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LB30057 Inhibits Platelet Aggregation and Vascular Relaxation Induced by Thrombin

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Previous study showed that an amidrazonophenylalanine derivative, LB30057, which has high water solubility, inhibited the catalytic activity of thrombin potently by interaction with the active site of thrombin. In the current investigation, we examined whether LB30057 inhibited platelet aggregation and vascular relaxation induced by thrombin. Treatment with LB30057 to platelet-rich plasma (PRP) isolated from human blood resulted in a concentration-dependent inhibition of thrombin-induced aggregation. Values for IC_{50} and IC_{100} were 54 ± 4 nM and 96 ± 3 nM, respectively. This inhibition was agonist (thrombin) specific, since IC_{50} values for collagen and ADP were much greater than those for thrombin. In addition, concentration-dependent inhibitory effects were observed on the serotonin secretion induced by thrombin in PRP. Consistent with these findings, thrombin-induced increase in cytosolic calcium levels was inhibited in a concentration-dependent manner. When LB30057 was treated with aortic rings isolated from rats, LB30057 resulted in a concentration-dependent inhibition of thrombin-induced vascular relaxation. All these results suggest that LB30057 is a potent inhibitor of platelet aggregation and blood vessel relaxation induced by thrombin.

Key words: LB30057, Platelet-rich plasma, Thrombin inhibitor.

INTRODUCTION

The clotting enzyme thrombin is generated at sites of vascular injury and is a central mediator of haemostasis and thrombosis (Ross, 1993). Not only thrombin catalyzes the cleavage of fibrinogen to fibrin, but also it activates platelets and various other cells via cell surface receptors. Platelets activated by thrombin undergo morphological and biochemical changes such as shape change, degranulation, aggregation and adhesion (Vanhoutte, 1983; Hughes and Pfaff, 1998). Thus, excessive production of thrombin induces significant platelet activation leading to thrombosis.

Since thrombin plays a important role in various vascular diseases most antithrombotic strategies are direct toward the thrombogenic effects of thrombin (Hirsch, 1991; Becker, 1992). For the treatment of thrombotic disease, heparin has been most widely used until recently. Heparin, however, has several limitations (Kelton *et al.*, 1988). It requires a physiological cofactor, antithrombin III, to inactivate free thrombin and is unable to inactivate thrombin that is bound to fibrin (Weitz, 1996). In addition, heparin showed the unpredictable pharmacokinetics because of binding to plasma proteins and to the vessel wall, and of being inactivated by heparinase and platelet factor IV which are released by activated platelets (Turpie *et al.*, 1995). So much effort has been expended to develop synthetic thrombin inhibitors that could be orally administrated (Ripka, 1997). Recently, direct thrombin inhibitors that directly bind to the catalytic site of thrombin have been developed. Presently, argatroban is the drug registered, and commercially available (Hursting *et al.*, 1997). Argatroban is used for intravenous treatment of arterial thrombosis and acute ischemic stroke. An example of direct thrombin inhibitor, melagatran, is currently under development for oral administration to cure thrombotic diseases (Ripka, 1997).

LB30057, an amidrazonophenylalanine derivative, was synthesized as a selective potent thrombin inhibitor (Oh *et al.*, 1998). Due to its high water solubility, it can be used as an oral anti-thrombotic agent. Although it was reported that LB30057 was a potent selective thrombin inhibitor, a

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little studies have been made to explain its mode of inhibition, which may be important in the efficacy of its anticoagulant and antithrombotic effects. The current study was to intend whether LB30057 could inhibit thrombin-induced physiological activities of platelets and blood vessels in an effort to develop a new thrombin inhibitor.

MATERIALS AND METHODS

Mateirals

LB30057,(S)-4-[3-(cyclopentylmethylamino)-2-[(2-naphthalenylsulfonyl)amino]-3-oxopropyl] benzenecarboximidic acid hydrazide (Z)-2-butenediote (1:1) salt was synthesized from Biotech Research Institute, LG Chemical (Taejon, Korea). Calcium chloride, sodium chloride, trisodium citrate, citric acid, thrombin, EDTA, trichloroacetic acid, *o*-phthalaldehyde, serotonin creatinine sulfate, anhydrous DMSO, EGTA, phenylephrine were purchased from Sigma Chemical Co. (St.Louis, USA). Calcium green-1/AM and pluronic F-127 were purchased from Molecular Probes Inc (Eugene, USA). All other chemicals were of the highest available purity and obtained from routine commercial sources.

Preparation of platelet-rich plasma and washed platelets

Human blood from 20~30 healthy male volunteers (6:1 in 85 mM ACD) was centrifuged 100 g for 10 min to obtain platelet rich plasma (PRP). After PRP was again centrifuged at 500 g for 10 min, the platelets were gently suspended in washing buffer (134 mM NaCl, 2.9 mM·KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 10.0 mM HEPES, 5.0 mM dextrose, 12.0 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 10% ACD, 0.3% bovine serum albumin, pH 7.4). After centrifugation at 150 g for 10 min, washed platelets (WP) were resuspended with suspension buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM $MgCl_2$ $6H_2O$, 10.0 mM HEPES, 5.0 mM dextrose, 12.0 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.3% bovine serum albumin, 1 mM $CaCl_2$, pH 7.4). The number of cells in PRP and WP was adjusted to 3.8×10^8 cells/ml.

Measurement of platelet aggregation and serotonin release

Platelet aggregation was measured turbidimetrically at 37°C using a lumi-aggregometer (Chrono-Log Co., USA). PRP was pretreated with LB30057 or saline, and thrombin (at concentrations which produced the maximum amount of aggregation) was added. The extent of aggregation was determined by measuring the increase in amplitude of the light transmission. The percentage of aggregation inhibition was calculated from the ratio of the extent of aggregation in samples with LB30057 compared to control samples.

Serotonin release was determined by the fluorimetric method. Platelets were treated with LB30057 for 3 min, and then thrombin was added. A 0.7 ml aliquot of platelets was mixed with 5 mM EDTA in ice, and centrifuged at 12,000 g for 2 min. The supernatant (0.7 ml) was mixed with 0.14 ml of 6 M trichloroacetic acid (TCA) and centrifuged at 12,000 g for 2 min. A 0.6 ml aliquot of TCA supernatant was mixed with 2.4 ml of the solution (0.5% *o*-phthalaldehyde in ethanol diluted 1:10 with 8 N-HCl), placed in a boiling water bath for 10 min, and then cooled in ice. The excess TCA was extracted with chloroform and fluorophore was measured at the wavelength of excitation (360 nm) and emission (475 nm). The results are expressed as percentage fractions of 0.08 unit/ml of thrombin.

Measurement of lactate dehydrogenase leakage

Leakage of lactate dehydrogenase (LDH) from platelets was measured by spectrophotometry. After various times of incubation, platelets were centrifuged. A 0.05 ml aliquot of resulting supernatant was added to 2 ml of Tris-EDTA-NADH buffer (56 mM Tris, 5.6 mM EDTA, 0.17 mM NADH, pH 7.4) and then incubated for 10 min at 37°C. After incubation, 0.2 ml of 14 mM pyruvate solution that had been preincubated at 37°C was added to the incubation vessel. The decrease of absorbance at 340 nm resulting from conversion of NADH to NAD^+ was measured. The extent of LDH leakage was expressed as the % of total enzyme activity measured from a control incubation lysed with 0.3% Triton X-100.

Measurement of cytosolic calcium in platelets

The methods for preparation of platelets loaded with calcium green-1 are the same as previously described (Lee *et al.*, 1999). Calcium green-1 loaded platelets in a fluorimeter cuvette were preincubated for 1 min and then pretreated with LB30057 for 5 min. Platelet aggregation was induced by addition of thrombin (0.1 U/ml) and then measured for 3 min at the excitation wavelength (506 nm) and the emission wavelength (533 nm). The fluorimeter was calibrated as follows: Washed platelets containing 1 mM calcium were treated with 4 mM EGTA and 30 μM Tris in order to chelate extracellular calcium. After lysing the platelets with digitonin, the fluorescence intensity was measured as F_{min}. $CaCl_2$ (4 mM) was then added to the cuvette and fluorescence intensity was measured as F_{max}. Cytosolic calcium concentration was calculated by the following equation; $[Ca^{2+}] = Kd\ (F - F_{min})/(F_{max} - F)$, Kd = 190 nM.

Measurement of vasorelaxation

Rats were euthanized by decapitation, the thoracic aorta between the aortic arch and diaphragm was rapidly removed and placed in cold Krebs-Ringer (KR) solution (pH 7.4, 115.5 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.026 mM

calcium-d sodium EDTA and 11.1 mM glucose). The aorta was cleaned of all fat and connective tissues and was cut into ring segments of 3~4 mm in length. The ring was mounted on stainless-steel hooks in 10 ml tissue baths filled with KR solution continuously gassed with 95% O_2/5% CO_2 at 37°C. Tension was measured isometrically using Grass FTOS force tranceducers (Grass Instrument Co., Quincy, MA) and was recorded using Acknowledge III computer program (BIOPAC system Inc., Goleta, CA). The rings were stretched gradually to an optimal resting tension at 1.0 g and equilibrated for 30 min. The aortic rings were treated with LB30057 for 5 min and then phenylephrine was added to precontract. After adding thrombin cumulatively, the relaxation of aortic rings was determined.

Statistical analysis

The means and the standard errors of means were calculated for all the experiment groups. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncans multiple range test, to determine whether the means differed significantly from each other or from the control. A p-value of <0.05 was used in all cases to specify significance.

RESULTS AND DISCUSSION

Pretreatment with LB30057 to platelet-rich plasma (PRP) isolated from human caused a concentration-dependent inhibition of thrombin-induced aggregation (Fig. 1). IC_{50} and IC_{100} values of LB30057 for thrombin-induced aggregation were 54 ± 4 nM and 96 ± 3 nM, respectively (Table I). However IC_{50} values of LB30057 for platelet aggregation induced by collagen and ADP were 81 μM and 303 μM, respectively, which reflects the pronounced specificity of LB30057 for thrombin. Compared to Ki value (0.38 nM) of LB30057 against thrombin activity (Oh *et al.*, 1998), IC_{50} value for platelet aggregation was much higher, suggesting that LB30057 are capable of binding to plasma protein in PRP.

Serotonin release from dense granules occurs following platelet aggregation by agonists. Treatment with LB30057 to PRP decreased thrombin-induced serotonin secretion (Fig. 2) in a concentration-dependent manner. These data are consistent with IC_{50} value of platelet aggregation in PRP as shown in Table I and indicate that inhibition of platelet aggregation by LB30057 is accompanied by suppression of serotonin release from dense granules.

Cytosolic calcium levels increases significantly following platelet aggregation by agonists such as thrombin (Authi, 1992). Fig. 3 shows that thrombin challenge to platelets

Table I. Effects of LB30057 on agonist-induced aggregation in platelet-rich plasma isolated from human

Agonist	PRP	
	IC_{50} (μM)	IC_{100} (μM)
Thrombin	0.054 ± 0.004	0.096 ± 0.003
Collagen	81 ± 10	149 ± 8
ADP	303 ± 36	536 ± 40

Platelets rich plasma (PRP) isolated from human blood was incubated with saline (vehicle) or LB30057 at 37°C for 3 min and then each agonist was added. Concentrations of thrombin, collagen, and ADP in PRP system were 0.6 U/ml, 1.5 μg/ml, and 10 μM, respectively. Data represent means ± SEM (n = 3).

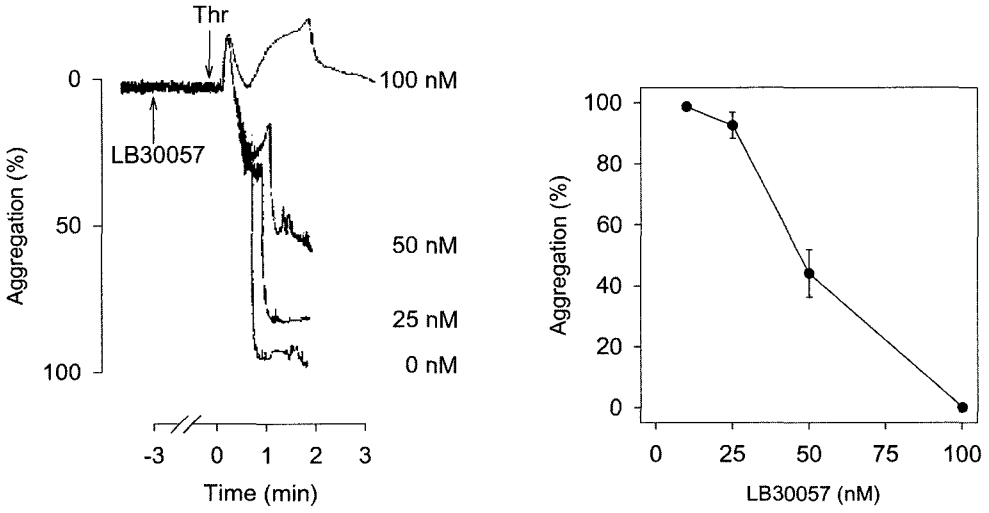

Fig. 1. Effect of LB30057 on thrombin-induced aggregation in human platelet-rich plasma. Platelet rich plasma (PRP) isolated from human was preincubated with saline (vehicle) or various concentrations of LB30057 for 3 min at 37°C. Thrombin (Thr; 0.6 U/ml) was added at time 0 to initiate platelet aggregation. Changes in light transmission were detected by lumiaggregometer. Data are the representative tracings of 3-5 independent experiments.

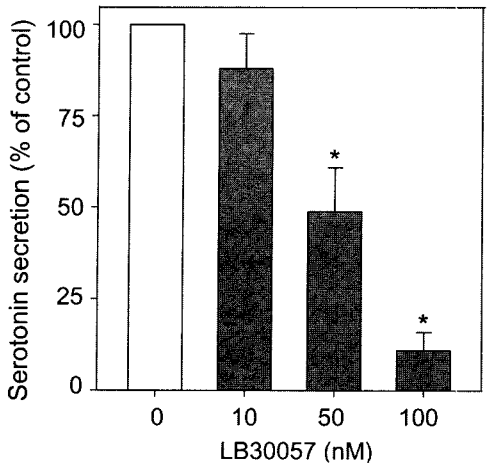

Fig. 2. Effect of LB30057 on thrombin-induced serotonin secretion from human PRP. PRP was incubated with saline (vehicle) or LB30057 at 37 °C for 3 min prior to addition of thrombin (0.08 U/ml), and then the reaction was stopped by addition of 5 mM ice-cold EDTA. The extent of serotonin secretion was determined as described in Methods section. Data represent means ± SEM of 3 independent experiments. *represent significant differences from thrombin alone ($p<0.05$).

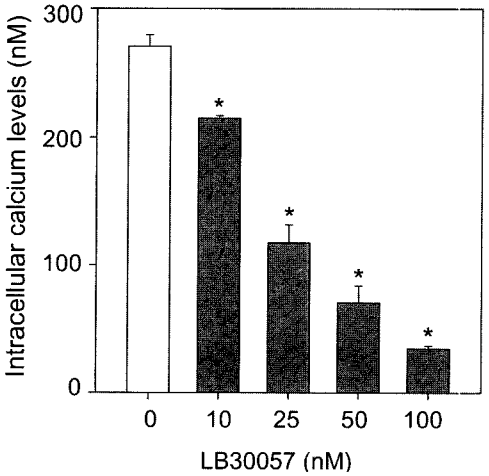

Fig. 3. Effect of LB30057 on thrombin-induced intracellular calcium level in human platelets. Platelets isolated from human were loaded calcium green-1 dye, and then preincubated with saline (vehicle) or LB30057 for 3 min at 37°C followed by addition of thrombin (0.08 U/ml). Data represent means ± SEM of 3 independent experiments. *represent significant differences from thrombin alone ($p<0.05$).

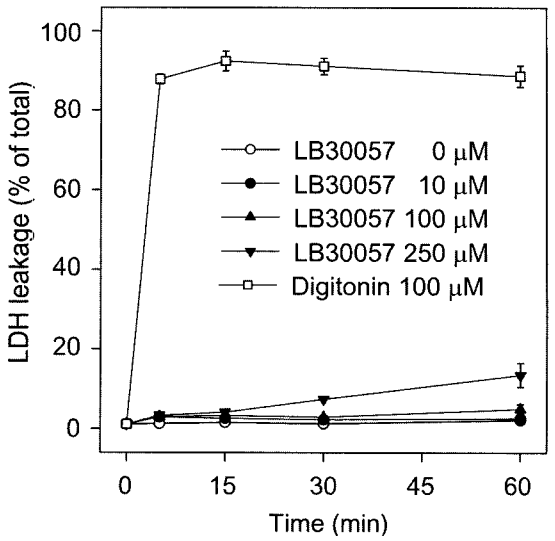

Fig. 4. Effect of LB30057 on lactate dehydrogenase (LDH) release from platelets. WP was incubated with saline (vehicle) or LB30057 at 37°C. Cytotoxicity, as determined by LDH leakage, was assessed at various time points. Digitonin (100 μM) was used as a positive control. Data represent means ± SEM of 3 independent experiments.

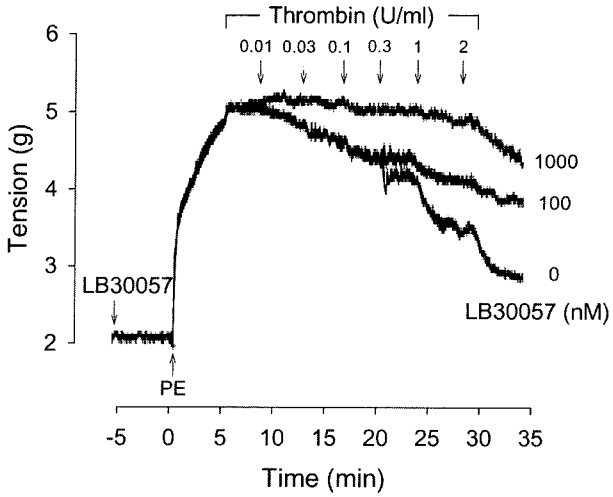

Fig. 5. Inhibitory effect of LB30057 on thrombin-induced relaxation in rat aortic rings. Aortic rings isolated from rats were incubated with saline (vehicle) or LB30057 at 37°C for 5 min, and then phenylephrine (PE) was added to precontract. Thrombin was then cumulatively added to relax the blood vessels. Data are the representative tracing of 3 independent experiments.

resulted in significant elevation of cytosolic calcium levels. LB30057 treatment, however, decreased thrombin-induced cytosolic calcium levels in a concentration dependent manner. These results suggest that LB30057 inhibited thrombin-induced calcium levels in platelets, possibly leading to suppression of platelet aggregation. Whether LB30057 was cytotoxic to platelets or not, LDH leakage from cells was determined (Fig. 4). Digitonin as a positive control demonstrated significant cytotoxicity in platelets. On the other hand, LB30057 up to 250 μM did not induce cytotoxicity at all, suggesting that overall inhibition by LB30057 was not due to cytotoxicity to platelets.

It has been demonstrated that thrombin itself induced receptor-mediated vasorelaxation of aortic rings isolated from rats (Eidt *et al.*, 1989). The rat aortic rings in organ bath system were pretreated with LB30057, and then thrombin was cumulatively added. A representative dose-

dependent curve was shown in Fig. 5. LB30057 inhibited vasorelaxation induced by thrombin in a concentration dependent manner, suggesting LB30057 could interfere the signaling pathway mediated by thrombin in rat blood vessels as well.

In platelets, thrombin induced serotonin release followed by platelet aggregation. Serotonin is stored in dense granules of resting platelets, and is released after platelet activation (Vanhoutte and Houston, 1985). Serotonin itself is a strong vasoconstrictor and potentiates platelet aggregation induced by other pro-aggregatory factors (Van Nueten *et al.*, 1984). Our results showed that LB30057 inhibited thrombin-induced serotonin release from dense granules in a concentration-dependent manner (Table I). Consistent with this finding, treatment with LB30057 suppressed thrombin-induced platelet aggregation to a similar extent (Fig. 1). These data suggest that LB30057 inhibited not only platelet aggregation as a popular marker but also interative events induced by degranulation of serotonin.

Recently, argatroban and melagatran have been developed as a direct thrombin inhibitor. Argatroban is used parenterally for the treatment of thrombosis in Japan (Matsuo *et al.*, 1997). LB30057 was about 100 times more potent than argatroban, when Ki value for thrombin was compared *in vitro* (0.38 nM vs 30 nM). On the other hand, melagatran could be administrated orally, but LB30057 inhibited thrombin about 5 times more potent than melagatran (Ki = 2 nM) (Gustafsson *et al.*, 1998). However, the potency of inhibition of platelet aggregation *in vivo* among these three compounds may be dependent upon their capabilities of protein binding in plasma. This premise is currently under investigation. In any case, our unpublished data suggest that significant fractions of LB30057 tend to bind to plasma protein within a short period of time in PRP system.

In conclusion, LB30057 is a potent, reversible and competitive inhibitor of thrombin that demonstrates higher selectivity over other biologically important serine proteases. *In vitro* studies show that LB30057 is also a potent inhibitor of platelet activation and vessel relaxation mediated by thrombin. Our data indicate that LB30057 could be served as a direct thrombin inhibitor for the treatment of various thrombotic disorders.

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REFERENCES

Authi, K. S., Localization of the [^{32}P]IP3 binding site on human platelet intracellular membranes isolated by high-voltage free-flow electrophoresis. *FEBS Lett.*, 298, 173-176 (1992).

Becker, R. C., Thrombin antagonists and antiplatelet agents. *Am. J. Cardiol.*, 69, 39A-51A (1992).

Eidt, J. F., Allison, P., Noble, S., Ashton, J., Golino, P., McNatt, J., Buja, L. M. and Willerson, J. T., Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. *J. Clin. Invest.*, 84, 18-27 (1989).

Gustafsson, D., Antonsson, T., Bylund, R., Eriksson, U., Gyzander, E., Nilsson, I., Elg, M., Mattsson, C., Deinum, J., Pehrsson, S., Karlsson, O., Nilsson, A. and Sorensen, H., Effects of melagatran, a new low-molecular-weight thrombin inhibitor, on thrombin and fibrinolytic enzymes. *Thromb. Haemost.*, 79, 110-118 (1998).

Hirsh, J., Heparin. *N. Engl. J. Med.*, 324, 1565-1574 (1991).

Hughes, P. E. and Pfaff, M., Integrin affinity modulation. *Trends Cell Biol.*, 8, 359-364 (1998).

Hursting, M. J., Alford, K. L., Becker, J. C., Brooks, R. L., Joffrion, J. L., Knappenberger, G. D., Kogan, P. W., Kogan, T. P., McKinney, A. A. and Schwarz, R. P., Jr., Novastan (brand of argatroban): a small-molecule, direct thrombin inhibitor. *Semin. Thromb. Hemost.*, 23, 503-516 (1997).

Kelton, J. G., Sheridan, D., Santos, A., Smith, J., Steeves, K., Smith, C., Brown, C. and Murphy, W. G., Heparin-induced thrombocytopenia: laboratory studies. *Blood*, 72, 925-930 (1988).

Lee, S. K., Lee, J. Y., Lee, M. Y., Chung, S. M. and Chung, J. H., Advantages of calcium green-1 over other fluorescent dyes in measuring cytosolic calcium in platelets. *Anal. Biochem*, 273, 186-191 (1999).

Matsuo, T., Koide, M. and Kario, K., Development of argatroban, a direct thrombin inhibitor, and its clinical application. *Semin. Thromb. Hemost.*, 23, 517-522 (1997).

Oh, Y. S., Yun, M., Hwang, S. Y., Hong, S., Shin, Y., Lee, K., Yoon, K. H., Yoo, Y. J., Kim, D. S., Lee, S. H., Lee, Y. H., Park, H. D., Lee, C. H., Lee, S. K. and Kim, S., Discovery of LB30057, a benzamidrazone-based selective oral thrombin inhibitor. *Bioorg. Med. Chem. Lett.*, 8, 631-634 (1998).

Ripka, W. C., New thrombin inhibitors in cardiovascular disease. *Curr. Opin. Chem. Biol.*, 1, 242-253 (1997).

Ross, R., The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, 362, 801-809 (1993).

Turpie, A. G., Weitz, J. I. and Hirsh, J., Advances in antithrombotic therapy: novel agents. *Thromb. Haemost.*, 74, 565-571 (1995).

Van Nueten, J. M., Leysen, J. E., de Clerck, F. and Vanhoutte, P. M., Serotonergic receptor subtypes and vascular reactivity. *J. Cardiovasc. Pharmacol.*, 6 Suppl 4, S564-S574 (1984).

Vanhoutte, P. M., 5-hydroxytryptamine and vascular disease. *Fed. Proc.*, 42, 233-237 (1983).

Vanhoutte, P. M. and Houston, D. S., Platelets, endothelium, and vasospasm. *Circulation*, 72, 728-734 (1985).

Weitz, J. I., Biological rationale for the therapeutic role of specific antithrombins. *Coron. Artery Dis,*. 7, 409-419 (1996).

Zimmerman, G. A., Elstad, M. R., Lorant, D. E., McIntyre, T. M., Prescott, S. M., Topham, M. K., Weyrich, A. S. and Whatley, R. E., Platelet-activating factor (PAF): signalling and adhesion in cell-cell interactions. *Adv. Exp. Med. Biol.,* 416, 297-304 (1996).