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European Journal of Pharmacology 527 (2005) 37–43
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2-(2-Br-phenyl)-8-methoxy-benzoxazinone (HPW-RX2), a direct thrombin inhibitor with a suppressive effect on thromboxane formation in platelets

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Received 11 August 2005; received in revised form 6 October 2005; accepted 18 October 2005

Abstract

2-(2-Br-phenyl)-8-methoxy-benzoxazinone (HPW-RX2), a newly synthetic benzoxazinone derivative, has previously been shown to inhibit rabbit platelet aggregation caused by thrombin and arachidonic acid. In the present study, the mechanism for the antiplatelet effect of HPW-RX2 was further investigated. In human platelets, HPW-RX2 concentration-dependently inhibited platelet aggregation, ATP release, P-selectin expression, and intracellular calcium mobilization caused by thrombin. In contrast, HPW-RX2 had no significant effect on either SFLLRN- or GYPGKF-induced platelet aggregation, indicating that HPW-RX2 did not interfere with platelet thrombin receptors. Moreover, HPW-RX2 inhibited the amidolytic activity of thrombin and prolonged the fibrinogen clotting time. These results suggest that the inhibitory effect of HPW-RX2 on thrombin-induced platelet aggregation is via direct inhibition of thrombin proteolytic activity. Besides the inhibition on thrombin, HPW-RX2 also prevented platelet aggregation, ATP release, and increase in $[Ca^{2+}]_i$ caused by arachidonic acid and low concentration collagen. In a parallel manner, both arachidonic acid-induced thromboxane B_2 and prostaglandin D_2 formations were decreased in platelets treated with HPW-RX2. This indicates that HPW-RX2 is able to inhibit the arachidonic acid cascade at the cyclooxygenase level. This is the first report of a benzoxazinone derivative possessing both thrombin and cyclooxygenase inhibitory properties. The dual effect of HPW-RX2 might provide extra therapeutic benefits for treatment of arterial thrombosis.

Keywords: Antiplatelet agent; Thrombin; Thromboxane A_2

1. Introduction

Arterial thrombosis accounts for most heart attacks and strokes, which are the main causes of death in the developed countries. Although the established antiplatelet agents aspirin, clopidogrel, and dipyridamole, and anticoagulant agents heparin and warfarin have been shown to be beneficial in the treatment of thromboembotic disease, these drugs have considerable limitations (CAPRIE Steering Committee, 1996; Antithrombotic Trialists' Collaboration, 2002). Thus, there remains an unmet need for the development of more effective and safe anti-thrombotic agents.

Platelets play a central role in the process of arterial thrombosis. Following endothelial damage or disruption of atherosclerotic plaque in blood vessels, platelets rapidly adhere to newly exposed sub-endothelial matrix, such as collagen and von Willebrand factor. Adhesion is followed by platelet shape change, spreading, and release of ADP and thromboxane A_2, which act as secondary agonists to recruit more circulating platelets to the site of injury. Activated platelets facilitate thrombin generation by providing a catalytic surface on which coagulation activation occurs. Thrombin is not only responsible for the formation of fibrin but also an extreme platelet activator. The growing mound of activated platelets is eventually stabilized by crosslinked fibrin and results in the formation of a platelet-rich thrombus (Ross, 1993; Ruggeri, 2002). The interactions between platelets with various adhesive proteins and soluble agonists may provide potential targets for developing antiplatelet agents (Bhatt and Topol, 2003; Jackson and Schoenwaelder, 2003).

The 2-substituted benzoxazinones isolated from *Dianthus* species of Caryophyllaceae possess in vitro antifungal activity and are considered as phytoalexin (Kurosaki and Nishi, 1983; Ponchet et al., 1984). Some synthetic 2-substituted benzoxazinones have been shown to exhibit interesting pharmacological

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doi:10.1016/j.ejphar.2005.10.023

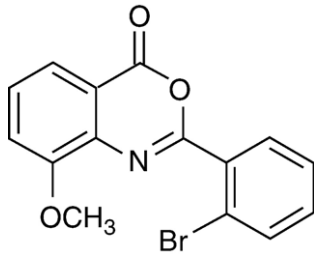

Fig. 1. Chemical structure of HPW-RX2.

activity including antiviral and hypolipidemic effects (Abood et al., 1997; Hsieh et al., 2004; Fenton et al., 1989). In addition, 2-substituted benzoxazinones have been characterized as inhibitors of a variety of serine proteases through an alternate-substrate mechanism involving a stable acyl enzyme complex (Teshima et al., 1982; Spencer et al., 1986). In the course of our search for new antiplatelet agents, a series of 2, 8-disubstituted benzoxazinones were synthesized and tested for their inhibitory activities on rabbit platelet aggregation. Among them, 2-(2-Br-phenyl)-8-methoxy-benzoxazinone (HPW-RX2, Fig. 1) showed the most potent inhibitory effect on rabbit platelet aggregation elicited by thrombin and arachidonic acid (Hsieh et al., 2005). In the present study, we further examined the effects of HPW-RX2 on platelet aggregation and other activation processes, such as ATP release, P-selectin expression, and intracellular calcium mobilization, in human platelets. We also investigated the mechanism for the antiplatelet activity of HPW-RX2. Our data suggest that HPW-RX2 inhibits platelet activation by dual inhibition of thrombin activity and thromboxane production in platelets.

2. Materials and methods

2.1. Materials

HPW-RX2 was synthesized base on the methods described previously (Hsieh et al., 2005). Bovine α-thrombin, arachidonic acid, U46619 (9, 11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$), collagen (type I, bovine Achilles tendon), D-phenylalanyl-prolyl-arginine-chloromethylketone (PPACK), 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., USA. The synthetic peptide SFLLRN-NH_2 and GYPGKF-NH_2 were purchased from Bachem Co., USA. The thromboxane B_2 enzyme immunoassay kit and the prostaglandin D_2 enzyme immunoassay kit were purchased from Amersham Co., USA and Cayman Co., USA, respectively. FITC-conjugated mouse anti-CD62P (P-selectin) and FITC-conjugated mouse IgG_1 were obtained from Serotec Inc., USA. All other chemicals were purchased from Sigma Chem. Co., USA.

2.2. Preparation of washed human platelets

Human blood anticoagulated with acid citrate dextrose was obtained from healthy human volunteers who have 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., 2004). 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.

2.3. 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 stimulators. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of stimulators.

2.4. 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 (Sigma Co., USA) in a microplate luminometer (Lucy 1, Anthos Labtec Instruments, Austria).

2.5. Measurement of P-selectin expression 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 (0.2 U/ml) in the presence of excessive amounts of FITC-conjugated anti-CD62P or FITC-conjugated IgG_1 (isotype negative control) 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, and the presence of the monoclonal antibody against P-selectin (anti-CD62P) was used to determine the percentage of platelets expressing P-selectin.

2.6. Thromboxane B_2, prostaglandin D_2, and cyclooxygenase assay

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 thromboxane B_2 and prostaglandin D_2 in the supernatants were assayed using enzyme immunoassay kits according to the procedure described by the manufacturer.

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

Platelets pelleted from platelet-rich plasma were resuspended in Ca^{2+}-free Tyrode's solution, 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 pre-incubated with test compounds in the presence of 1 mM extracellular calcium at 37 °C for 3 min prior to the addition of the platelet stimulators. Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo, Japan).

2.8. Measurement of the enzymatic activity of thrombin

Thrombin clotting activity was assayed as described by Hofmann et al. (1983). The clotting time of rabbit fibrinogen (2.5 mg/ml) in Tyrode's solution containing 2 mM $CaCl_2$ was measured after addition of thrombin (0.1 U/ml, final concentration) pre-incubated at 37 °C for 3 min with DMSO or HPW-RX2.

To assay amidolytic activity, thrombin was diluted to 0.1 U/ml final concentration in 50 mM Tris–HCl and 175 mM NaCl, pH 7.9, in the presence of DMSO or HPW-RX2. After 3 min incubation at 37 °C, the residual activity was measured by adding the chromogenic substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-1-L-arginine-*p*-nitroaniline. 2HCl). The increase in absorbance at 405 nm was recorded in a microplate reader and the initial rate of *p*-nitroaniline liberation determined.

2.9. 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.

3. Results

3.1. Effects of HPW-RX2 on human platelet aggregation caused by various stimulators

In washed human platelets, thrombin (0.1 U/ml), arachidonic acid (200 μM), collagen (2 and 10 μg/ml), and U46619 (2 μM, a stable thromboxane A_2-mimetic) all elicited about 80–90% aggregation. HPW-RX2 inhibited thrombin-, arachidonic acid- and low concentration (2 μg/ml) collagen-induced platelet aggregation in a concentration-dependent manner with IC_{50} values 3.6±0.9, 6.5±1.9 and 9.7±1.4 μM, respectively (Fig. 2A). In contrast, HPW-RX2 (up to 20 μM) had no or little effect on platelet aggregation caused by U46619 and 10 μg/ml collagen (Fig. 2B).

As shown in Fig. 2B, D-phenylalanyl-prolyl-arginine-chloromethylketone (PPACK), a potent and irreversible thrombin inhibitor, inhibited thrombin-induced platelet aggregation with an IC_{50} value of 0.01±0.002 μM, but did not affect those by arachidonic acid, collagen and U46619 (n=5). In contrast,

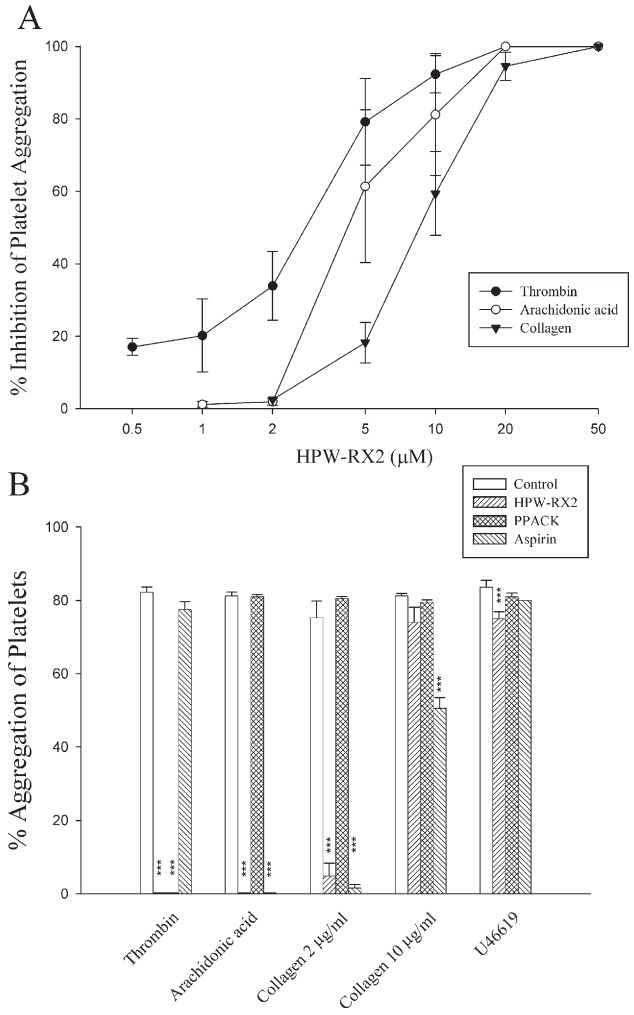

Fig. 2. (A) Concentration–inhibition curves for HPW-RX2 on human platelet aggregation. Washed human platelets were incubated with DMSO (vehicle control) or HPW-RX2 at 37 °C for 3 min, then thrombin (0.1 U/ml), arachidonic acid (200 μM) or collagen (2 μg/ml) was added to trigger platelet aggregation. (B) Effects of HPW-RX2, PPACK and aspirin on platelet aggregation caused by various stimulators. Washed human platelets were incubated with DMSO (control), HPW-RX2 (20 μM), PPACK (0.5 μM), or aspirin (200 μM) at 37 °C for 3 min, then thrombin (0.1 U/ml), arachidonic acid (200 μM), collagen (2 or 10 μg/ml), or U46619 (2 μM) was added to trigger platelet aggregation. Results are presented as mean±S.E.M. (n=5). ***P<0.001 as compared with the control.

aspirin, a cyclooxygenase inhibitor, prevented arachidonic acid- and collagen-induced platelet aggregation with IC_{50} values of 138.5±1.5 and 80.0±14.1 μM, respectively (n=5), but did not affect thrombin stimulation.

3.2. Effects of HPW-RX2 on ATP release and P-selectin expression in human platelets

The secretion of dense granule and α-granule contents in platelets were evaluated by ATP release and P-selectin expression, respectively. HPW-RX2 concentration-dependently prevented ATP release from activated platelets by thrombin, collagen and arachidonic acid (Table 1). HPW-RX2 also inhibited thrombin-induced P-selectin expression on platelets in a manner similar to that observed with the inhibition of ATP release (Fig. 3).

Table 1
Effects of HPW-RX2 on ATP release from activated platelets

	Released ATP (ng/3×10^8 platelets)			
	Thrombin	Arachidonic acid	Collagen	U46619
DMSO (control)	7.9±1.1	6.9±1.6	14.7±1.3	7.3±1.9
HPW-RX2				
2 μM	6.2±0.8	4.6±0.7	–	–
5 μM	1.9±0.8[a]	2.4±1.1	–	–
10 μM	0.5±0.0[b]	0.45±0.0[a]	8.9±1.3[a]	–
20 μM	–	–	5.8±2.1[a]	–
50 μM	–	–	2.8±1.3[b]	4.2±2.0
PPACK				
0.02 μM	0.5±0.0[b]	4.3±1.0	–	–
0.5 μM	–	–	13.3±3.3	3.6±1.3
Aspirin				
200 μM	8.6±1.7	0.5±0.0[b]	5.7±2.1[b]	–

Washed human platelets were incubated with DMSO (vehicle control), HPW-RX2, PPACK or aspirin at 37 °C for 3 min, then thrombin (0.1 U/ml), arachidonic acid (200 μM), collagen (2 μg/ml) or U46619 (2 μM) was added to trigger ATP release. Released ATP was measured by a luciferase/luciferin kit as described in Materials and methods. Results are presented as mean±S.E.M. (n=3). [a]P<0.01, [b]P<0.001 as compared with the respective control.

PPACK only inhibited thrombin-induced ATP release and P-selectin expression, while aspirin inhibited both arachidonic acid- and collagen-induced ATP release without affecting thrombin stimulation (Table 1 and Fig. 3).

3.3. Effects of HPW-RX2 on intracellular Ca^{2+} mobilization in platelets

In fluo-3-loaded platelets, thrombin, arachidonic acid, collagen, and U46619 all caused a marked increase of intracellular free calcium. As shown in Fig. 4, in the presence of 1 mM of extracellular calcium, pretreatment of platelets with HPW-RX2 largely prevented calcium signal elicited by these inducers except U46619. In contrast, PPACK only inhibited the increase in $[Ca^{2+}]_i$ caused by thrombin (data not shown).

3.4. Effects of HPW-RX2 on thrombin receptors and thrombin enzymatic activity

There are two protease-activated receptors (PARs)—PAR1 and PAR4—existing in human platelets (Vu et al., 1991; Kahn et al., 1998). Activation of PARs by thrombin involves cleavage of the exodomain of the receptor to create a new amino terminus, which can then act as a tethered ligand to activate the receptor. In addition to proteolytic activation, PAR1 and PAR4 can also be activated by synthetic peptides corresponding to their tethered ligand. To assess whether HPW-RX2 is able to interfere with the thrombin receptor PAR1 and PAR4 in human platelets, we examined the effects of HPW-RX2 on platelet aggregation induced by SFLLRN (a PAR1-activating peptide) and GYPGKF (a PAR4-activating peptide). At the concentration of 20 μM that completely abolish thrombin stimulation, HPW-RX2 only slightly decreased SFLLRN (10 μM)-induced platelet aggregation (% aggregation of platelets: 70.3±0.3 vs control 77.3±1.6, n=3, P<0.01) and did not affect that induced by 1 mM GYPGKF (% aggregation of platelets: 80.2±1.1 vs control 82.3±0.3, n=3, P>0.05). In contrast, SFLLRN- and GYPGKF-induced platelet aggregation were completely prevented by the PAR1 antagonist SCH-79797 (2 μM) (Ahn et al., 2000) and the PAR4 antagonist YD-3 (0.5 μM) (Wu et al., 2002, 2003), respectively (data not shown).

To examine if the action of HPW-RX2 on thrombin-induced platelet aggregation is via direct inhibition of thrombin enzymatic activity, the effect of HPW-RX2 on thrombin-induced fibrinogen clotting was tested. HPW-RX2 (0.5–20 μM) concentration-dependently prolonged thrombin (0.1 U/ml)-induced fibrinogen clotting time to about 6-fold of the control value (Fig. 5A). Furthermore, in the amidolytic assay, the thrombin activity was also inhibited by HPW-RX2 in a concentration-dependent manner (Fig. 5B). The concentrations of HPW-RX2 required to prolong clotting time or to inhibit amidolytic activity were comparable to those required to inhibit thrombin-induced platelet aggregation.

3.5. Effects of HPW-RX2 on thromboxane B_2 and prostaglandin D_2 formation

In order to investigate if HPW-RX2 interferes with the arachidonic acid metabolism, exogenous arachidonic acid (200 μM) was added as the substrate and thromboxane A_2/B_2, the major product of cyclooxygenase in platelets, was measured. As shown in Table 2, HPW-RX2 inhibited thromboxane B_2 formation caused by arachidonic acid in a concentration-dependent manner. In addition, treatment of platelets with 20 μM HPW-RX2 largely decreased thrombin (0.1 U/ml)- and collagen (10 μg/ml)-induced thromboxane B_2 formation by 98.5% and 77.6%, respectively (n=3, P<0.001).

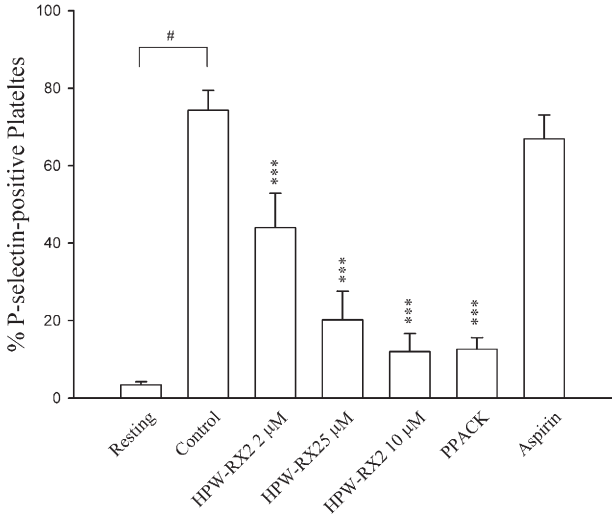

Fig. 3. Effect of HPW-RX2 on P-selectin expression. Washed human platelets were pre-incubated with DMSO or test compounds for 5 min, and then treated with or without thrombin (0.2 U/ml) in the presence of excessive amounts of FITC-conjugated anti-CD62P or FITC-conjugated IgG_1 (isotype negative control) 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). ***P<0.001 as compared with the control. P<0.001 as compared with the resting value.

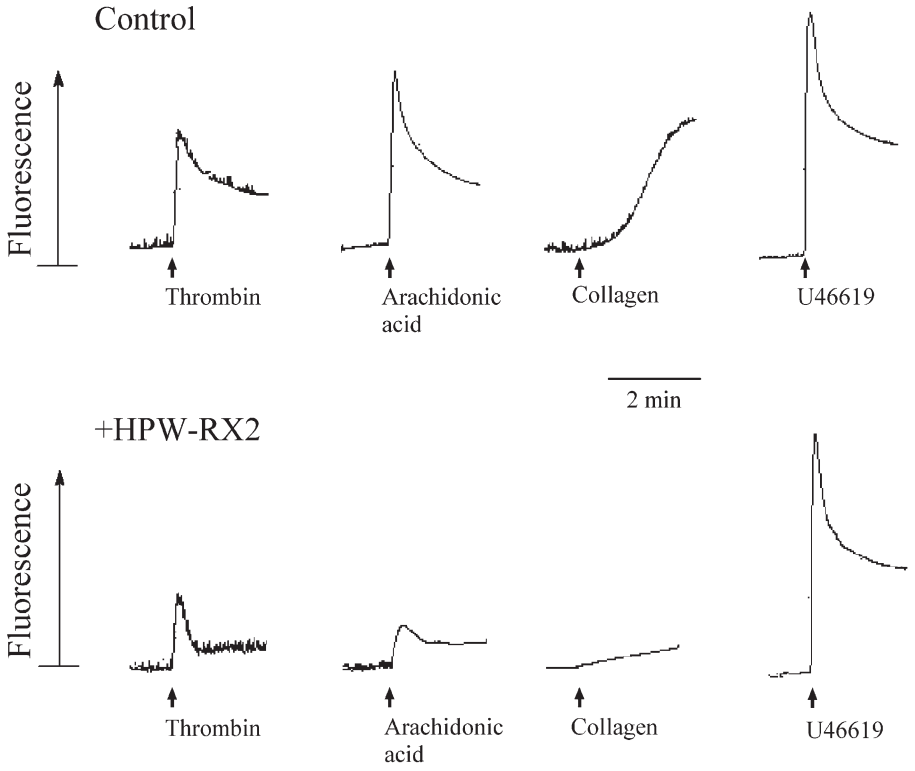

Fig. 4. Effect of HPW-RX2 on intracellular calcium mobilization in human platelets. Fluo-3-loaded human platelets were incubated with DMSO or HPW-RX2 (20 μM) at 37 °C for 3 min in the presence of 1 mM extracellular Ca^{2+}; thrombin (0.1 U/ml), arachidonic acid (200 μM), collagen (2 μg/ml), or U46619 (2 μM) was then added to trigger the increase of $[Ca^{2+}]_i$ (fluorescence, F). Similar results were obtained in three separate experiments.

We next examine the effect of HPW-RX2 on PGD_2 levels in platelets, which is another product of cyclooxygenase pathway. As shown in Table 2, HPW-RX2 concentration-dependently decreased arachidonic acid-induced prostaglandin D_2 formations in parallel with its inhibition of thromboxane B_2 formation. Similarly, aspirin (200 μM) also prevented both thromboxane B_2 and prostaglandin D_2 formations.

4. Discussion

In the present study, we showed that HPW-RX2, a newly synthetic benzoxazinone derivative, inhibited human platelet aggregation, granule release, intracellular calcium mobilization caused by thrombin, arachidonic acid, and a low concentration of collagen. Our data demonstrate that the effect of HPW-RX2 on thrombin-induced platelet activation resulted from a direct inhibition on the proteolytic activity of thrombin. First, HPW-RX2 concentration-dependently prolonged thrombin-mediated fibrinogen clotting time in the absence of any components of plasma. Second, HPW-RX2 inhibited thrombin activity in an amidolytic assay using a chromogenic peptide substrate, which only interacts with the active site. Third, HPW-RX2, even at concentrations that completely abolish thrombin stimulation, had no significant effect on platelet aggregation caused by either SFLLRN or GYPGKF, indicating that HPW-RX2 does not antagonize both PAR1 and PAR4. On the other hand, HPW-RX2 also inhibited platelet activation caused by arachidonic acid and low collagen, suggesting in addition to the anti-thrombin effect, there are other effects of HPW-RX2 in human platelets. It is known that platelet stimulation by low concentration collagen is dependent on the release of arachidonic acid from membrane phospholipids and the formation of thromboxane A_2, and therefore can be prevented by cyclooxygenase inhibitors, such as aspirin (Pollock et al., 1986). The similarity between HPW-RX2 and aspirin in inhibiting arachidonic acid- and collagen-induced platelet activation suggests that HPW-RX2 may also interfere with the metabolism of arachidonic acid. In fact, HPW-RX2 concentration-dependently inhibited exogenous arachidonic acid-induced thromboxane production in platelets. Furthermore, this inhibition was associated with a decrease in prostaglandin D_2 levels. These results indicate that HPW-RX2 suppressed thromboxane formation in platelets by inhibition of cyclooxygenase rather than thromboxane synthase, since thromboxane synthase inhibitors, such as imidazole, can produce a re-diversion of arachidonic acid metabolism toward prostaglandin D_2, which is the major prostaglandin in platelets (Anhut et al., 1978; Bertele et al., 1984). In addition, the possibility that HPW-RX2 had an effect on antagonism of the thromboxane receptor can also be ruled out, as HPW-RX2 did not affect platelet aggregation, platelet dense granule release, and intracellular Ca^{2+} mobilization caused by the thromboxane A_2-mimetic U46619.

Substitute benzoxazinones have been characterized as mechanism-based inhibitors of serine proteases (Powers et al., 2002). The mechanism of inhibition of serine proteases by benzoxazinones involves the nucleophilc reaction with the active site

serine to form a stable acyl enzyme intermediate (Spencer et al., 1986). Although the effects of benzoxazinones have been examined in several biological systems, they have never been explored in platelets. Therefore, this study is the first report of a benzoxazinone derivative possessing both anti-thrombin and anti-cyclooxygenase activities in human platelets. It has been known that the Ser 529 residue of platelet cyclooxygenase is critical for the enzyme activity, since acetylation of this serine residue by aspirin leads to an irreversible inhibition of cyclooxygenase (Roth et al., 1975; DeWitt and Smith, 1988). Therefore, it may be speculated that the benzoxazinone derivative HPW-RX2 is able to acylate the serine residue in platelet cyclooxygenase by a mechanism similar to that in thrombin. This speculation is supported by the fact that the inhibition of arachidonic acid-induced platelet aggregation by HPW-RX2 is irreversible (data not shown). However, further experiments are needed to establish the precise mechanism of this effect of HPW-RX2.

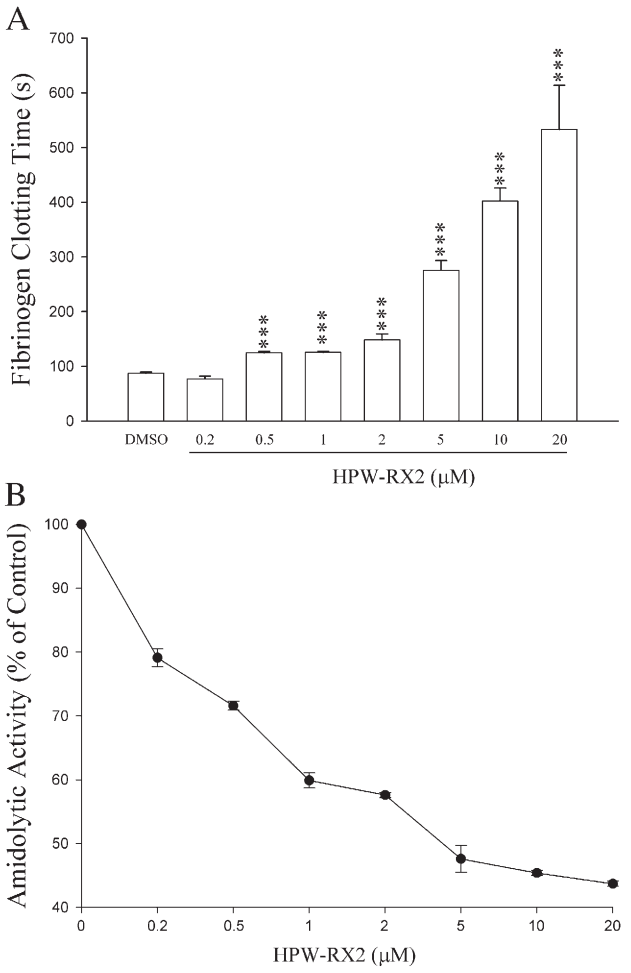

Fig. 5. (A) Effects of HPW-RX2 on thrombin-induced fibrinogen clotting time. Fibrinogen (2.5 mg/ml) in Tyrode's solution containing 2 mM $CaCl_2$ was pre-incubated with DMSO or various concentrations of HPW-RX2 at 37 °C for 3 min, then thrombin (0.1 U/ml) was added to trigger clot formation. Results are presented as mean±S.E.M. ($n=3$). ***$P<0.001$ as compared with the control. (B) Effect of HPW-RX2 on the amidolytic activity of thrombin. Thrombin (0.1 U/ml) was pre-incubated with DMSO or various concentrations of HPW-RX2 at 37 °C for 3 min before adding S-2238. The absorbance at 405 nm was monitored. Data are presented as the percent of thrombin amidolytic activity with DMSO ($n=3$).

Table 2
Effects of HPW-RX2 on the thromboxane B_2 (TxB_2) and prostaglandin D_2 (PGD_2) formations in washed human platelets caused by arachidonic acid

	TxB_2 (ng/3×10^8 platelets)	PGD_2 (ng/3×10^8 platelets)
DMSO (control)	621.7±160	4.4±0.5
HPW-RX2		
2 μM	300.0±39.4	3.8±1.0
5 μM	102.4±57.3[a]	2.4±0.6
10 μM	90.1±24.1[a]	2.0±0.8[b]
20 μM	68.9±24.4[b]	0.8±0.2[c]
Aspirin		
200 μM	31.7±6.1[c]	1.1±0.1[c]

Washed human platelets were pre-incubated with DMSO, HPW-RX2 or aspirin at 37 °C for 3 min and then arachidonic acid (200 μM) was added. Reactions were terminated by EDTA (2 mM) and indomethacin (50 μM) 5 min after the addition of AA. The level of TxB_2 and PGD_2 in resting platelets was 0.7±0.1 and 0.1±0.0 ng/3×10^8 platelets, respectively. Values are presented as mean±S.E.M. ($n=3$). [a]$P<0.01$, [b]$P<0.05$, [c]$P<0.001$ as compared with control.

Platelet activation during the progress of arterial thrombosis involves many factors, including adhesive proteins (such as collagen and von Willebrand factor) and soluble agonists (such as thrombin, thromboxane A_2, ADP, platelet-activating factor (PAF), serotonin, and epinephrine) that stimulate platelets through different receptors and signaling pathways. Thus, it is no wonder that antiplatelet agents affecting only one pathway of platelet activation, for example, aspirin and clopidogel, have limited efficacy in the treatment of arterial thrombotic diseases. In contrast, the combinations of antiplatelet drugs with different mechanisms of action have been proved to be more effective than single-agent therapy in preventing thrombus formation (Herman, 1998). In the case of the anti-thrombin agents, they are able to efficiently inhibit thrombin-induced fibrin formation and platelet aggregation, but have no effect on platelet activation caused by collagen and the metabolites of arachidonic acid; thus their anti-thrombotic efficacy can be enhanced when aspirin is administered together with them (Andre et al., 2003). In the present study, although the anti-thrombin potency of HPW-RX2 is much lower than the current anti-thrombin agents, it shows a broader spectrum of antiplatelet activity via an additional inhibition on thromboxane synthesis in human platelets. The dual inhibition of thrombin and thromboxane formation by HPW-RX2 may provide a lead for the development of new antiplatelet drugs.

Acknowledgments

This work was supported by grants from National Science Council of Taiwan (NSC 93-2320-B-037-034).

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