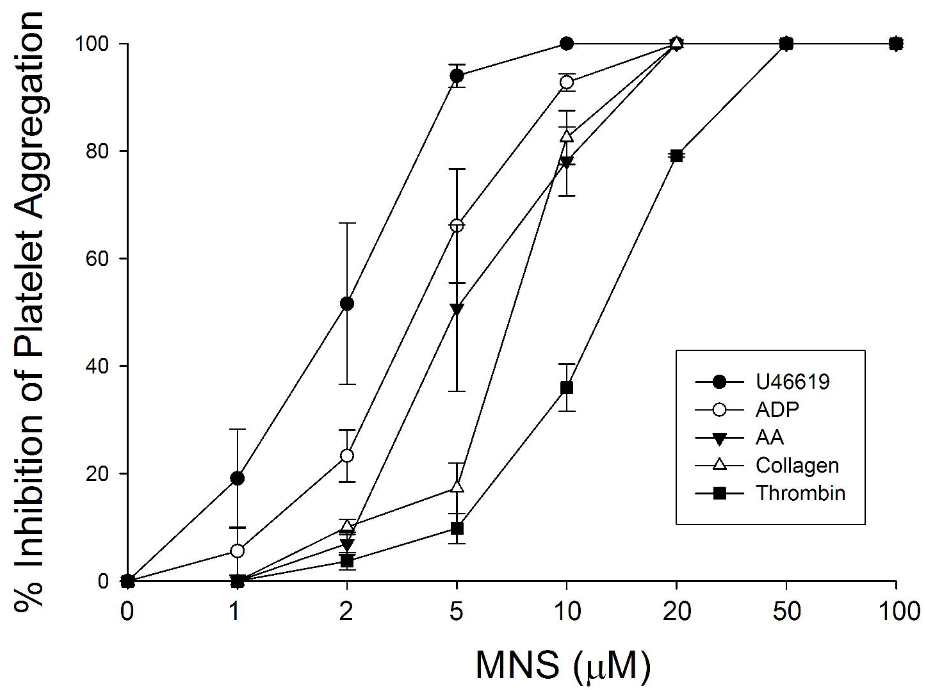

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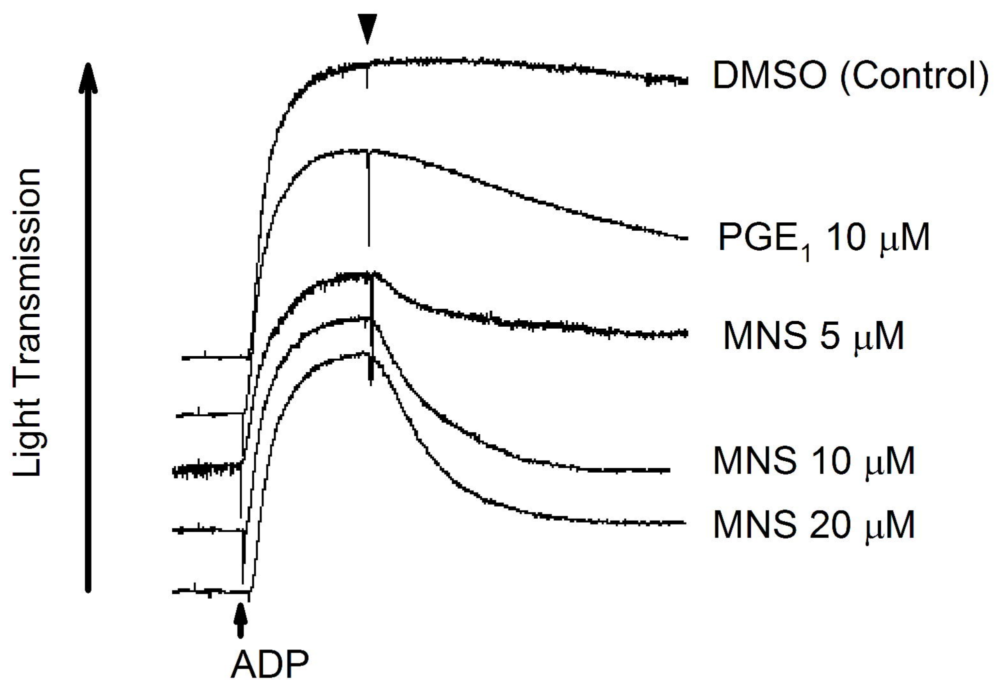

Fig.2

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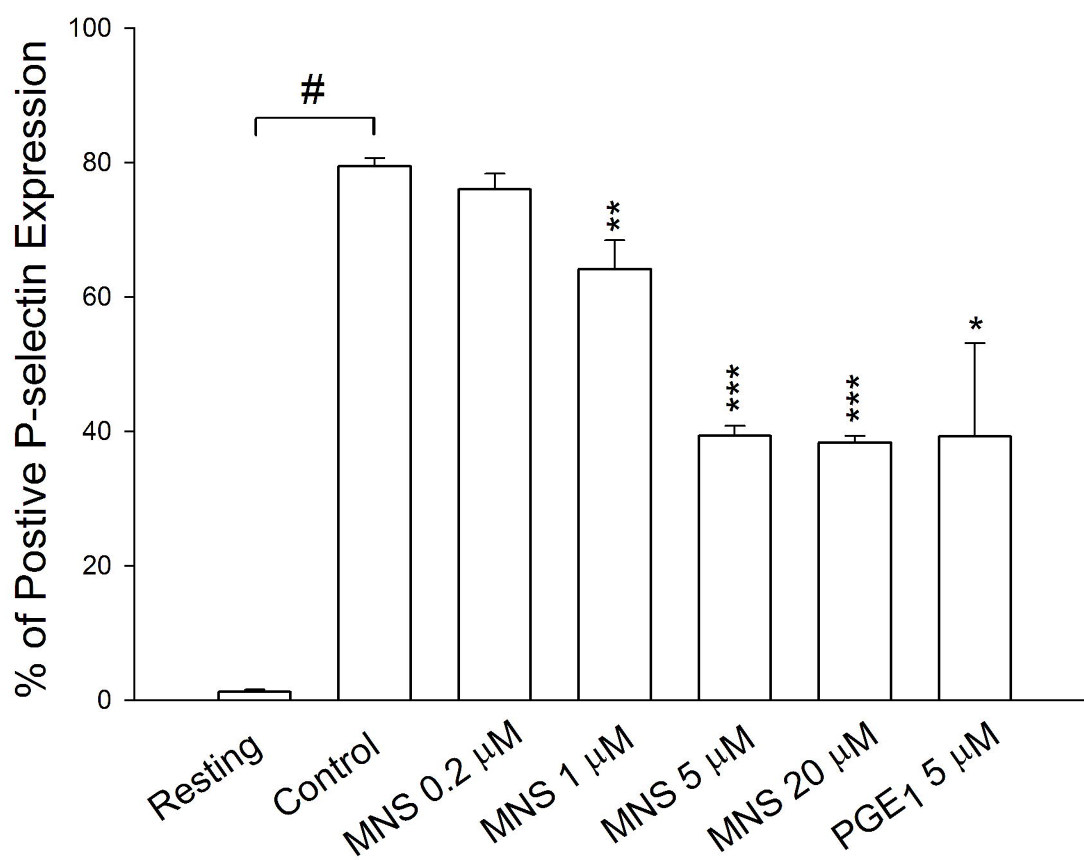

Fig.3

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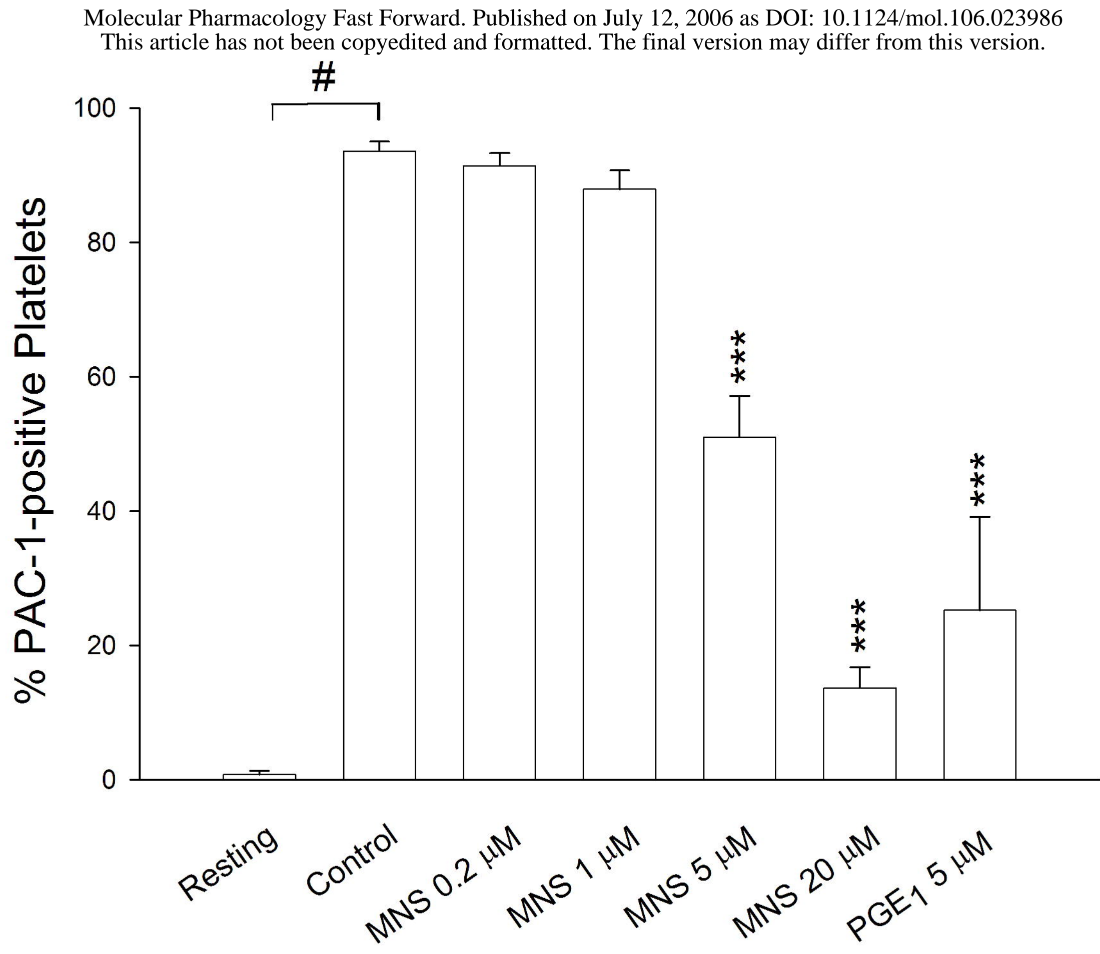

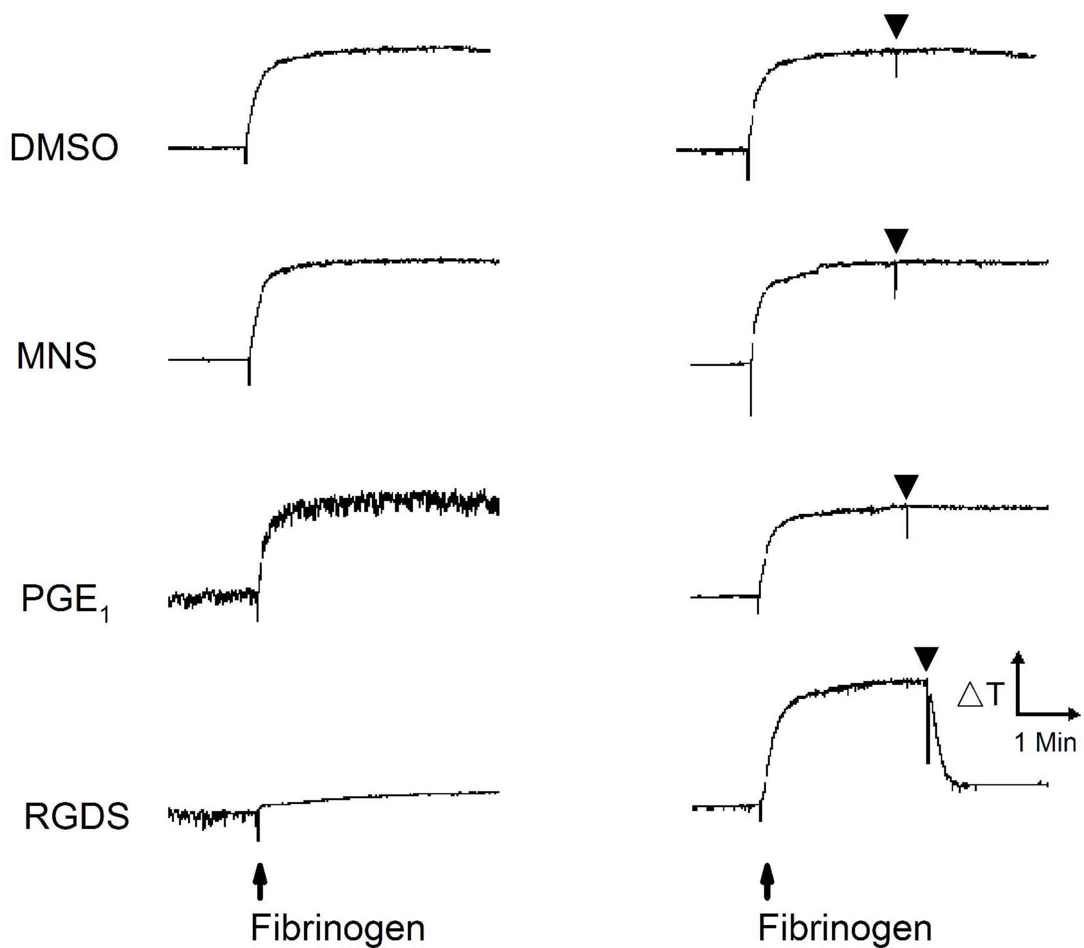

Fig. 4

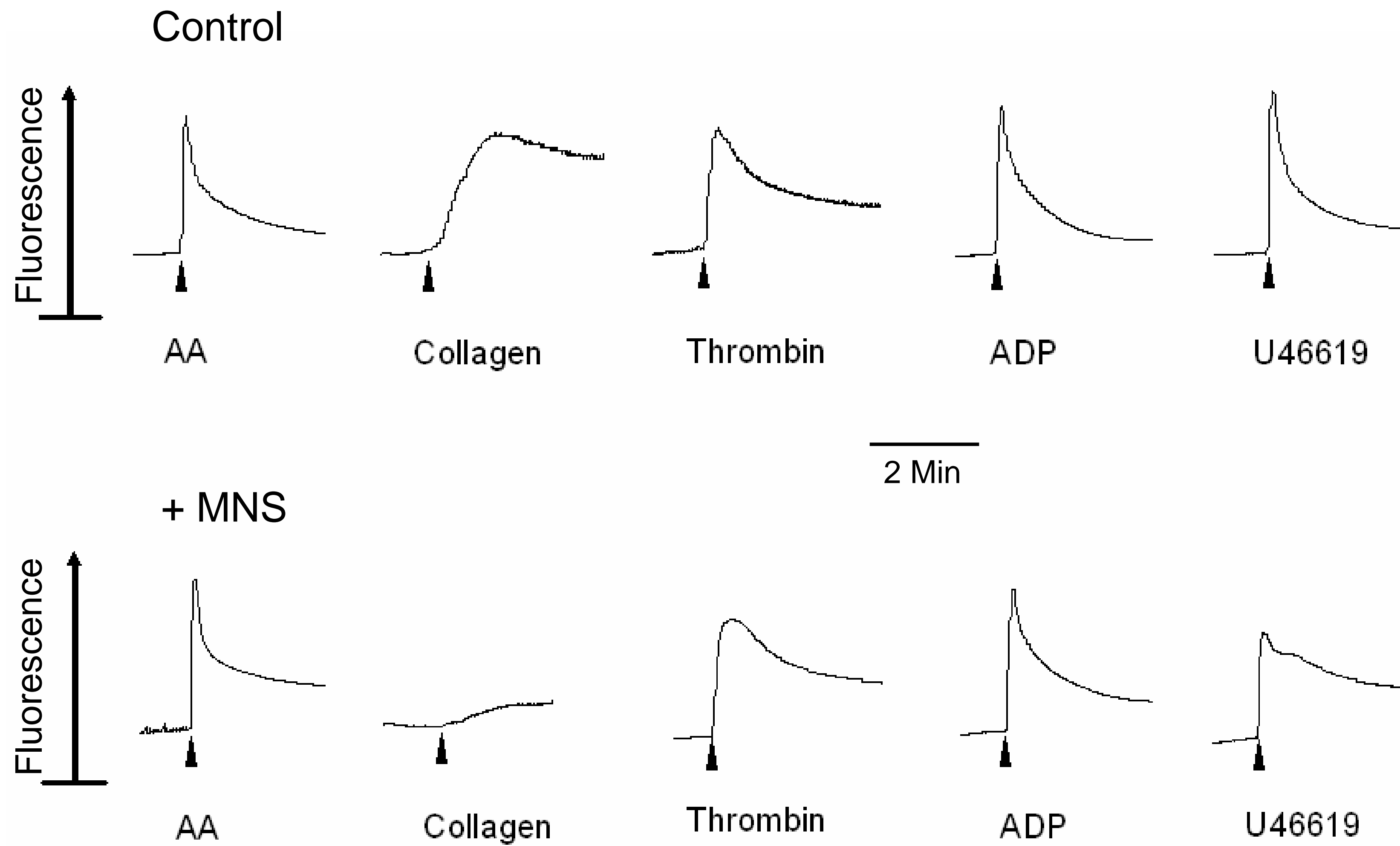

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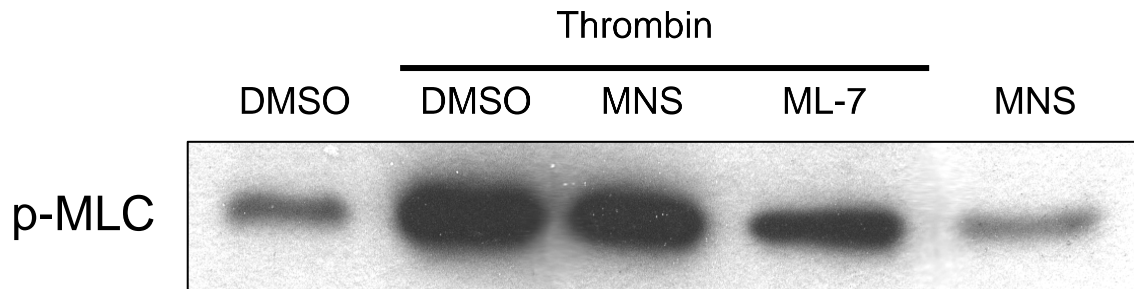
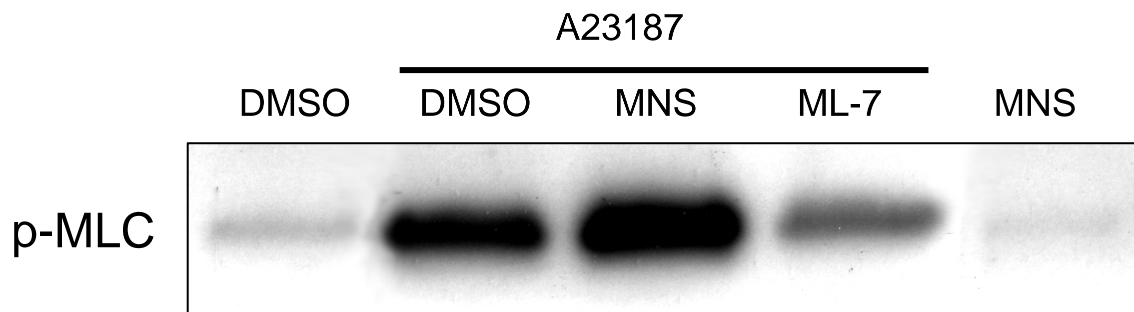
Fig.5

A





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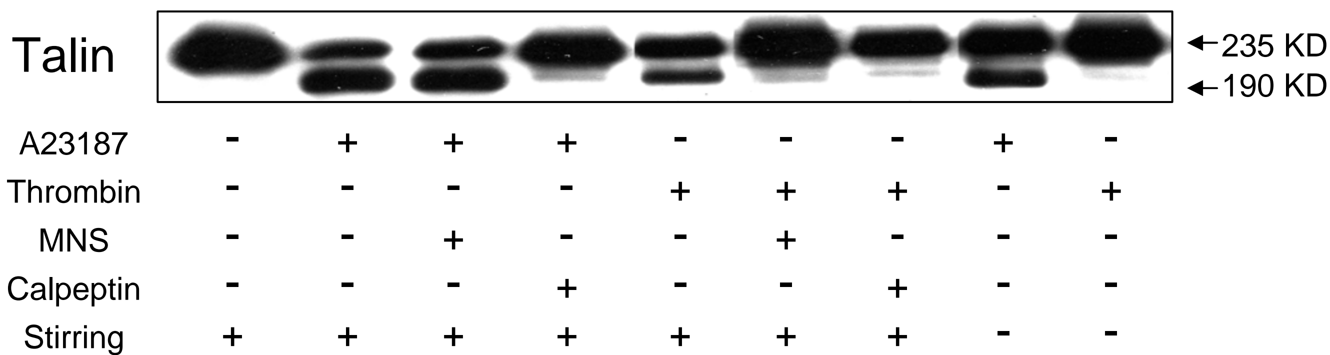

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Fig.6

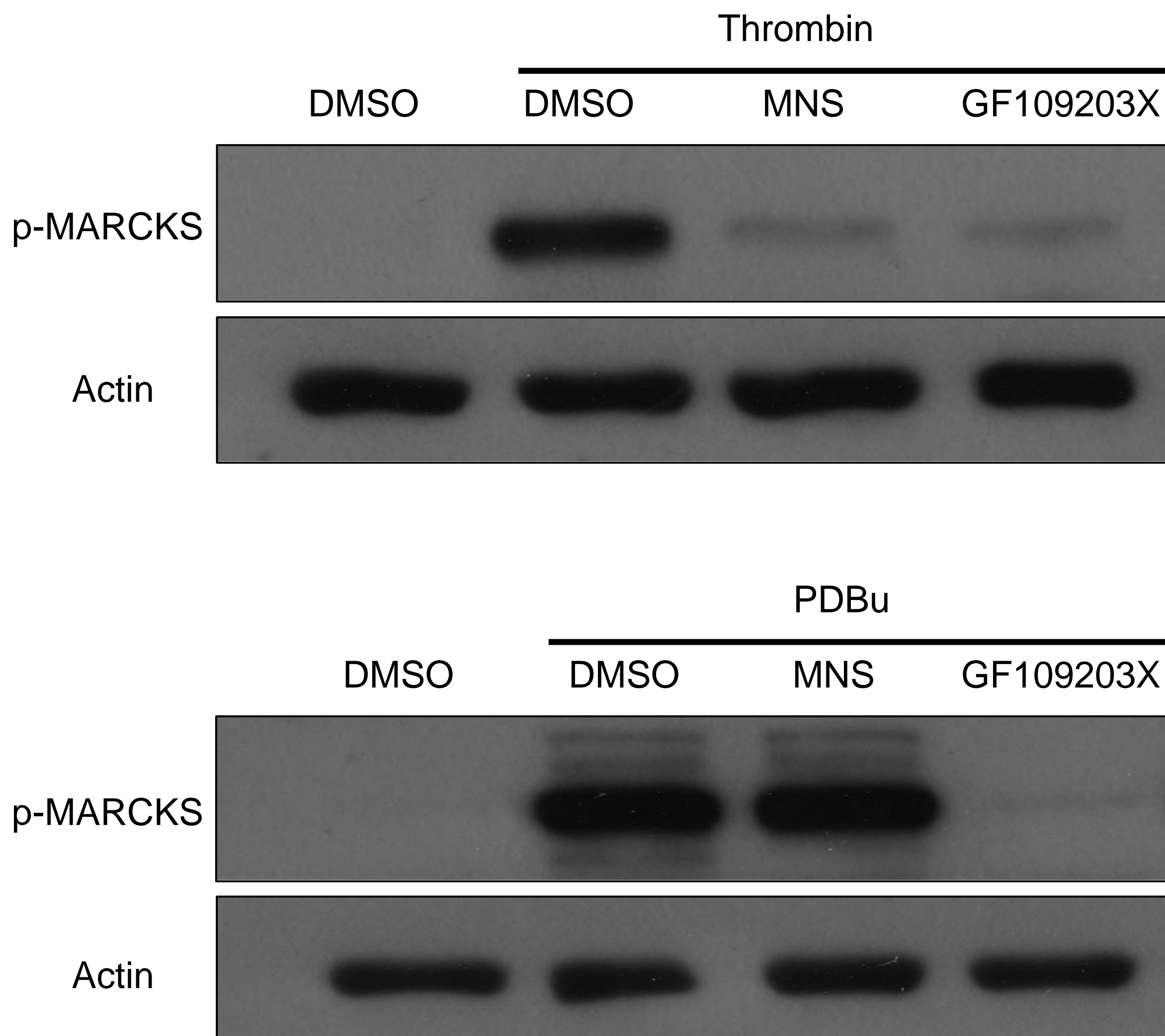

Fig.7
A

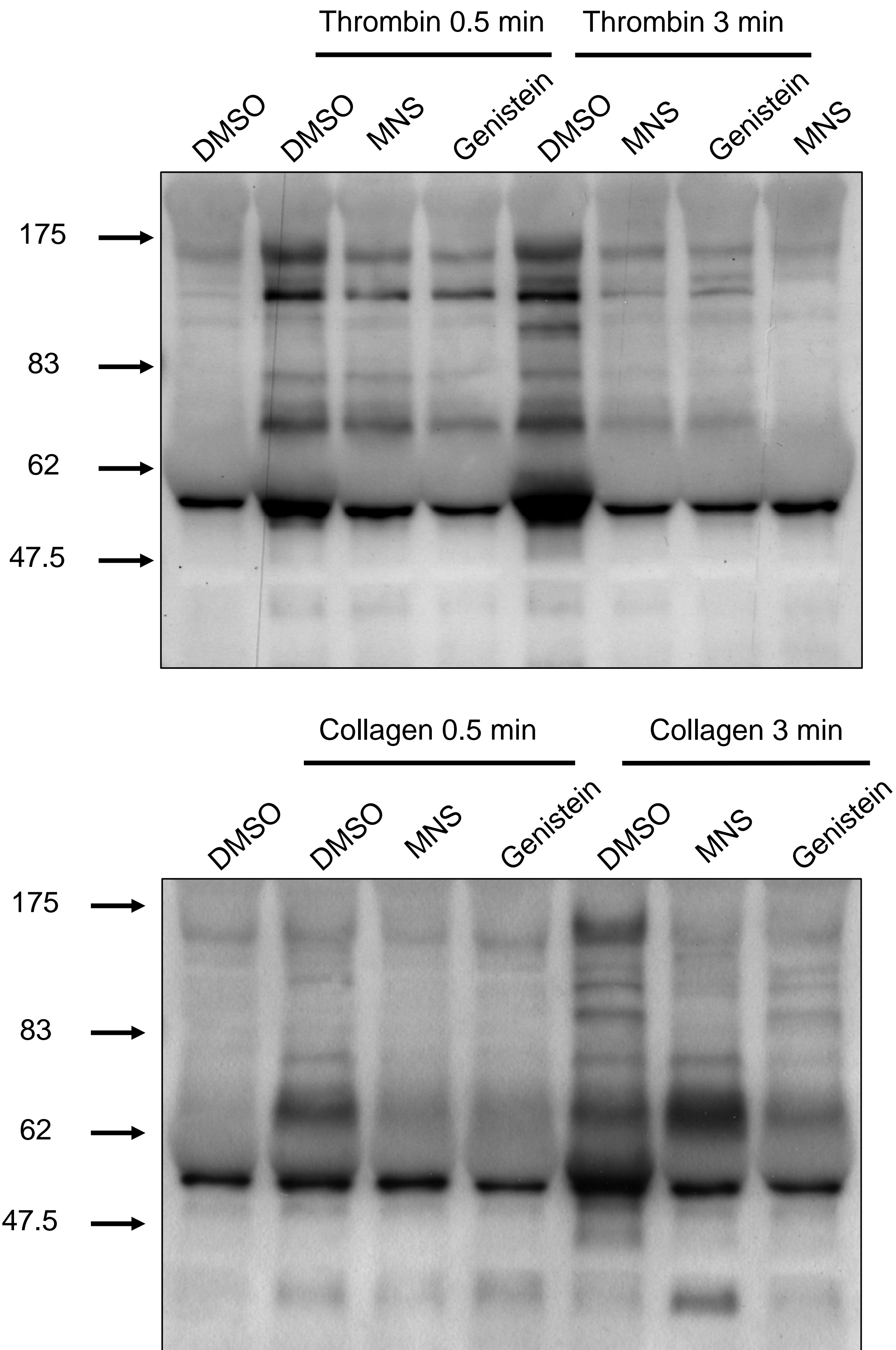

Fig.7B

Thrombin 0.5 min
Thrombin 3 min
DMSO
DMSO
MNS
Genistein
DMSO
MNS
Genistein
175
83
62
47.5

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Fig. 8

A

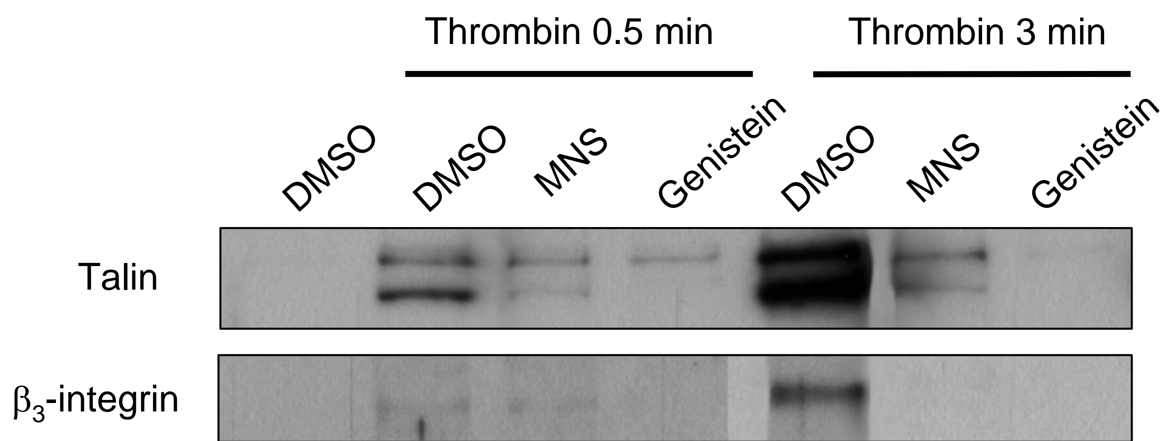

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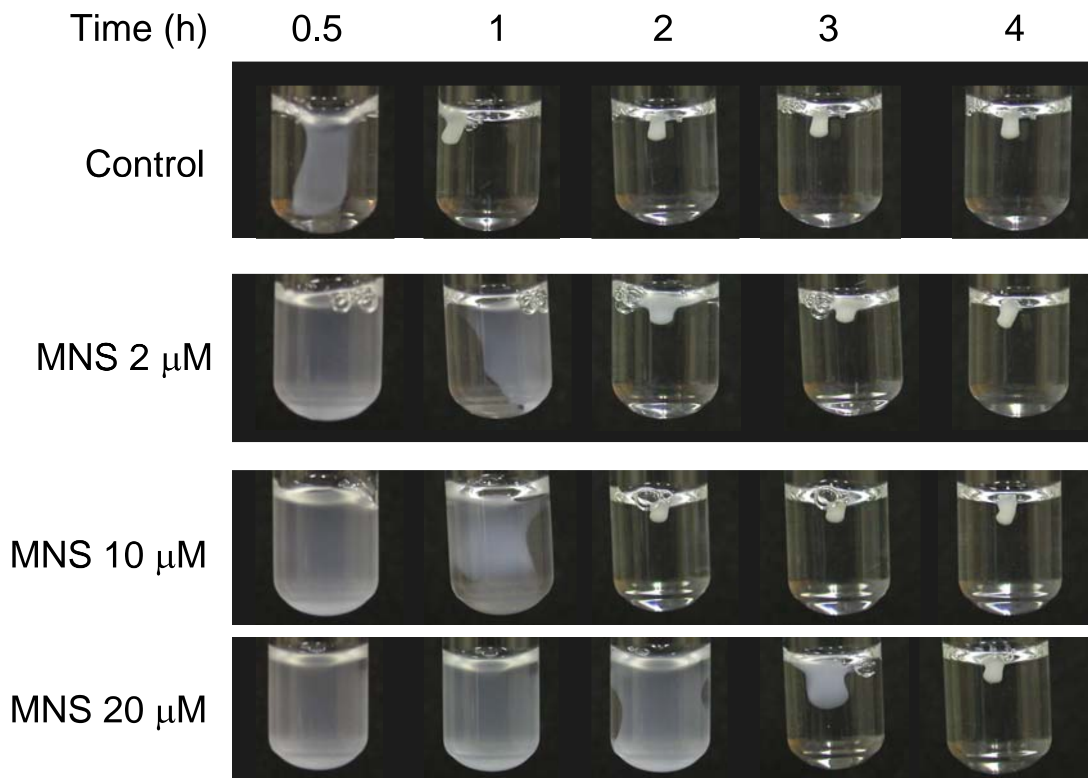
Fig. 8
B



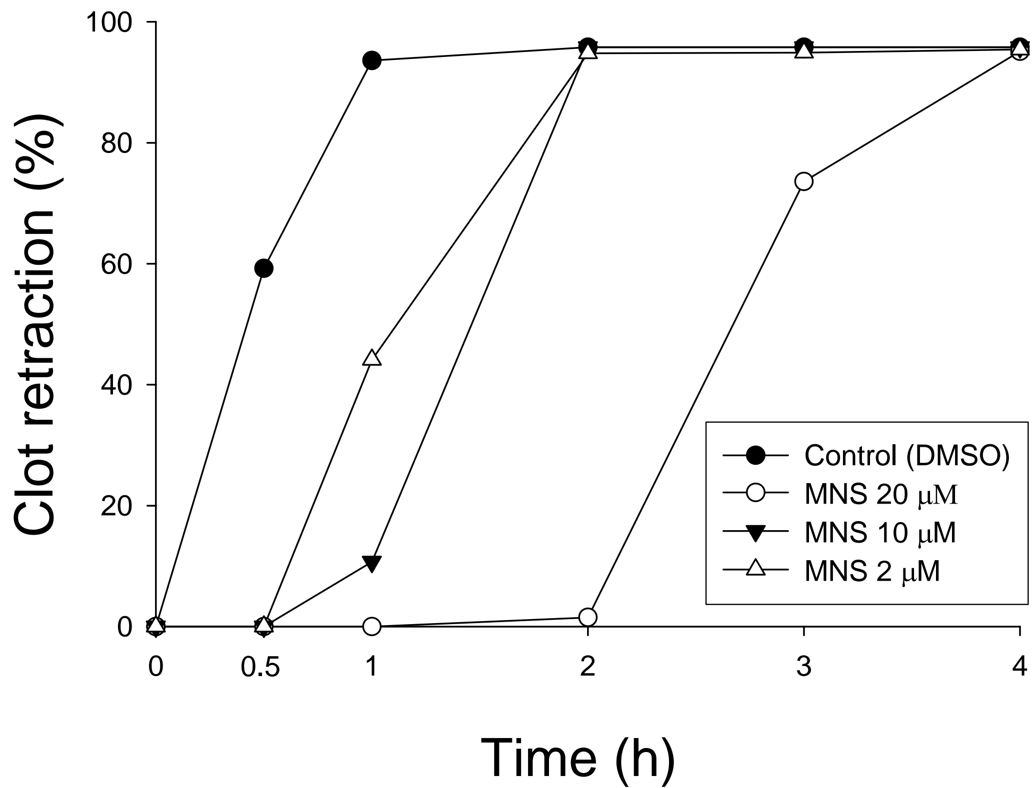
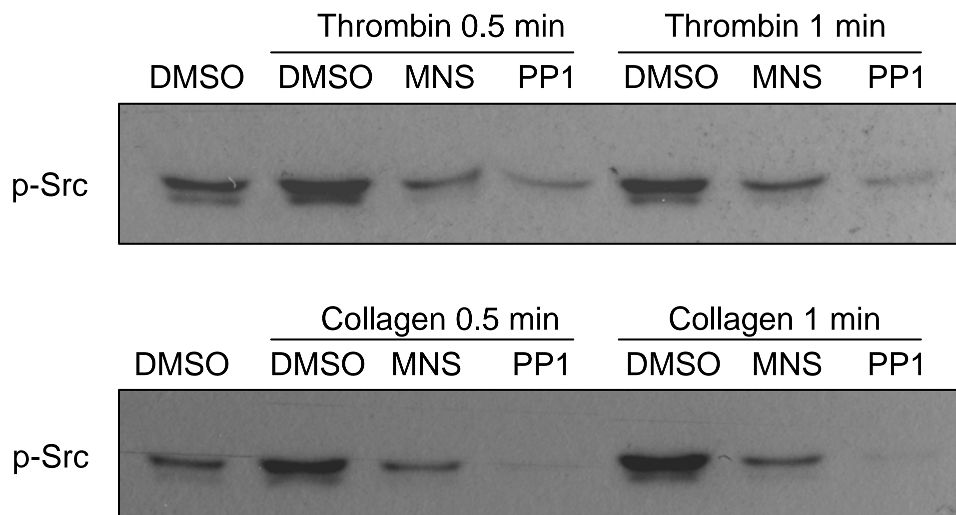


Fig.9

A



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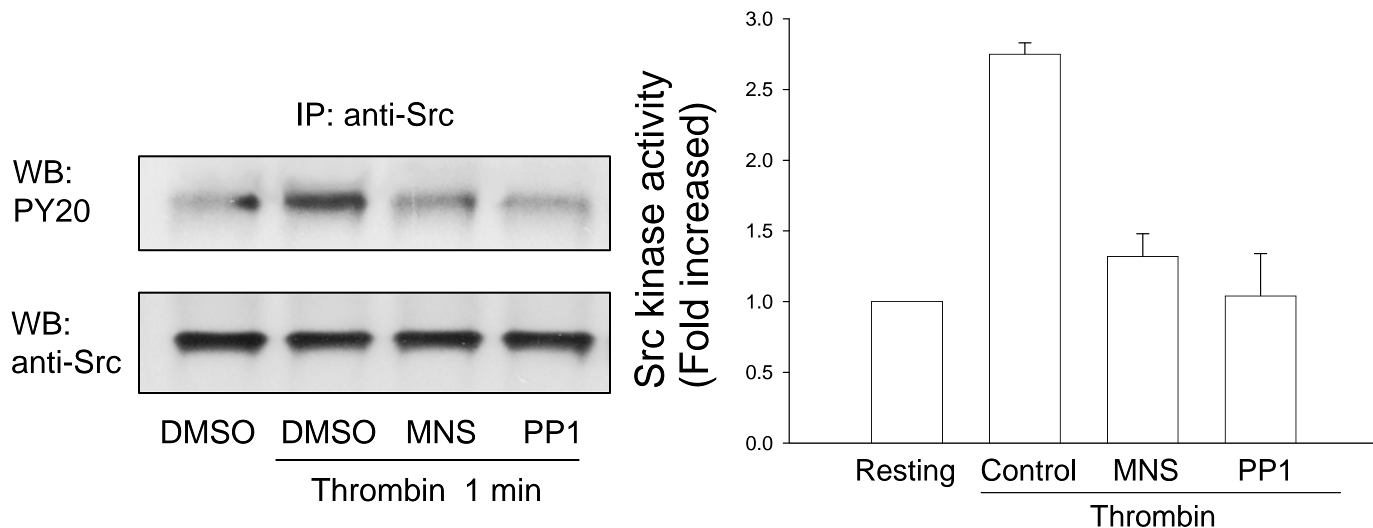
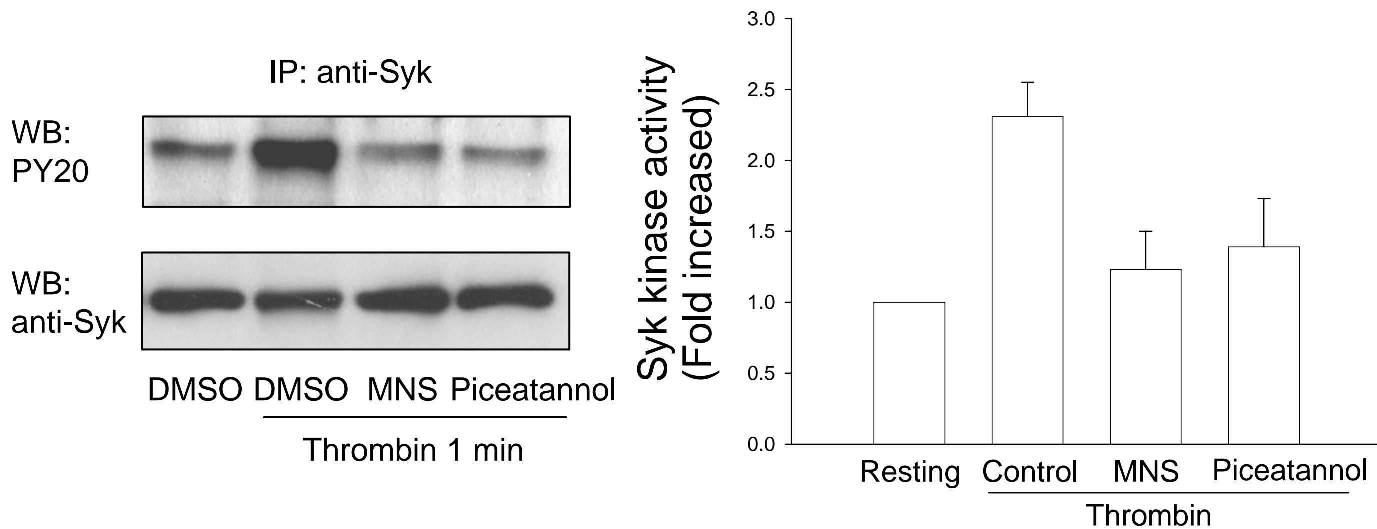


Fig. 9
C



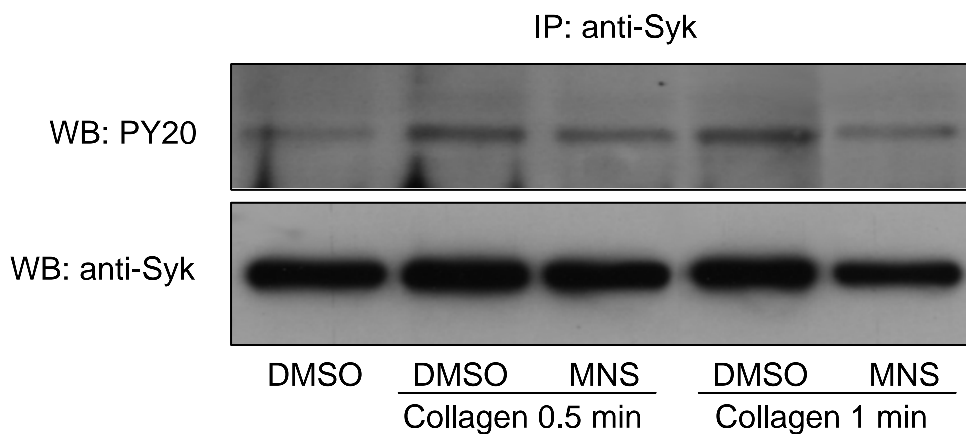


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Fig.9
D