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EFFECTS OF A POTENT THROMBIN INHIBITOR, NO. 407, ON DIC MODELS#

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INDEXING WORDS

synthetic thrombin inhibitor; drug effect; animal model; DIC; endotoxin; lactic acid

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 effects of No. 407 on DIC were studied in several typical animal models. No. 407 was effective in preventing the local and generalized Shwartzman reactions at doses of 25 mg/kg s.c. and above, and endotoxin shock in mice at 50 mg/kg s.c. No. 407 was also effective in a lactic acid-induced pulmonary thrombosis in rats at doses of 10 mg/kg i.v. and above. These findings prove that No. 407 is substantially useful in the prevention of DIC.

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

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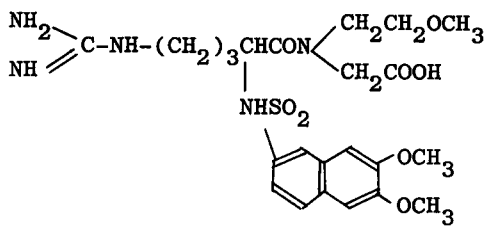
INTRODUCTION

Disseminated intravascular coagulation (DIC) occurs as a complication of basic underlying diseases such as malignant tumors, septicemia or obstetric complication[15] and DIC is recognized as the disease of damage of major organs (especially the kidney) and the skin and of bleeding accompanied by multiple coagulation defects. DIC is characterized by the intravascular formation and deposition of fibrin and platelet aggregates caused by the intravascular generation of thrombin, which is formed through the extrinsic pathway started from the entry of tissue thromboplastin into the circulation or the intrinsic pathway started from the activation of FXII.[16] It is well-known that deposition of fibrin formed by direct action of thrombin plays an important role in the development of DIC. On the other hand platelet aggregation induced by thrombin is also an unnegligible factor for the development of DIC. Another role of platelets in causing DIC is one for the acceleration in the coagulation system, besides platelet aggregation by thrombin.[16] Aggregated platelets provide the binding sites for FXa, which are presumed to consist of a FV-like lipoprotein. FXa bound to the platelet surface activates prothrombin 60,000 fold faster than FXa in solution, generating thrombin concentrated around platelet aggregates, and platelet aggregation proceeds further and further to make a bigger mass.[10] Fibrin is also generated around platelet aggregates by the action of thrombin and makes a network among them. Thus thrombin plays an important role in DIC generation through fibrin formation and platelet aggregation. Accordingly inhibitor of thrombin is considered to be effective in the prevention of DIC.

This report describes the preventive effect on DIC of a new potent and selective inhibitor of thrombin, N^{α}-(6,7-dimethoxynaphthalene-2-sulfonyl)-L-Arginyl-N-(2-methoxyethyl)glycine (No. 407, depicted below), which is one of low toxic compounds of a series of potent thrombin inhibitory arginine derivatives such as No. 189 and No. 205.[6,12] In this report, we selected the local and generalized Shwartzman reactions, endotoxin shock and a lactic acid-induced thrombosis as DIC models.



No. 407

MATERIALS AND METHODS

Local Shwartzman Reaction.

Effects of No. 407 on the local Shwartzman reaction were studied according to the method of Arman et al.[1] Male rabbits weighing 1.6 - 2.0 kg were shaved on the abdomen and intradermal preparatory injections of 50 μg each (0.5 ml, in 0.9% saline) of bacterial endotoxin (Lipopolysaccharide, E. Coli 0127:B8, Sigma Chem.) were made in each rabbit. Twenty-four hours later, the eliciting injections of 100 μg of the endotoxin (1 ml each, 0.9% saline) were made intravenously. No. 407 and indomethacin were dissolved in 0.9% saline in various concentrations adjusted to give a volume of 1 ml/kg of body weight and were injected subcutaneously at 30 minutes before and 6, 23.5 and 30 hours after the preparatory injection. Another 24 hours later, all rabbits were sacrificed and the lesion spots at endotoxin-injected sites were measured. The score of the local Shwartzman reaction (LSR) was calculated as follows:

Score of LSR = length (mm) x width (mm) x color value

Color value
- 0: no detectable response
- 1: pinkish
- 2: red
- 3: dark red
- 4: black

Generalized Shwartzman Reaction.

Male rabbits weighing 1.9 - 2.1 kg were injected intravenously

with two sequential doses (100 μg) of the endotoxin 24 hours apart. No. 407 was administered subcutaneously at 30 minutes before, and 6, 23.5 and 30 hours after the first endotoxin injection. Urea concentration in plasma, which was prepared by centrifugation from the citrated blood, was measured with "Urea Color Test" (Boeringer Manheim Yamanouchi). Weight of the kidneys was measured and the renal cortical necrosis was observed macroscopically.

Effect on Endotoxin Schock in Mice.

Male mice in a group of 7 weighing 22 - 25 g were given No. 407, aspirin or 0.9% saline (control group) subcutaneously 60 minutes before intravenous administration of endotoxin (E. Coli serotype No. 0127: B8, Difco.) in a dose of 25 mg/kg. Preventive ability against death was evaluated 24 hours after the endotoxin injection.[3)]

Effect on Lactic Acid-Induced Pulmonary Thrombosis in Rats.

Effects of No. 407 on a lactic acid-induced pulmonary thrombosis were studied according to the method of Tomikawa et al.[18)] Male wister rats weighing 260 - 280 g were anesthetized with pentobarbital (40 mg/kg body weight) by an intraperitoneal injection. Twenty minutes after anesthetization, 0.7 M lactic acid was infused via the femoral vein at the rate of 1.4 ml/100 g body weight/hr for 1 hour. The test drugs were injected intravenously at 5 minutes before the start of lactic acid infusion. Control animals received 0.9% saline in a similar way. Immediately after the stop of infusion, the lungs were removed and fixed with formaline solution for histological examination of thrombi. Paraffin sections were stained with hematoxylin-eosin. The severity of a lactic acid-induced thrombosis was represented as the number of total thrombi (above 25 micron diameter) counted in each of five sections from the lungs (superior lobe, middle lobe, post caval lobe, inferior lobe and left lung).

Incidence of thrombi (%):

$$\frac{\text{the number of thrombi}}{\text{the number of the vessels}} \times 100$$

Percent inhibition (%):

$$\left(1 - \frac{\text{Averaged incidence of thrombi in rats treated with drugs}}{\text{Averaged incidence of thrombi in untreated rats}}\right) \times 100$$

RESULTS

Local Shwartzman Reaction.

As shown in Table 1, it was found that No. 407 strongly prevented the local Shwartzman reaction at subcutaneous doses of 50 and 25 mg/kg, but not at 12.5 mg/kg. Indomethacin showed strong preventive effect as well.

Table 1 The effect of No. 407 and indomethacin on the local Shwartzman reaction.

Drug	Dose (mg/kg s.c.)	Score					
		Exp. 1		Exp. 2		Exp.3	
Control		3283	2761	3452	1724	1835	1119
No. 407	12.5					974	1257
	25			692	496	604	
	50	0	0	666	231		
Indomethacin	5	321		1366		0	

Generalized Shwartzman Reaction.

Table 2 The preventive effect of No. 407 on the generalized Shwartzman reaction.

Drug	Dose (mg/kg s.c.)	Renal cortical necrosis		Urea concentration in plasma ± S.E. (mg/ml)	Kidney weight ± S.E. (g)
		Frequency	(%)		
Control		7/7	100	2.00 ± 0.36	15.21± 1.25
No. 407	12.5	3/3	100	1.93 ± 0.24	16.33± 2.14
	25	2/6*	33	1.01 ± 0.19	13.72± 1.07
	50	1/4*	25	1.36 ± 0.41	11.63± 0.83

Data were analyzed by Fisher's exact method.
*: $P<0.05$ (versus control).

The effect of various doses of No. 407 is shown in Table 2. When No. 407 was given subcutaneously in doses of 25 or 50 mg/kg, the incidence of the generalized Shwartzman reaction was prevented.

No protective effect was observed by a dose of 12.5 mg/kg; the incidence of the reaction in this group was comparable to that in the control group. Moreover, observations were made on the increase in urea concentration in plasma and kidney weight which are considered to reflect the development of the generalized Shwartzman reaction. The results evaluated by these factors also confirmed the protective effect of No. 407 indicated by the ratio of incidence of the generalized Shwartzman reaction. Therefore it turned out that the degree of renal cortical necrosis in the generalized Shwartzman reaction could be determined by the measurement of urea concentration in plasma or kidney weight.

Effect on Endotoxin Shock in Mice.

No. 407 or aspirin was subcutaneously given to mice at doses of 25 and 50 mg/kg or at dose of 50 mg/kg respectively. As shown in Table 3, No. 407 substantially reduced mortality at a dose of 50 mg/kg. On the other hand, aspirin was not effective in preventing the endotoxin shock. It was observed that hypothermia caused by endotoxin injection was relatively prevented by No. 407.

Table 3 The effect of No. 407 and aspirin on endotoxin shock in mice.

Drug	Dose (mg/kg s.c.)	Mortality rate (24 hr)
Control		8/10
No. 407	25	7/10
	50	3/10*
Aspirin	100	6/10
	300	7/10

Data were analyzed by Fisher's exact method.
*: $P<0.05$ (versus control).

Effect on Lactic Acid-Induced Pulmonary Thrombosis in Rats.

No. 407 or aspirin was given to rats at intravenous doses of 10 and 40 mg/kg or at a dose of 50 mg/kg respectively before the lactic acid infusion. As shown in Table 4, No. 407 showed the decrease in the incidence of thrombi at a dose of 10 mg/kg as well as 40 mg/kg. The plasma concentration at this dose was presumed

Table 4 The preventive effect of No. 407 and aspirin on lactic acid-induced pulmonary thrombosis in rats.

Drug	Dose (mg/kg i.v.)	Incidence of thrombi ± S.E. (%)	Inhibition (%)
Control		24.2 ± 5.56 (5)	
No. 407	10	9.68 ± 1.57* (5)	60.0
	40	6.97 ± 1.75**(5)	71.2
Aspirin	50	9.68 ± 2.24 (5)	60.0

Data were analyzed by Student's t-test.
*: $P<0.05$, **: $P<0.02$ (versus control).
Figures in the parentheses are the number of rats used.

to be about 40 μM at the start of lactic acid infusion and 2.5 μM at the discontinuation of lactic acid infusion (unpublished observations). Aspirin also was effective in preventing thrombus formation, but it was less effective than No. 407.

In the microscopical observation of the slices from the lungs, the thrombi were found mainly in the veins and slightly in the arteries. The number of the formed thrombi differed among vessels of different sizes: a lot of thrombi were observed in the small vessels such as the arterioles or the capillaries. It was microscopically found that this thrombus consisted of fibrin only or platelets and fibrin, with a little contamination by erythrocytes or leucocytes. The other pathological changes in the lung were as follows: congestion and edema of the pulmonary parenchyma and extension of the alveolar duct. The changes in the pulmonary parenchyma were reduced in parallel with the inhibition of thrombus formation.

DISCUSSION

DIC is characterized by the intravascular formation and deposition of fibrin and platelet aggregates, which are precipitated by the intravascular generation of thrombin. No. 407 is a competitive thrombin inhibitor having extremely potent and highly

selective action with I_{50} of 0.3 μM. In addition, No. 407 shows potent inhibitory action to platelet aggregation induced by thrombin with I_{50} of 0.2 μM.[13] The results of this study indicated that No. 407 could markedly prevent some typical DIC models such as the local and generalized Shwartzman reactions, endotoxin shock or a lactic acid-induced pulmonary thrombosis in which fibrin clotting and platelet aggregation were involved.

No. 407 was effective on the local and generalized Shwartzman reactions at doses over 25 mg/kg s.c. Although it has been reported that the Shwartzman reactions result from the enhancement of platelet aggregation and blood coagulation system and are suppressed by heparin, warfarin and platelet aggregation inhibitors such as indomethacin and aspirin,[4,7,9,14] our results confirmed that the thrombin inhibitor, No. 407, suppressed the Shwartzman reactions. The effectiveness of heparin has been shown by its treatment at early stages within 4 hours after the provocation of endotoxin. The blood concentration of No. 407 was 40 μM at the provocation of endotoxin and 8 μM, 2 hours after the provocation.[11] Since I_{50} of No. 407 for in vitro clotting time was 0.3 μM, higher plasma concentration exceeding at least 10 times the I_{50} of No. 407 was required for the suppression of the reactions.

No. 407 prevented the mortality in endotoxin shock in mice significantly at 50 mg/kg s.c. Aspirin did not prevent the mortality rate at 24 hours, although it showed the prolongation of the survival time (data not shown). This result in aspirin is consistent with the reported results.[20] In the Shwartzman reaction aspirin did not suppress the reaction by the administration around the second endotoxin injection, but by the administration at the preparatory injection.[9] Conventional mice used in our experiment are considered to be sensitized more or less with intrinsic endotoxin. Therefore the endotoxin injection in the endotoxin shock experiment may correspond to the provocation injection of the Shwartzman reactions.[2,17] Ineffectiveness of aspirin on endotoxin shock may reflect the considerations described above.

A lactic acid-induced pulmonary thrombosis is accompanied by DIC and prevented by platelet aggregation inhibitors such as aspirin and ticlopidin and anticoagulants such as heparin.[19] The thrombi formed by this method consist of platelet aggregates

and fibrin. No. 407 showed 60% suppressive effect in this model at 10 mg/kg i.v. and the effect of No. 407 was higher than that of aspirin. The blood concentration of No. 407 was about 40 μM and 2.5 μM at the starting and ending of lactic acid infusion, respectively (data not shown). In this model, it was reported that heparin inhibited 50% at a dose of 2.5 mg/kg i.v. in which the whole blood clotting time was prolonged by more than twice during the experiment.[19] However, No. 407 showed the strong inhibitory effect at the concentration that it doubled the recalification time (about 5 μM, data not shown). On the other hand, it was observed that No. 407 was effective at lower plasma concentration (0.2 μM) in the experimental DIC caused by direct thrombin infusion.[5] This discrepancy in effective plasma concentrations may be due to the difference in mode of the generation of DIC. In addition, it was also found that No. 407 substantially inhibited the formation of the arterial platelet thrombi caused by acetic acid at 5 - 10 μM.[8]

As platelet aggregation is considered to play an important role in the formation of thrombi of these models, it seems that the antithrombin agent, No. 407, prevents thrombus formation effectively through its inhibitory action to platelet aggregation as well as its suppressive action to hypercoagulability. Moreover, it would be thought that thrombin plays an important role in the formation of platelet thrombi, because No. 407 inhibited strongly thrombin induced platelet aggregation, but weakly collagen, ADP or arachidonate induced platelet aggregation in vitro (I_{50} 600 μM, 1,000 μM, and 10,000 μM for collagen, ADP and arachidonate, respectively).[5]

As mentioned above, No. 407 is expected to be applicable to DIC in human use.

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