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(Citation)
The Kobe journal of the medical sciences,26(1):47-60

(Issue Date)
1980-03

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

Kobe J. Med. Sci. 26, 47 - 60, March 1980

EFFECT OF A POTENT THROMBIN INHIBITOR, NO. 407, ON PLATELET FUNCTION IN VITRO AND IN VIVO#

Hiroto HARA*, Yoshikuni TAMAO*, Ryoji KIKUMOTO*,
Yoshinori FUNAHARA**, Akiko HIJIKATA** and Shosuke OKAMOTO**

*Central Research Laboratories
Mitsubishi Chemical Industries Ltd.

**1st Department of Physiology
Kobe University School of Medicine

INDEXING WORDS

synthetic thrombin-inhibitor; arginine derivative; platelet; collagen; ADP; thrombin; DIC

SYNOPSIS

No. 407 (N^{α}-(6,7-dimethoxynaphthalene-2-sulfonyl)-L-Arginyl-N-(2-methoxyethyl)glycine) is a new thrombin inhibitor that shows a potent and highly selective inhibitory effect. The effect of No. 407 on platelet function was studied in vitro and in vivo. No. 407 showed a potent inhibitory action on thrombin-induced platelet aggregation with I_{50} of 0.2 μM. On collagen-induced platelet aggregation and secondary aggregation of guinea-pig platelets, No. 407 was found to be a weak inhibitor, but no inhibition was observed on ADP- or arachidonate-induced platelet aggregation. No. 407 showed a weak inhibition of platelet adhesiveness to glass beads and clot retraction. At doses of 12.5 mg/kg s.c. and above, the micropuncture bleeding time in mice was prolonged significantly, and the prolongation was also observed at oral doses of 300 mg/kg and above. No. 407 prevented the decrease in platelet count

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This will be a part of the dissertation of Hiroto HARA submitted to Kobe University School of Medicine for the requirement of Doctor of Medical Sciences.

Received for publication October 12, 1979

Authors' names in Japanese: 原 啓人　玉尾嘉邦　菊本亮二　船原芳範
土方明子　岡本彰祐

potently in the experimental DIC caused by thrombin infusion. Its effective plasma level was about 0.2 μM. These findings imply that No. 407 may be a useful antithrombotic drug and, especially, in DIC.

INTRODUCTION

Thrombin participates in the clotting of blood and also the aggregation of platelets. We aimed at a low molecular weight thrombin inhibitor as new antithrombotic drug, and have synthesized many N^{α}-arylsulfonyl arginine derivatives. First candidates, No. 189 and No. 205, have both potent thrombin inhibitory effects, in vitro and in vivo, and high specificity for thrombin.[5,8,12,13,14,19] However, these compounds are not applicable for the antithrombotic drug because of their high toxicities. Many attempts to lower the toxicity were made, and a compound, No. 407, that had not only high anti-thrombin activity but also low toxicity was selected.[15] No. 407 showed the thrombin inhibitory effect. Its I_{50} value for thrombin time was 0.3 μM, but it was less inhibitory to other coagulation enzymes and some serine proteases. In acute toxicity study, No. 407 was found to be less toxic, showing LD_{50}'s in mice of 377 mg/kg i.v. and 890 mg/kg i.p. Therefore, No. 407 was selected as a candidate for the development of an antithrombotic drug among many arginine derivatives.

Salzman and Chambers showed in 1964 that ADP-induced platelet aggregation was inhibited by a group of synthetic substrates for esterases.[18] After this observation, several studies on the inhibitory effects of synthetic substrates or inhibitors of proteases on platelet function were made.[1,4,7,9,16] These compounds inhibited collagen-induced platelet aggregation and the secondary wave of ADP-induced platelet aggregation at the concentration of 10^{-5} to 10^{-3} M. The inhibition of aggregation was accompanied by that of the release of platelet serotonine. The possible participation of proteases in platelet aggregation has been explored through these studies.

In this paper, we describe the effect of No. 407 on the platelet function in vitro and in vivo.

MATERIALS AND METHODS

Materials.

Thrombin (Bovine, topical) was obtained from Mochida Pharm. and stored as a 100 u/ml solution in 50% glycerol Tris buffered saline (TBS: 0.05 M Tris-HCl, pH 7.2, 0.9% NaCl) at -20°C. Immediately before use, thrombin was diluted in saline to a desired concentration. Adenosine-5'-diphosphate (ADP, Grade I), collagen (from bovine achilles tendon) and arachidonic acid (Grade I) were purchased from Sigma Chem. ADP was dissolved in TBS. Arachidonic acid was dissolved in water containing a small amount of 1 N NaOH and stored as a 5 mg/ml solution at 4°C. This solution was diluted in TBS to a desired concentration before use. Collagen was suspended in saline with Polytron homogenizer, and the suspension was diluted in TBS before use. No. 407 was dissolved in TBS or saline.

Preparation of Platelet-Rich Plasma (PRP).

Rabbits Male Japanese white rabbits (1.8 - 2.5 kg body weight) were anesthetized with sodium pentobarbital and blood was collected from the carotide artery through a polyethylene cannula into 0.1 volume of 3% sodium citrate. Platelet-rich plasma (PRP) obtained by the low speed centrifugation was adjusted to 5×10^5 platelets/mm^3 with platelet-poor plasma (PPP) which was obtained by the high speed centrifugation. Rabbit washed platelets were prepared by gel filtration by the column (2.5 x 15 cm) of sepharose 2B.

Guinea-Pigs Blood was withdrawn into 0.1 volume of 3.8% sodium citrate with polypropylene syringe from the vena cava of male guinea-pigs (300 - 400 g body weight) anesthetized with ethyl ether. PRP and PPP were obtained by the differential centrifugation and platelet count in PRP was adjusted to 4×10^5 platelets/mm^3 with PPP.

Platelet Aggregation.

Washed platelets (WP) were used in thrombin-induced platelet aggregation and platelet-rich plasma (PRP) in others. 0.8 ml aliquot of PRP (or WP) was added to a cuvette containing 0.1 ml of 10 mM $CaCl_2$-saline and 0.05 ml of No. 407 in Tris-buffered saline. The mixture was stirred at 1300 rpm in Bryston aggregometer with continuous recording of light transmission. After the incubation

for 3 minutes, 0.05 ml aliquot of platelet aggregation inducer was added. The final concentrations of the inducers were as follows: thrombin, 0.06 NIH u/ml; ADP, 1 μM (in rabbit platelets), 0.3 μM (in guinea-pig platelets); collagen, 15 μg/ml; arachidonate, 0.04 mM. Temperature in test system was 37°C in thrombin-, collagen- and arachidonate-induced aggregation and 25 - 26°C in ADP-induced aggregation.

Platelet Adhesiveness to Glass Beads.

The mixture of 2 ml of rabbit citrated blood and 20 μl of No. 407 was incubated at 37°C for 3 minutes and passed through a glass beads column (containing 1.3 g glass beads, Eiken Kizai) in 50 ± 4 sec. The platelet counts before and after passing through glass beads were determined, and platelet adhesiveness was calculated. In this experiment, the normal adhesiveness of rabbit platelets was 50 - 70%.

Clot Retraction.

The mixture of 2 ml of rabbit blood and 20 μl of No. 407 in saline was put into a glass tube which had a clot supporting rod and incubated at 37°C for 30 minutes. After incubation, the clot supporting rod with blood clot was drawn up carefully. Volume of serum remaining in the tube was measured, and the value of clot retraction was calculated.

Micropuncture Bleeding Time of Mouse Mesentric Vein.

Micropuncture bleeding time in mice was determined according to the method of Harrmann et al.[3] No. 407 in saline was injected either subcutaneously at the back or orally (0.1 ml/10 g body weight). The determination of bleeding time was started from 30 minutes after the administration.

DIC Caused by Thrombin Infusion in Rabbits.

Disseminated intravascular coagulation (DIC) was caused by thrombin infusion in rabbits and the decrease in platelet count was determined. Male rabbits, 2.0 - 2.2 kg, were used for this experiment and anesthetized by subcutaneous injection of urethane, 1.5 g/kg. The femoral veins were exposed and thrombin solution was infused into a femoral vein at a rate of 10.5 NIH units/kg/min (1.5 ml/min) for 4 minutes. No. 407 in saline was infused continuously into another femoral vein from either 5 minutes before

or 2 minutes after the start of thrombin infusion. Blood was collected from carotid artery through a polyethylene cannula. Platelet count in blood was determined according to the method of Brecker and Cronkite.[2)]

RESULTS

In Vitro Platelet Aggregation Studies.

Rabbit Platelets Results of the inhibitory effects of No. 407 on platelet aggregation induced by various agents are summarized in Table 1. Platelet aggregation induced by thrombin was potently inhibited when No. 407 was added at a final concentration as low as 10^{-7} M order to washed platelet suspension. Weak inhibition was seen on collagen-induced platelet aggregation. The I_{50} value for the latter platelet aggregation was 600 μM. But neither ADP- nor arachidonate-induced platelet aggregation was affected by No. 407.

Table 1 Effect of No. 407 on rabbit platelet aggregation in vitro.

Inducer of Platelet Aggregation	Drugs	I_{50}
Thrombin (0.06 NIH u/ml)	No. 407	0.2 μM
	Heparin	0.07 u/ml
Collagen (15 μg/ml)	No. 407	600 μM
	Aspirin	40 μM
ADP (1 μM)	No. 407	>1000 μM
Arachidonate (0.04 mM)	No. 407	>1000 μM
	Indomethacin	0.5 μM

Guinea-Pig Platelets When ADP was added to PRP prepared from the blood of guinea-pig, a two phase aggregation was observed as shown in Fig. 1. At 500 μM of No. 407, platelet aggregation was increased. But No. 407 completely inhibited only the secondary phase of aggregation at 1000 μM.

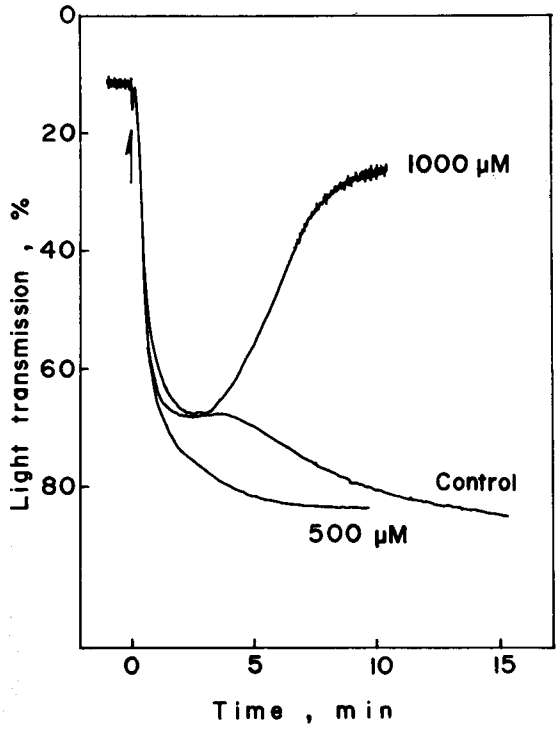

Fig. 1 Inhibitory effect of No. 407 on ADP-induced aggregation of guinea-pig platelets. ADP (0.4 μM) was added at the 0 time indicated by the arrow.

Effect on Platelet Adhesiveness.

Platelet adhesiveness is regarded as a prior reaction to platelet aggregation and the first step of the thrombi formation. To determine the effect of No. 407 on platelet adhesiveness, we examined it with the glass beads column method. As shown in Table 2, No. 407 scarcely inhibited the adhesion of platelet to glass beads.

Effect on Clot Retraction.

After the blood clotting, a phenomenon that blood clot contracts and forces serum out, namely clot retraction, is observed. Table 2 shows the effect of No. 407 on clot retraction. No. 407 did not show any effect at 1 μM and 10 μM, but produced 68%

inhibition at 100 μM. This 68% inhibition, however, may not have been caused by inhibition of the platelet function by No. 407, but by its inhibition of blood coagulation factors because there is a possibility that the decrease in clot retraction is caused secondarily by a prolongation of blood clotting time and a decrease in the amount of generated fibrin.

Table 2 Effect of No. 407 on platelet adhesiveness and clot retraction.

No. 407 (μM)	% Inhibition*	
	Adhesiveness	Clot Retraction
1	18.2 ± 6.01	25.2 ± 9.32
10	21.8 ± 10.3	18.7 ± 8.98
100	37.0 ± 0.56	68.6 ± 17.4

* Results are the mean ± S.D. of 3 experiments.

Micropuncture Bleeding Time in Mice.

In minor bleeding, the bleeding time depends on the activity of platelet function. Table 3 shows the effect of No. 407 on micropuncture bleeding time in mice. No. 407 significantly prolonged the bleeding time at subcutaneous doses of 12.5 mg/kg and 25 mg/kg. In these experiments, the plasma levels of No. 407 in each dose were presumed 20 μM and 40 μM, respectively. Since it was demonstrated that No. 407 could be absorbed at oral administration,[10] the bleeding time by the oral route was measured. After oral administration of No. 407, the bleeding time was significantly prolonged at doses of 300 mg/kg and 1000 mg/kg.

DIC Caused by Thrombin Infusion in Rabbits.

When thrombin was infused into femoral vein of rabbits at a rate of 10.5 NIH u/kg/min for 4 minutes, platelet count in blood rapidly decreased and reached 30 - 40% at 5 minutes after the start of thrombin infusion. After the end of the thrombin infusion, platelet count gradually returned to 60 - 70%. The effects of No. 407 were examined in this experimental DIC. As shown in Fig. 2, when No. 407 was continuously infused from 5 minutes before the thrombin infusion, the decrease in platelet count

Table 3 Micropuncture bleeding time following administration of No. 407 in mice.

Dose (mg/kg)	Route	n	Bleeding Time* (sec.)	P Value
None	——	14	15.7 ± 0.27	——
6.25	s.c.	6	16.7 ± 0.31	<0.05
12.5	s.c.	6	19.4 ± 0.98	<0.01
25.0	s.c.	5	29.2 ± 1.05	<0.01
None	——	20	18.5 ± 0.93	——
100	p.o.	8	21.1 ± 2.65	<0.05
300	p.o.	8	24.5 ± 5.11	<0.05
1000	p.o.	10	28.9 ± 8.66	<0.01

* Results are the mean ± S.E. of n experiments.

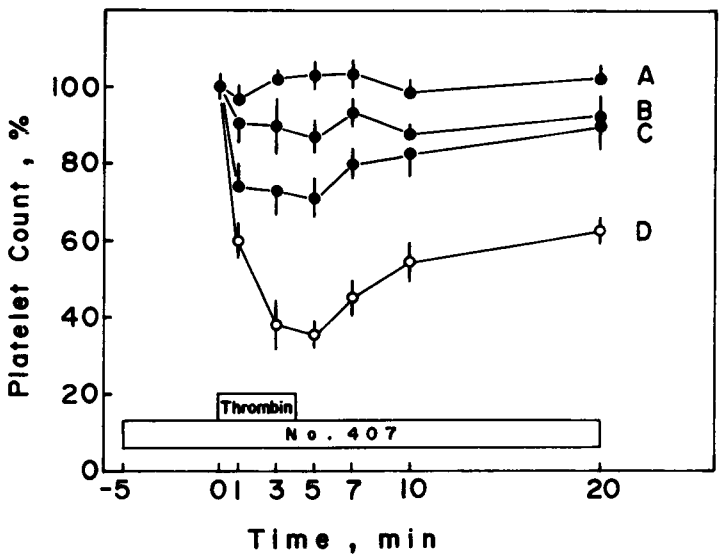

Fig. 2 Suppressing effect of No. 407 on the decrease in platelet count by thrombin infusion in rabbits. No. 407 was infused continuously from 5 minutes before the start of thrombin infusion at the following rates. A: 75 μg/kg/min, B: 15 μg/kg/min, C: 3.75 μg/kg/min, D: control. Results are the mean ± S.E.

caused by thrombin was prevented almost completely at doses of 75 μg/kg/min and 15 μg/kg/min. At 3.75 μg/kg/min, inhibition of the platelet count decrease was about 50%. The presumed plasma levels of No. 407 in each dose were 4 μM, 0.8 μM and 0.2 μM, respectively. When No. 407 was continuously infused from 2 minutes after the thrombin infusion, platelet count which had decreased for the first two minutes rapidly recovered (Fig. 3).

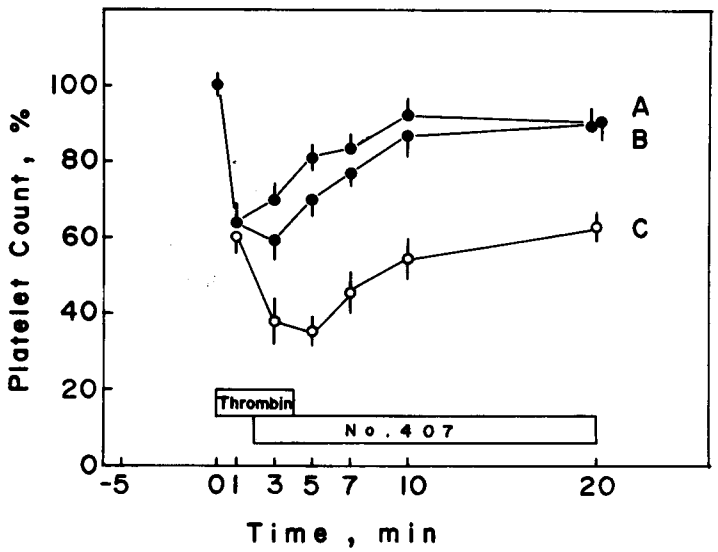

Fig. 3 Effect of No. 407 infusion started from 2 minutes after thrombin infusion on the decrease in platelet count by thrombin infusion. No. 407 was infused at the following rates. A: 75 μg/kg/min, B: 15 μg/kg/min, C: control. Results are the mean ± S.E.

DISCUSSION

No. 407 is a potent and highly selective inhibitor of thrombin. We studied the effects of No. 407 on the platelet function. On platelet aggregation, when thrombin was used as aggregation inducer, No. 407 showed a potent inhibitory action. Its I_{50} value was 0.2 μM. I_{50} value for collagen-induced platelet aggregation was 600 μM, and the inhibition on the secondary aggregation of guinea-pig platelets was also shown in the concentration of

above 500 μM. This inhibitory effect of No. 407 on collagen-induced platelet aggregation and the secondary wave of ADP-induced platelet aggregation was nearly equal to those of some other inhibitors of proteases.[1,4,7,9,16,18] But, on thrombin-induced platelet aggregation, No. 407 showed much more potency than these protease inhibitors. In regard to the inhibition by these protease inhibitors of collagen-induced platelet aggregation or release reaction, Pickett et al. suggested that protease inhibitors inhibit platelet aggregation by blocking a platelet trypsin-like protease involved in the activation of phospholipase A_2 in platelet membrane.[17] No. 407 shows a weak inhibitory effect on trypsin and its I_{50} value is 310 μM (1 mM Benzoyl arginine p-nitroanilide as substrate). The order of this value is equal to that of the I_{50} for collagen-induced platelet aggregation, and so there is a possibility that No. 407 inhibits collagen-induced platelet aggregation by the inhibition of the trypsin-like protease. On the other hand, Aoki et al. showed that the inhibitory effects of protease inhibitors on collagen-induced platelet aggregation did not always depend on their inhibitory potencies on trypsin activity.[1] Therefore, this possible mechanism cannot explain all of the effects of No. 407 on collagen-induced platelet aggregation and release reaction. No. 407 may not inhibit the prostaglandin biosynthesis in which arachidonic acid is converted to cyclic endoperoxides and thromboxane A_2, potent inducers of platelet aggregation, because no inhibitory effect on arachidonate-induced platelet aggregation was shown by No. 407. On platelet adhesiveness and clot retraction, No. 407 was weakly inhibitory. Thus, No. 407 may not affect the adhesion of platelet to the injured vessel wall and the platelet contractile process.

In order to observe the antiplatelet action of No. 407 in vivo, the effects on micropuncture bleeding time and experimental DIC by thrombin infusion were examined. No. 407 prolonged this bleeding time significantly at plasma level of 20 μM and above. At oral administration, the prolongation of bleeding time was also observed. No. 407 showed the potent suppressing effect on the decrease in count of platelets in the experimental DIC caused by thrombin infusion. The results for this experiment indicated that a plasma level of 0.2 μM was effective on this DIC. Thus, this

effective plasma level was the same concentration as I_{50} values for thrombin time and thrombin-induced platelet aggregation. In the previous study, the continuous infusion of No. 189 suppressed the decrease in platelet count completely in this experimental DIC.[5] The plasma level at that dose was about 1 μM. In the additional experiments, the plasma level of 0.1 - 0.2 μM of No. 189 gave 50% inhibition of the decrease in platelet count. While, I_{50} values of No. 189 for thrombin time and thrombin-induced platelet aggregation were 0.3 μM and 0.08 μM, respectively. When these three values of No. 189, that is, I_{50}'s for thrombin time and thrombin-induced platelet aggregation and the effective plasma level in experimental DIC, were compared with those of No. 407, the similar tendency among these three values between No. 407 and No. 189 was noticed. Effect of heparin on the same experimental DIC was also examined (data not shown). At a dose of 1 u/kg/min, the decrease in platelet count was prevented by about 50%. This inhibitory effect of heparin was comparable to that of No. 407 at a plasma level of 0.2 μM. When heparin was infused continuously at 1 u/kg/min to rabbits, the plasma recalcification time was prolonged by 1.5 - 1.8 times that of the control. Clinically heparin is often injected to give double the blood coagulation time of the control for therapeutic purposes. Therefore, if plasma level of No. 407 can be kept at 0.2 μM, the equal therapeutic effect will be obtained. Furthermore, No. 407 at the plasma level as low as 10^{-7} M order would have influence on neither blood coagulation time nor bleeding time, and so it may be suggested that No. 407 has less hemorrhage tendency at the therapeutic range than heparin.

In this study, No. 407 showed the inhibitory effect on bleeding time and experimental DIC. It was also found that No. 407 inhibited some experimental thrombosis.[6,11] The formation of platelet thrombi was suppressed by the administration of No. 407 in these experiments, although the effective plasma levels of No. 407 ranged between 10^{-7} and 10^{-5} M. At this range of concentration, No. 407 inhibited thrombin-induced platelet aggregation, but not collagen- or other agents-induced platelet aggregation, in vitro. If platelet thrombi were formed by the similar mechanism to in vitro collagen-induced platelet aggregation, no inhibition of experimental thrombi formation by No. 407, a potent thrombin-

specific inhibitor, may be observed. Therefore, it seems likely that thrombin plays a greater role in platelet thrombi formation in vivo than ever expected from the results of in vitro platelet aggregation studies.

ACKNOWLEDGEMENT

We are indebted to Dr. S. Hattori, the director of Bio-Science Laboratory, Mitsubishi Chemical Industries Ltd., for his interest and encouragement.

REFERENCES

1. Aoki, N., Naito, K. and Yoshida, N. Blood 1978. 52. 1/12. Inhibition of platelet aggregation by proteases. Possible involvement of proteases in platelet aggregation.
2. Brecher, G. and Cronkite, E.P. J. Applied Physiol. 1950. 3. 365. Morphology and enumeration of human blood platelets.
3. Harrmann, R.G., Frank, J.D. and Marlett, D.L. Proc. Soc. Exp. Biol. Med. 1968. 128. 960/964. An in vivo technique for assessing the formation of a hemostatic platelet plug.
4. Henson, P.M., Gould, D. and Becker, E.L. J. Exp. Med. 1976. 144. 1657/1673. Activation of stimulus-specific serine esterases in the initiation of platelet secretion. I. Demonstration with organophosphorus inhibitors.
5. Hijikata, A., Okamoto, S., Mori, E., Kinjo, K., Kikumoto, R., Tonomura, S., Tamao, Y. and Hara, H. Thromb. Res. Suppl. II. 1976. 8. 83/89. In vitro and in vivo studies of a new series of synthetic thrombin-inhibitors.
6. Ikoma, H., Ohtsu, K., Tamao, Y., Kikumoto, R., Mori, E., Funahara, Y., and Okamoto, S. Kobe J. Med. Sci. 1980. 26. 33/45. Effect of a potent thrombin inhibitor, No. 407, on novel experimental thrombosis generated by acetic acid.
7. Jobin, F., Tremblay, F. and Morisseti, M. Thromb. Diath. Haemorrh. 1970. 23. 110/119. Platelet reactions and immune processes. III. The inhibition of platelet aggregation by chymotrypsin substrates and inhibitors.
8. Kikumoto, R., Okamoto, S., Tamao, Y. and Hijikata, A. XXIIth Annual Meeting, Inter. Commit. Thromb. Haemost. Sept.

1976, Kyoto. Proc. of Discuss. Group on Synthetic Substrates and Inhibitors of Coagulation and Fibrinolysis. p. 11. Structure and activity relationships of OM-inhibitors.

9. Kilburn, E.P. and Firkin, B.G. Thromb. Diath. Haemorrh. 1968. 20. 366/376. A study of human platelet esterases.
10. Oda, K., Ohtsu, K., Tamao, Y., Kikumoto, R., Hijikata, A., Kinjo, K. and Okamoto, S. Kobe J. Med. Sci. 1980. 26. 11/31. Comparison of plasma levels and excretory routes between No. 189 and No. 407, potent thrombin inhibitors.
11. Ohtsu, K., Tamao, Y., Kikumoto, R., Ikezawa, K., Hijikata, A. and Okamoto, S. Kobe J. Med. Sci. 1980. 26. 61/71. Effects of a potent thrombin inhibitor, No. 407, on DIC models.
12. Okamoto, S., Hijikata, A., Kinjo, K., Kikumoto, R., Ohkubo, K., Tonomura, S. and Tamao, Y. Kobe J. Med. Sci. 1975. 21. 43/51. A novel series of synthetic thrombin-inhibitors having extremely potent and highly selective action.
13. Okamoto, S., Hijikata, A., Ikezawa, K., Kinjo, K., Kikumoto, R., Tonomura, S. and Tamao, Y. Thromb. Res. Suppl. II. 1976. 8. 77/82. A new series of synthetic thrombin-inhibitors (OM-inhibitors) having extremely potent and selective action.
14. Okamoto, S., Hijikata, A., Kikumoto, R., Ikezawa, K. and Mori, E. XXIIth Annual Meeting, Intern. Commit. Thromb. and Haemost. Sept. 1976, Kyoto. Proc. of Discuss. Group on Synthetic Substrates and Inhibitors of Coagulation and Fibrinolysis. p.5. A new series of synthetic thrombin-inhibitors (OM-inhibitors) having extremely potent and highly selective activities.
15. Okamoto, S., Hijikata, A., Kikumoto, R. and Tamao, Y. XVII Congress of the International Society of Hematology, Abstracts (I). July 1978, Paris. P. 301. An improved synthetic thrombin inhibitor (No. 407) suitable for animal experiments.
16. Musterd, J.F., Packham, M.A. and Senyi, A. J. Lab. Clin. Med. 1967. 70. 1003/1004. The effect of DFP on platelet function in vitro and in vivo.
17. Pickett, W.C., Jesse, R.L. and Cohen, P. Biochem. J. 1976. 160. 405/408. Trypsin-induced phospholipase activity in human platelets.

18. Salzman, E.W. and Chambers, D.A. Nature. 1964. 204. 698/700. Inhibition of ADP-induced platelet aggregation by substituted amino-acids.
19. Yamada, K., Meguro, T., Hara, H. and Okamoto, S. Japanese J. Clin. Hemat. 1976. 17. 149. Effect of an arginine derivative on Factor XIII.