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Synthesis and pharmacological evaluation of novel β-nitrostyrene derivatives as tyrosine kinase inhibitors with potent antiplatelet activity

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ABSTRACT

Protein tyrosine kinases have been known to be involved in regulation of platelet aggregation, suggesting a potential target for antiplatelet therapy. Our previous study showed that 3,4-methylenedioxy-β-nitrostyrene (MNS) prevented platelet aggregation caused by various stimulators, and this action was accompanied by inhibition of tyrosine kinases. In the present study, in order to examine the structural determinants required for the actions of MNS and to develop more potent tyrosine kinase inhibitors and antiplatelet agents, a new series of β-nitrostyrene derivatives were synthesized and pharmacologically characterized. The β-nitrostyrene derivatives inhibited thrombin- or collagen-induced human platelet aggregation, ATP secretion, GPIIb/IIIa activation and protein tyrosine phosphorylation. In recombinant enzyme assay, some β-nitrostyrene derivatives also demonstrated potent inhibition of Src and/or Syk kinase activity. Furthermore, there was a good correlation between the inhibitory potency of these compounds on tyrosine kinases and on platelet activation/aggregation. Among them, a benzoyl ester derivative (compound **10**) possess up to 8-fold greater potency than MNS and over two orders of magnitude greater potency than genistein or tyrphostin A47 in inhibiting platelet responses to thrombin. Our data suggest that β-nitrostyrenes may represent a new class of tyrosine kinase inhibitors with potent antiplatelet activity.

1. Introduction

Blood platelets contain high levels of different non-receptor tyrosine kinases, suggesting an important role for these enzymes in platelet functions [1]. Upon stimulation of platelets, multiple proteins become phosphorylated at tyrosine residues [2,3]. This process is mediated by at least four families of tyrosine kinases, Src, Syk, FAK, and JAK [4]. Many functional responses of platelets are regulated by protein tyrosine phosphorylation, including aggregation, which has been shown to be positively regulated by tyrosine kinases in general, as suggested by its potentiation by protein tyrosine phosphatase inhibitors (e.g., pervanadate) [5] and its inhibition by broad-spectrum inhibitors of tyrosine kinases (e.g., genistein and tyrphostins) [6,7].

Because of the importance of tyrosine kinases for regulating platelet aggregation and platelet activation, these enzymes

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Abbreviations: ATP, adenosine 5′-triphosphate; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; GP, glycoprotein; MARCKS, myristoylated alanine-rich C kinase substrate; MNS, 3,4-methylenedioxy-β-nitrostyrene; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; RGDS, Arg-Gly-Asp-Ser; SAR, structure–activity relationship; U46619, 9,11-dideoxy-9α,11α-methanoepoxy PGF2$_\alpha$

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may represent a potential target for the development of new antiplatelet drugs [8]. Several tyrosine kinase inhibitors have been reported to exhibit antiplatelet effects; however, these compounds usually suffer limitations, such as lack of potency, specificity and/or cell permeability [9–12]. In the previous study [13], we have shown that a small-molecule compound, 3,4-methylenedioxy-β-nitrostyrene (MNS) is a potent tyrosine kinase inhibitor and a broad-spectrum antiplatelet agent. MNS is much more potent than genistein in inhibiting platelet aggregation and protein tyrosine phosphorylation. Moreover, MNS had no direct effects on other signaling pathways required for platelet activation, such as protein kinase C, myosin light kinase, calpain and the metabolism of arachidonic acid, suggesting its high specificity for tyrosine kinases. In the current study, in order to examine the structural determinants required for the actions of MNS and to develop more potent tyrosine kinase inhibitors and antiplatelet agents, a new series of β-nitrostyrene derivatives have been synthesized and pharmacologically characterized.

2. Materials and methods

2.1. Chemicals and reagents

3,4-Methylenedioxy-β-nitrostyrene (MNS), 3,4-methylenedioxy cinnamic acid (compound **1**), isosafrole (compound **2**), *trans*-β-nitrostyrene (compound **3**), and β-nitro-4-hydroxy-3-methoxystyrene (compound **5**) were purchased from Sigma Chem. Co., USA. 3,4-Dimethoxy-β-nitrostyrene (compound **4**) was from Alfa Aesar Inc., USA. Bovine-α-thrombin, U46619 (9,11-dideoxy-9α,11α-methanoepoxy PGF_2), 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), and adenosine 5′-triphosphate (ATP) bioluminescent assay kit were obtained from Sigma Chem. Co., USA. Baculovirus produced human Src and Syk were purchased from Upstate Biotech. Inc., USA. Human recombinant FAK was from Biosource International Inc., USA. Tyrosine kinase activity assay kit was from Chemicon International Inc., USA. FITC-conjugated PAC-1 was purchased from BD Biosciences, USA. Anti-phosphotyrosine monoclonal antibody (PY20), anti-phospho-MARCKS, anti-MARCKS, anti-Src, anti-Syk, and anti-FAK were purchased from Santa Cruz Biotechnologies, USA. Anti-phospho-Src (Tyr416) antibody was obtained from Cell Signaling Technology, USA. Anti-phospho-FAK (Tyr397) antibody was from Abcam Inc., USA. All other chemicals were purchased from Sigma Chem. Co., USA.

2.2. Synthesis of β-nitrostyrene derivatives and related agents

Preparations of compounds **6–10** were carried out by the reaction of β-nitro-4-hydroxy-3-methoxystyrene (195 mg, 1.0 mmol) with corresponding acid anhydrides in pyridine solution. The reaction mixture was stirred overnight at room temperature for 24 h. The reaction was quenched by ice water, and then partitioned between ice water/ethyl acetate. The ethyl acetate fraction was evaporated at reduced pressure, and further subjected to purification by silica column chromatography or by re-crystallization. All products were fully characterized using spectral data.

2.3. Preparation of washed human platelets

Human blood anticoagulated with acid citrate dextrose was obtained from healthy human volunteers who had not taken any drugs within the last 2 weeks. The platelet suspension was then prepared according to the washing procedure described previously [14]. 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.

2.4. Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., USA). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or test compounds at 37 °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.

2.5. Measurement of ATP release from activated platelets

Washed human platelets were pre-incubated with DMSO or test compounds for 3 min, and then treated with platelet activators for additional 5 min. The reactions were stopped with EDTA (5 mM), and the samples were immediately centrifuged at 4 °C. ATP was measured in the supernatants by the addition of a luciferase/luciferin reagent in a microplate luminometer (Lucy 1, Anthos Labtec Instruments, Austria).

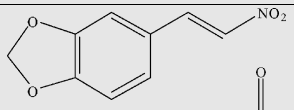
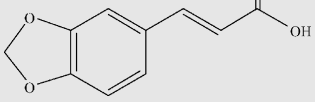
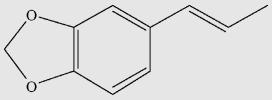
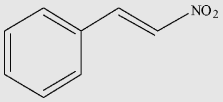
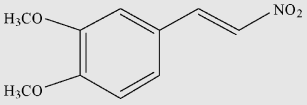
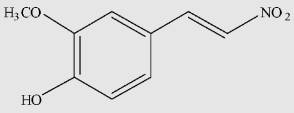
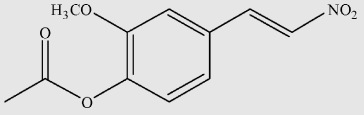
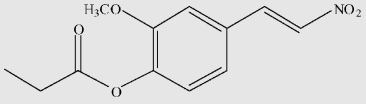
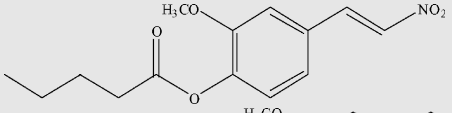
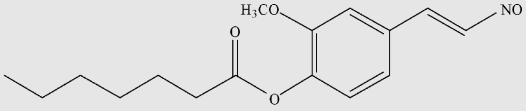
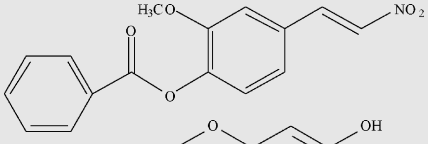
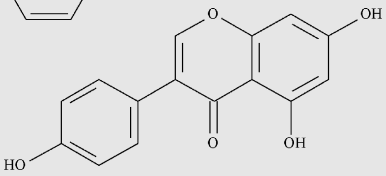
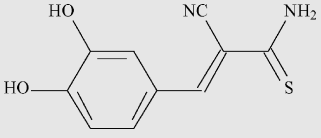
2.6. Measurement of PAC-1 binding by flow cytometry

Washed human platelets (3×10^7 platelets/ml) were pre-incubated with DMSO or test compounds for 5 min, and then treated with or without thrombin (0.1 U/ml) in the presence of FITC-conjugated PAC-1 monoclonal antibody for 15 min at room temperature. The samples were then fixed at 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 PAC-1 binding were expressed as the percentages of cells positive for PAC-1.

2.7. Western immunoblotting

To prepare whole platelet lysates, the reaction was terminated at the indicated time points by addition of 5× 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. Platelet lysates 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

Table 1 – Chemical structures and anti-aggregatory activities of β-nitrostyrene derivatives

Compounds	Structure	IC_{50} (μM)		
		Thrombin	U46619	Collagen
MNS		14.0 ± 2.1	7.4 ± 1.2	7.2 ± 0.7
1		>100	>100	>100
2		>100	>100	38. ± 4.3
3		17.8 ± 2.6	7.3 ± 1.1	4.9 ± 0.7
4		12.8 ± 1.0	7.6 ± 1.4	4.5 ± 1.0
5		66.5 ± 8.8	28.6 ± 6.2	16.4 ± 0.5
6		24.8 ± 2.1	13.1 ± 1.4	10.2 ± 0.6
7		15.7 ± 0.8	12.0 ± 1.7	7.7 ± 0.4
8		14.7 ± 0.7	9.9 ± 1.5	7.6 ± 0.2
9		13.1 ± 1.7	6.4 ± 0.3	7.4 ± 0.5
10		2.4 ± 0.6	1.9 ± 0.3	0.91 ± 0.04
Genistein		>600	61.7 ± 6.5	13.2 ± 0.6
Tyrphostin A47		>600	211.1 ± 3.2	30.2 ± 4.6

Washed human platelets were incubated with DMSO (vehicle control) or test compounds at 37 °C for 3 min. After incubation, platelet aggregation was triggered by addition of thrombin (0.1 U/ml), collagen (10 μg/ml) or U46619 (2 μM). Values are presented as mean ± S.E.M. ($n = 4$).

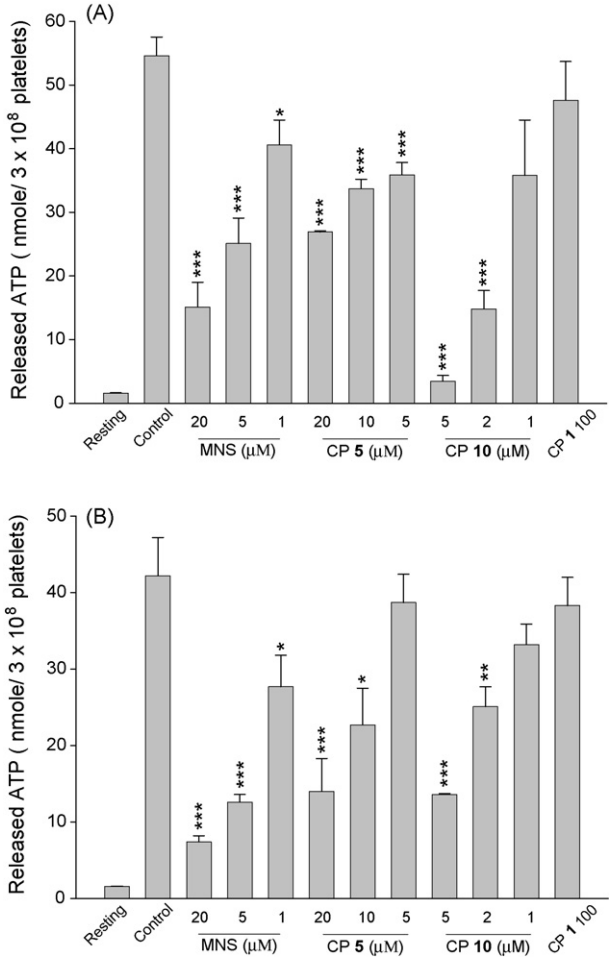


Fig. 1 – Effects of β-nitrostyrene derivatives on ATP release. Washed human platelets were incubated with DMSO (vehicle control) and tested compounds at 37 °C for 3 min, then thrombin (0.1 U/ml, A) or collagen (10 μg/ml, B) was added to trigger ATP release. Released ATP was measured by a luciferase/luciferin kit as described in Section 2. Results are presented as mean ± S.E.M. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared with the respective control.

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. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After washing with TBST, protein bands on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (Western Lightning®, Perkin-Elmer, USA).

2.8. Measurement of tyrosine kinase activity

In order to examine the effects of β-nitrostyrene derivatives on tyrosine kinase activity, recombinant human Src, Syk or FAK was used and treated with DMSO or test compounds for 5 min. The activity of 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 min. 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 chromogenic substrate reaction.

2.9. Measurement of intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} mobilization of platelets was measured by the method described previously [14]. In brief, 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. 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 pre-incubated with test compounds in the presence of 1 mM extracellular calcium at 37 °C for 3 min prior to the addition of thrombin. Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo, Japan).

2.10. Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M.) and comparisons were made using

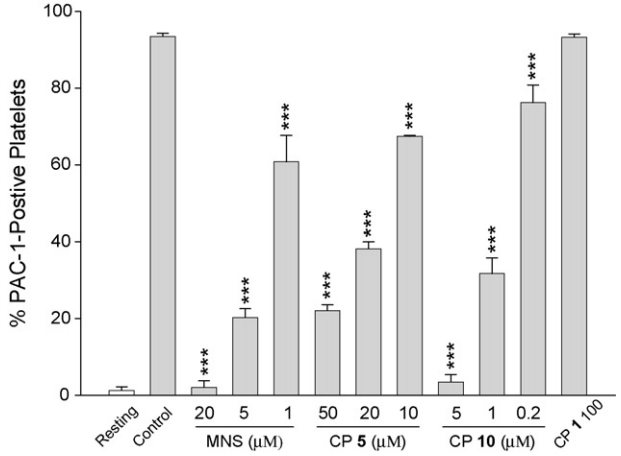


Fig. 2 – Effects of β-nitrostyrene derivatives on GPIIb/IIIa activation. Washed human platelets were pre-incubated with DMSO, MNS, compound (CP) 1, 5 or 10 for 3 min, and then treated with or without thrombin (0.1 U/ml) in the presence of FITC-conjugated PAC-1 for 15 min at room temperature. The samples were then fixed with 1% paraformaldehyde. The percentage of PAC-1 positive platelets was analyzed by flow cytometry as described in Section 2. Results are presented as mean ± S.E.M. ($n = 3$). ***$P <$ **0.001 as compared with the control.**

Student's *t* test. A probability of 0.05 or less was considered significant.

3. Results

3.1. *Effects of β-nitrostyrene derivatives on human platelet aggregation*

In order to investigate the structure–activity relationship (SAR) of β-nitrostyrenes, a series of analogues (compounds **1–10**, Table 1) were tested for their effect on human platelet aggregation caused by thrombin, collagen, and U46619 (a thromboxane A_2 mimic). As shown in Table 1, the replacement of the nitro group in MNS by carboxyl group (compound **1**) or methyl (compound **2**) led to complete or near complete loss of activity, suggesting that the nitro group is essential for the antiplatelet effect of MNS. In contrast, although the open-ring derivative of MNS (β-nitro-4-hydroxy-3-methoxystyrene, compound **5**) was less effective than MNS, both *trans*-β-nitrostyrene (compound **3**) and 3,4-dimethoxy-β-nitrostyrene (compound **4**) were equally potent as inhibitors of platelet aggregation as MNS, indicating that the 3,4-methylenedioxy ring of MNS is not necessary for its activity. We next examined the SAR of aromatic side-chain analogues of compound **5** (compound **6–9**) and found that the length of the carbon chain affects the antiplatelet activity in direct proportion. The optimal activity was obtained with a five or seven-carbon chain length. On the other hand, substitution of 4-hydroxyl group of compound **5** by a benzoyl group (compound **10**) led to a dramatic increase in its ability to inhibit platelet aggregation. Compound **10** expressed 28-fold greater potency than its parent compound (compound **5**), and up to 8-fold greater potency than MNS. In contrast, the tyrosine kinase inhibitors genistein and tyrphostin A47 were much weaker than either MNS or compound **10** in inhibiting human platelet aggregation.

3.2. *Effects of β-nitrostyrene derivatives on ATP release from activated platelets*

Platelet dense granules contain both ADP and ATP, and upon platelet stimulation with agonists, released ADP plays an important role in the potentiation of platelet activation [15]. In the present study, the secretion of dense granules in human platelets was evaluated by ATP release. The results presented in Fig. 1 show that MNS, compound **5**, and compound **10**

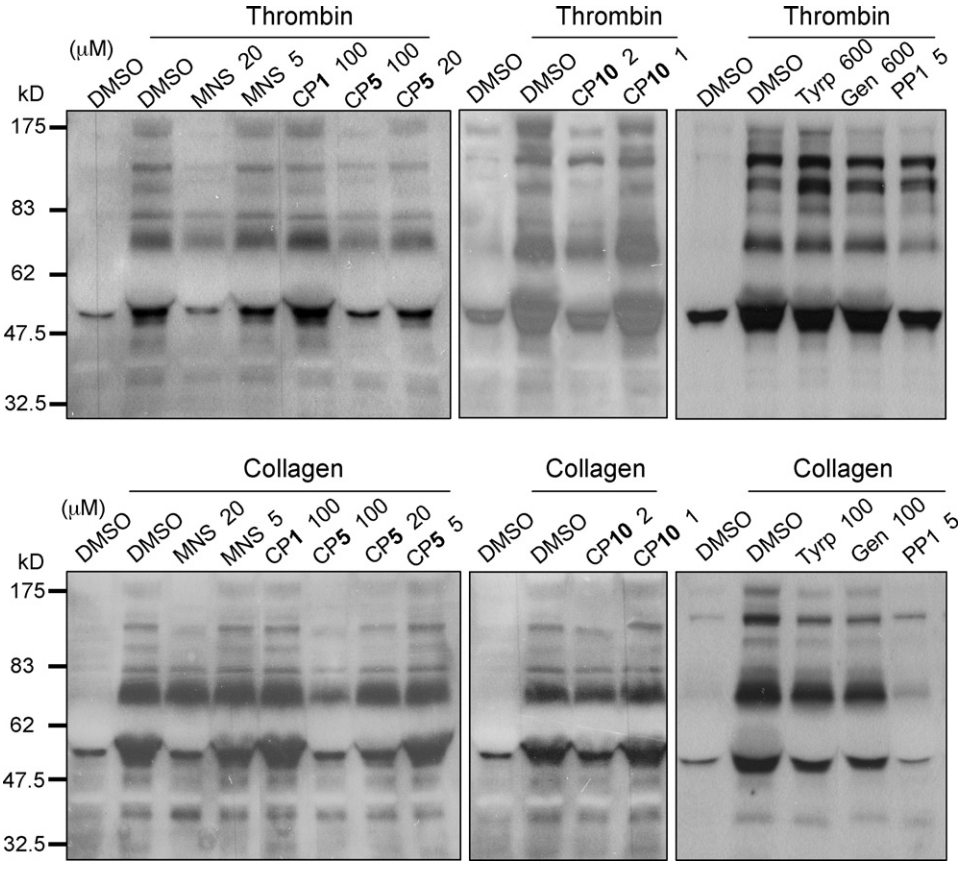


Fig. 3 – Effects of β-nitrostyrene derivatives on protein tyrosine phosphorylation. Human platelets were pre-incubated with DMSO, MNS, CP1, CP5, CP10, tyrphostin A47 (Tyrp), genistein (Gen) or PP1 at 37 °C for 3 min. And then, platelets were treated with or without thrombin (0.1 U/ml) or collagen (10 μg/ml) for 3 min. Western blot analysis was performed on whole platelet lysates using a monoclonal anti-phosphotyrosine antibody (PY20). Similar results were obtained in three separate experiments.

concentration-dependently prevented ATP release from platelets stimulated by thrombin or collagen. The concentrations of these compounds required to inhibit dense granule secretion were comparable to those required to inhibit platelet aggregation.

3.3. *Effects of β-nitrostyrene derivatives on platelet GPIIb/IIIa activation*

Upon platelet activation, the major platelet integrin GPIIb/IIIa undergoes a conformational change that dramatically

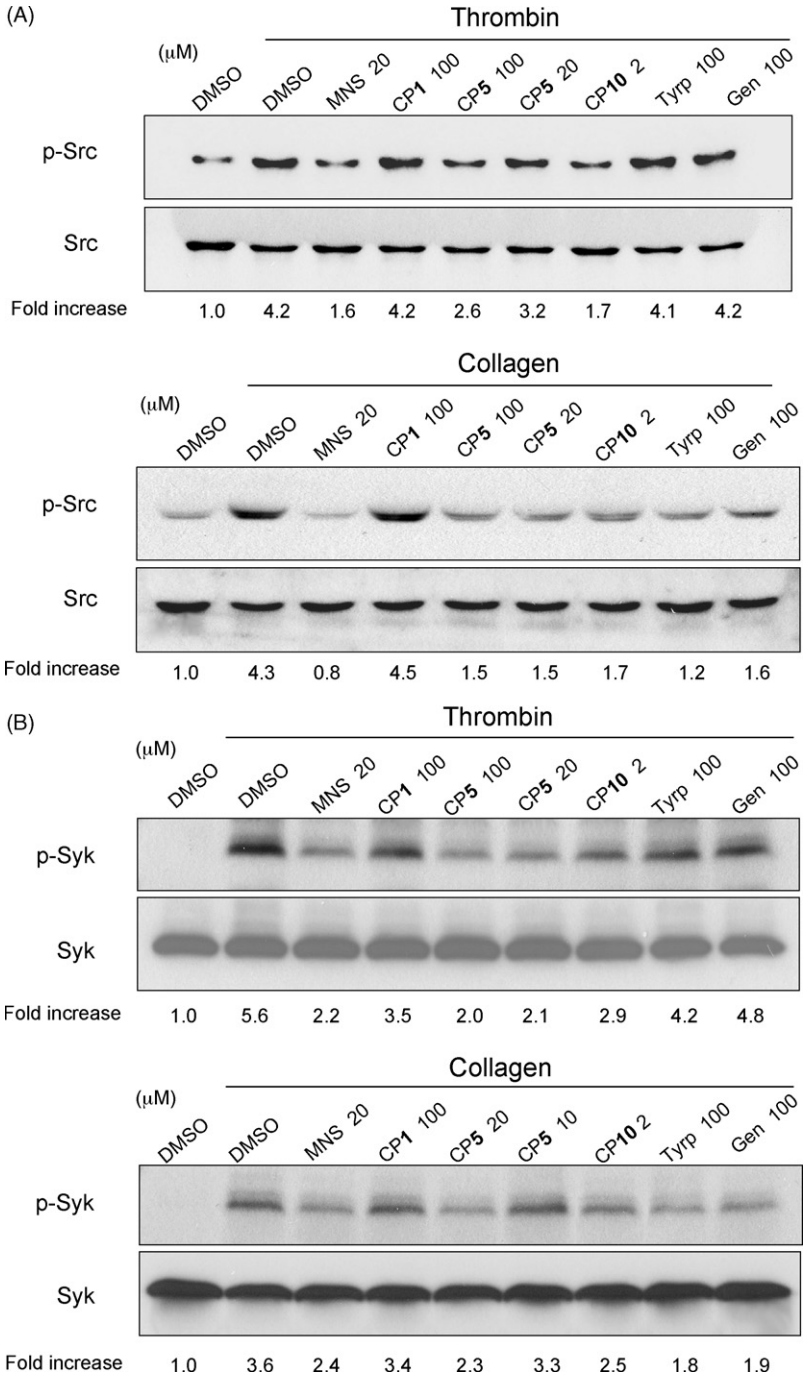


Fig. 4 – (A–D) Effects of β-nitrostyrene derivatives on Src, Syk, and FAK phosphorylation. Human platelets were pre-incubated with DMSO, MNS, CP1, CP5, CP10, tyrphostin A47 (Tyrp), genistein (Gen) or PP1 at 37 °C for 3 min. And then, platelets were treated with or without thrombin (0.1 U/ml) or collagen (10 μg/ml) for 1 min. Phosphorylation of Src, Syk, and FAK was determined by Western blot analysis using specific antibodies, respectively. Levels of phosphorylated proteins were determined by densitometry and normalized to their total protein levels. Similar results were obtained in three separate experiments.

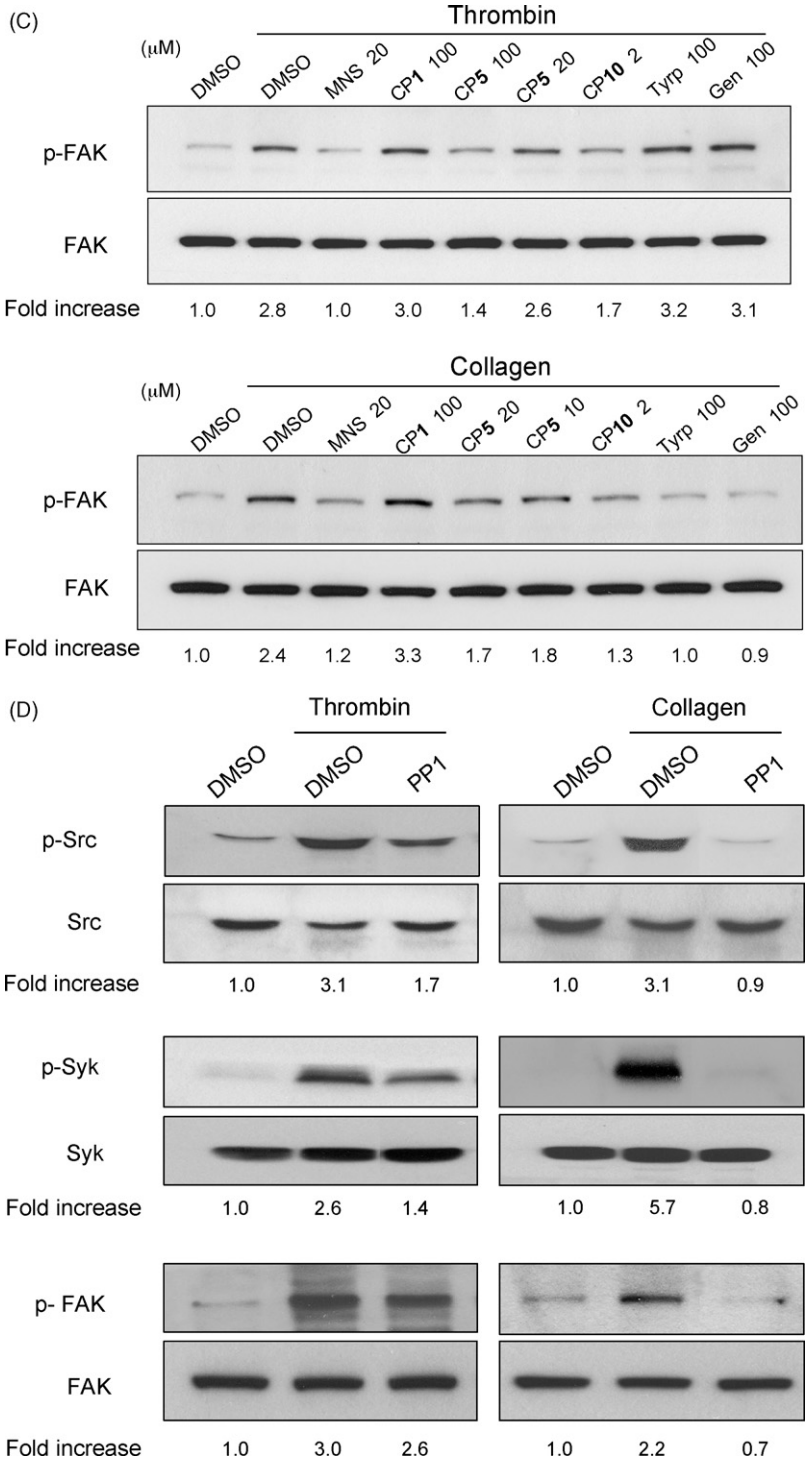


Fig. 4. (Continued).

increases its binding affinity for fibrinogen, resulting platelet aggregation [16]. In the present study, GPIIb/IIIa activation was monitored by the binding of FITC-conjugated PAC-1, since this monoclonal antibody only binds to the activated form of GPIIb/IIIa [17]. As shown in Fig. 2, MNS, compound **5**, and compound **10**, but not compound **1**, inhibited thrombin-induced PAC-1 binding to human platelets. In contrast, even at the highest concentration used (600 μM), no significant effect of genistein or tyrphostin A47 was observed on GPIIb/IIIa activation induced by thrombin (data not shown). These results were correlated with those observed in platelet aggregation experiments.

3.4. *Effects of β-nitrostyrene derivatives on protein tyrosine phosphorylation in platelets*

As shown in Fig. 3, both thrombin and collagen treatments induced marked protein tyrosine phosphorylation in platelets.

Pretreatment of platelets with MNS (20 μM), compound **5** (100 μM), or compound **10** (2 μM) significantly inhibited protein tyrosine phosphorylation caused by both inducers. However, compound **1** (100 μM) had no effect on protein tyrosine phosphorylation in stimulated platelets. The abilities of β-nitrostyrene derivatives to prevent protein tyrosine phosphorylation were well correlated with their potencies to inhibit platelet activation and aggregation. Although genistein, tyrphostin A47, and the Src inhibitor PP1 were effective in inhibiting collagen-induced protein tyrosine phosphorylation in platelets, they showed weaker inhibition on thrombin stimulation.

3.5. *Effects of β-nitrostyrene derivatives on the activation of Src, Syk, and FAK*

In order to investigate the mechanism of inhibition of agonist-induced protein tyrosine phosphorylation by β-nitrostyrenes, the activation of three major tyrosine kinases Src, Syk, and FAK in platelets was examined. As shown in Fig. 4, both thrombin- and collagen-induced Src autophosphorylations at Tyr416, a process indicative of Src activation, were markedly prevented by MNS (20 μM) and compound **5** (100 μM) as well as compound **10** (2 μM), but not by compound **1** (100 μM). Similar results were observed in experiments assaying Syk and FAK phosphorylation. Again, genistein, tyrphostin A47, and PP1 markedly inhibited collagen-induced phosphorylation of Src, Syk and FAK, whereas thrombin-induced phosphorylation of these tyrosine kinases was less sensitive to these compounds.

We next examined the direct inhibition of β-nitrostyrenes on tyrosine kinase activity by using recombinant human Src, Syk, and FAK. As shown in Table 2, Src kinase activity was inhibited by test compounds with the rank order of potency: compound **10** ≫ MNS > compound **1** = genistein = compound **5** > tyrphostin A47. The rank order of potency of these compounds in inhibiting Syk activity was MNS = compound **10** ≫ tyrphostin A47 = genistein > compound **5** > compound **1**. All of these compounds were less effective in inhibiting FAK activity.

Because the potencies of β-nitrostyrene derivatives in inhibiting recombinant FAK activity were much less than those in inhibiting FAK autophosphorylation, we would like to examine whether the action of β-nitrostyrenes in intact platelets was secondary to inhibition of platelet aggregation and subsequent outside-in signaling. Fig. 5 shows that in the unstirred condition and in the presence of the GPIIb/IIIa antagonist RGDS (200 μg/ml), thrombin failed to elicit FAK autophosphorylation in platelets; however, rapid tyrosine phosphorylation of Src and Syk was still observed in this condition.

3.6. *Effects of β-nitrostyrene derivatives on protein kinase C activation and calcium mobilization*

In the previous study, MNS has been shown not to directly inhibit protein kinase C (PKC) activation [13]. In order to examine the specificity of the new β-nitrostyrenes, we also assess the effects of these compounds on PDBu-induced PKC activation by measuring the phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), which is a major substrate of PKC [18]. Fig. 6A shows that GF109203X, a PKC inhibitor, but not β-nitrostyrenes inhibited MARCKS phosphorylation caused by PDBu. However, PDBu-induced platelet aggregation was abolished by compound **5**, compound **10** as well as MNS (data not shown).

Table 2 – Effects of β-nitrostyrene derivatives on the enzyme activities of recombinant human Src, Syk, and FAK

Compounds	IC_{50} (μM)		
	Src	Syk	FAK
MNS	27.3 ± 1.5	2.8 ± 0.8	97.6 ± 8.4
Compound **1**	56.0 ± 8.0	>100	>100
Compound **5**	71.5 ± 15.3	76.4 ± 0.6	>100
Compound **10**	2.7 ± 0.2	3.8 ± 0.2	19.0 ± 4.0
Tyrphostin A47	171.0 ± 24.4	37.7 ± 4.3	>300
Genistein	65.0 ± 7.0	39.4 ± 2.7	71.9 ± 6.7

The enzyme activities of these tyrosine kinases were measured by phosphorylation of biotinylated poly [Glu:Tyr] 4:1 substrate in the presence of test compounds as described in Section 2. Results are represented as mean ± S.E.M. ($n = 3$).

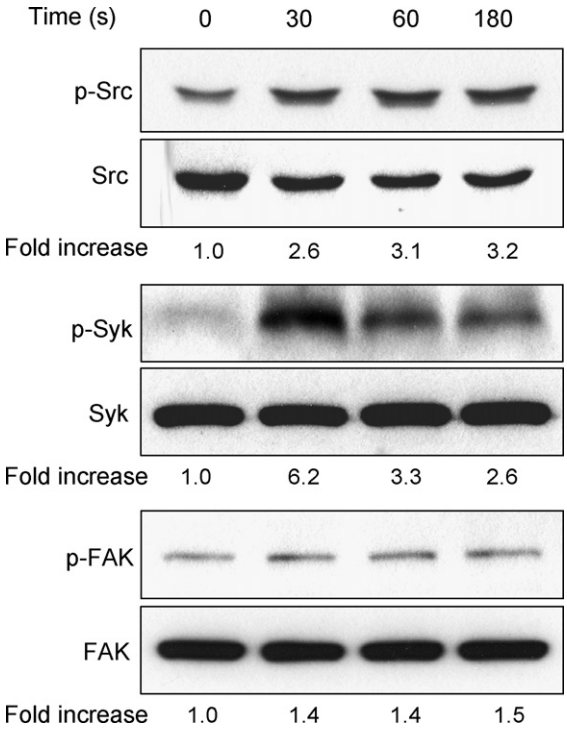


Fig. 5 – Thrombin induces the phosphorylation of Src and Syk, but not FAK in absence of platelet aggregation. Human platelets were stimulated with thrombin (0.1 U/ml) under unstirred condition for indicated times in the presence of RGDS (200 μg/ml). Phosphorylation of Src, Syk, and FAK was determined by Western blot analysis using specific antibodies, respectively. Levels of phosphorylated proteins were determined by densitometry and normalized to their total protein levels. Similar results were obtained in three separate experiments.

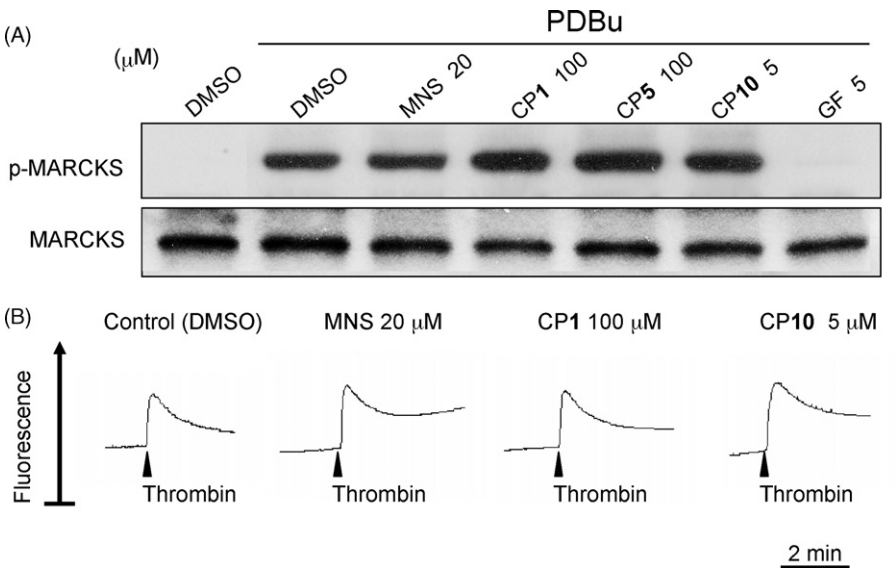


Fig. 6 – Effects of β-nitrostyrenes on PKC activation and intracellular Ca^{2+} mobilization in platelets. (A) Washed platelets were pre-incubated with DMSO, MNS, CP1, CP5, CP10 or GF109203X (GF) or 3 min at 37 °C, and then stimulated with PDBu (200 nM) for another 6 min. Western blot analysis was performed on platelet lysates using antibodies against phospho-MARCKS or total MARCKS. Levels of phospho-MARCKS were determined by densitometry and normalized to total MARCKS levels. Similar results were obtained in three separate experiments. (B) Fluo-3-loaded human platelets were incubated with DMSO or test compounds at 37 °C for 3 min in the presence of 1 mM extracellular Ca^{2+}, thrombin (0.1 U/ml) was then added to trigger the increase of $[Ca^{2+}]_i$ (fluorescence). Similar results were obtained in three separate experiments.

We next examine the effects of β-nitrostyrenes on intracellular Ca^{2+} mobilization in thrombin-stimulated platelets. It is known that thrombin-induced intracellular Ca^{2+} release is largely dependent on phospholipase C_β activation which is mediated by Gq protein but not tyrosine kinases. As shown in Fig. 6B, compound **10** and MNS had no inhibitory effect on thrombin-induced Ca^{2+} signal, even at the concentrations that inhibit platelet aggregation. Compound **5** was not tested in this experiment, because it caused fluorescence quenching of Fluo-3.

4. Discussion

In the present study, a series of β-nitrostyrene derivatives were compared with MNS for their antiplatelet activity and tyrosine kinase-inhibiting activity. These β-nitrotyrenes showed potent inhibition of platelet aggregation, secretion, GPIIb/IIIa activation, and protein tyrosine phosphorylation. We found a good correlation between the inhibitory potency of β-nitrostyrenes on protein tyrosine kinases and on platelet aggregation or PAC-1 binding. Moreover, similar to MNS, these compounds did not directly inhibit PKC activation and had no inhibitory effect on thrombin-induced intracellular Ca^{2+} increase in platelets. Therefore, the tyrosine kinase inhibitor activity of MNS and its analogues is apparently responsible for their antiplatelet effects.

In the SAR study, we found that the nitro group of MNS is essential for its activity in inhibiting both protein tyrosine phosphorylation and platelet aggregation. On the other hand, the absence of 3,4-methylenedioxy ring did not significantly affect MNS's action, β-nitro-4-hydroxy-3-methoxystyrene (compound **5**) thus was chosen as a lead compound for further structural modification and optimization. The acylation of the phenolic hydroxyl group of compound **5** resulted in an improvement in the antiplatelet activity, and the best activity was found in the compound with a benzoyl group (compound **10**). Compound **10** exhibited 28- and 8-fold greater potency in inhibiting platelet aggregation than compound **5** and MNS, respectively. In contrast, two widely used inhibitors of tyrosine kinase, genistein and tyrphostin A47 were much less potent than β-nitrostyrene derivatives in inhibiting protein tyrosine phosphorylation and tyrosine kinase activity. In parallel to their effect on tyrosine kinases, genistein and tyrphostin A47 also exhibited weak inhibition of platelet aggregation and PAC-1 binding caused by platelet stimulators, especially thrombin.

Human platelets contain different non-receptor tyrosine kinases that are activated during various stages of platelet activation. These include Src family kinases (Src, Fyn, Lyn, Hck, Yes), Syk, FAK family kinases (FAK, PYK), and JAK family kinases (JAK, TYK) [3,4]. As shown in the previous study [19] and in the current research, the tyrosine phosphorylation and activation of FAK are late events in platelet activation that require outside-in GPIIb/IIIa signaling and platelet aggregation. In contrast, although Src and Syk are able to be directly activated in response to fibrinogen binding to GPIIb/IIIa and play a central role in outside-in signaling [20,21], they also can be rapidly phosphorylated and activated in response to platelet agonists in the absence of platelet aggregation [22,23]. Therefore, Src and/or Syk are presumably involved in mediating inside-out activation of GPIIb/IIIa, although their

exact role still remains unclear [13,24,25]. In the present work, beta-nitrostyrenes potently inhibited the kinase activities and tyrosine phosphorylation of Src and Syk, and this effect paralleled their ability to inhibit platelet aggregation and activation, suggesting that Src and Syk are the major targets of beta-nitrostyrenes. In addition, our results suggest that blockade of both Src and Syk may provide more effective suppression of platelet aggregation than blockade of either kinase alone. This suggestion is based on two observations. First, compound **10** strongly inhibited *in vitro* activity of both Src and Syk and exhibited greater antiplatelet effect than MNS which preferentially inhibited the *in vitro* activity of Syk over that of Src. Secondly, PP1 alone did not significantly inhibit thrombin-induced platelet aggregation, whereas the combination of PP1 with the Syk inhibitor piceatannol showed synergistic anti-aggregatory effect (W.Y. Wang and C.C. Wu, unpublished data).

In summary, we have synthesized and tested a series of β-nitrostyrene derivatives as tyrosine kinase inhibitors and antiplatelet agents. Our results show that β-nitrostyrene derivatives inhibited human platelet aggregation, ATP secretion, GPIIb/IIIa activation and protein tyrosine phosphorylation. Furthermore, there was a good correlation between the inhibitory potency of these derivatives on protein tyrosine kinases and on platelet aggregation. Among these compounds, a benzoyl ester derivative (compound **10**) exhibited greater activity than MNS, genistein, and tyrphostin A47. Therefore, β-nitrostyrenes may represent a new class of tyrosine kinase inhibitors with potent antiplatelet activity, which should serve as useful tools to investigate protein tyrosine kinase and may have therapeutic potential for treating arterial thrombosis.

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