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New derivative of staphylokinase SAK-RGD-K2-Hirul exerts thrombolytic effects in the arterial thrombosis model in rats

Janusz Szemraj[1], Agnieszka Zakrzeska[2], George Brown[1], Adrian Stankiewicz[2], Anna Gromotowicz[2], Tomasz Grędziński[2], Ewa Chabielska[2]

[1]Department of Medical Biochemistry, Medical University of Łódź, Mazowiecka 6/8, PL 92-215 Łódź, Poland

[2]Department of Biopharmacy, Medical University of Białystok, Mickiewicza 2c, PL 15-222 Białystok, Poland

Correspondence: Janusz Szemraj, e-mail: jszemraj@csk.am.lodz.pl

Abstract:

SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul are recombinant proteins that are derivatives of r-SAK (recombinant staphylokinase). They are characterized by their fibrin-specific plasminogen activation properties and their antithrombin and antiplatelet activities. The difference between these proteins is the presence of the antithrombotic fragment (hirudin or hirulog) in the C-terminal portion of the r-SAK.

The aim of the present study was to examine the thrombolytic potentials of SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul in an electrically induced carotid artery thrombosis model in rats and to compare the potentials to that of r-SAK.

We determined that a bolus injection of SAK-RGD-K2-Hirul was more effective than one of r-SAK in the improvement and maintenance of carotid patency and in arterial thrombus weight reduction; however, it had the same potency as SAK-RGD-K2-Hir. The bleeding time, prothrombin time and activated partial thromboplastin time were significantly prolonged in the animals that were treated with either dose (1.5 or 3.0 mg/kg) of SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul, whereas no changes were observed in the plasma fibrinogen concentration or the α2 plasmin inhibitor level. r-SAK alone did not change the bleeding time or coagulation parameters.

In conclusion, our findings demonstrate the thrombolytic activity of intravenous bolus injection of the novel thrombolytic agent SAK-RGD-K2-Hirul in rats. Although this protein compares favorably with r-SAK, we were unable to show the presence of any beneficial effects of SAK-RGD-K2-Hirul over those of SAK-RGD-K2-Hir. Furthermore, our results suggest that high doses of SAK-RGD-K2-Hirul bear the risk of bleeding.

Key words:

recombinant protein, antiplatelet activity, thrombolytic agent, anticoagulant agent, arterial thrombosis

Abbreviations: APTT – activated partial thromboplastin time, BT – bleeding time, CR – cyclic reflow, FBF – final blood flow, IBF – initial blood flow, PO – persistent occlusion, PP – persistent patency, PT – prothrombin time, SAK-RGD-K2-Hir – recombinant protein containing the kringle 2 domain (K2), the Arg-Gly-Asp (RGD) peptide sequence and hirudin, SAK-RGD-K2-Hirul – recombinant protein containing the kringle 2 domain (K2), the Arg-Gly-Asp (RGD) peptide sequence and hirulog, r-SAK – recombinant staphylokinase, t-PA – tissue plasminogen activator, VEH – vehiculum

The contribution of first three autors are equivalent

Introduction

The current effective treatments of myocardial infarction and pulmonary embolism are based upon thrombolytic therapy. The most common scheme of thrombolytic therapy involves the activation of the fibrinolytic system by the intravenous infusion of plasminogen activators that convert plasminogen into the proteolytic en-

zyme plasmin and induce premature clot lysis [28]. Plasmin can also degrade circulating fibrinogen, which leads to "the lytic state" [7]. In addition, the therapeutic success rates do not exceed 70%. Therefore, it is important to identify thrombolytic agents that display a better thrombolytic potency, specific thrombolytic activity and improved fibrin selectivity [4, 30].

Staphylokinase (SAK, which is an extracellular protein that is produced by certain strains of *Staphylococcus aureus*) is a highly fibrin-specific plasminogen activator [32, 35]. Its high fibrin-specific properties have been known for more than fifty years. However, SAK has only recently attracted considerable attention in the search for an ideal thrombolytic agent [29]. Ideally, prolonged intravenous infusion regimens would be replaced with an intravenous bolus injection of newer and more specific thrombolytic drugs that display an active prolonged half-life. This possibility can be realized by the administration of recombinant SAK (r-SAK) in patients who have an acute myocardial infarction and who contain a demonstrated thrombolytic activity of this protein [1, 21].

During thrombolysis, a large amount of thrombin is released, which activates the coagulation system, promotes platelet activation, inhibits fibrinolysis and leads to vascular reocclusion [7, 30]. Therefore, the simultaneous administration of thrombolytics with antithrombotic and antiplatelet agents would be a logical step in the prevention of reocclusions [15, 33]. The large-scale application of molecular biological techniques in the 1980s initiated a dynamic development of research projects that were designed to "improve" the properties of natural proteins and to engineer new thrombolytic agents. These agents may consist of a thrombolytic portion to activate fibrinolysis, an antiplatelet portion to inhibit platelet aggregation and an antithrombotic peptide to inhibit thrombin activity. These features may potentially increase thrombolytic activity and simplify the administration of the agent to restore vessel patency.

We previously constructed the recombinant protein SAK-RGD-K2-Hir. This protein contains a fibrin-specific binding site of a tissue-type plasminogen activator, which is the kringle 2 domain (K2), an Arg-Gly-Asp (RGD) peptide sequence that prevents the aggregation of platelets and the hirudin antithrombotic agent that is located in the C-terminal portion of the r-SAK. We documented the thrombolytic potential of SAK-RGD-K2-Hir in an electrically induced carotid artery thrombosis model and a stasis induced venous thrombosis model in rats [24]. A weaker thrombolytic potency was observed in the arterial thrombosis model of SAK-RGD-K2-Hir due to the presence of hirudin (r-Hir) in the SAK-RGD-K2-Hir molecule, because SAK-RGD-K2 was the most effective treatment for maintaining carotid potency and reducing arterial thrombus mass.

Hirulog (20 amino acids in length) is a polypeptide analogue of hirudin (65 amino acids) that binds to thrombin at both the active site and the anion-binding exosite [18]. We hypothesized that the addition of the smaller-sized hirulog as a part of the recombinant protein would potentially increase the contact of hirulog (r-Hirul) with thrombin at the site of a thrombus. Therefore, we have constructed a new r-SAK variant, SAK-RGD-K2-Hirul, in which hirulog is fused to the C-terminal portion of r-SAK. Our *in vitro* studies have demonstrated that SAK-RGD-K2-Hirul is a fast-acting clot-dissolving agent that displays strong antithrombin, antiplatelet and fibrinolytic activities [14]. Our present *in vivo* study was designed to investigate the thrombolytic activity of SAK-RGD-K2-Hirul in an arterial thrombosis model in rats.

Materials and Methods

Agents

For the purpose of this study, we obtained the recombinant proteins SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir and r-SAK. The construction and production methods have been described previously [14, 25]. The proteins were dissolved in PBS (phosphate buffered saline, Sigma Aldrich, Poznań, Poland) before use.

Animals

Male Wistar rats (300–350 g) were used for this study. The animals were housed in a room with a 12 h light/dark cycle in cages that were appropriate for their size, and they were given tap water and fed a standard rat chow.

Twenty-four hours before the investigations began, the animals were deprived of food but allowed free access to water. All of the surgical and experimental protocols were approved by the local animal ethics committee in Białystok (Poland).

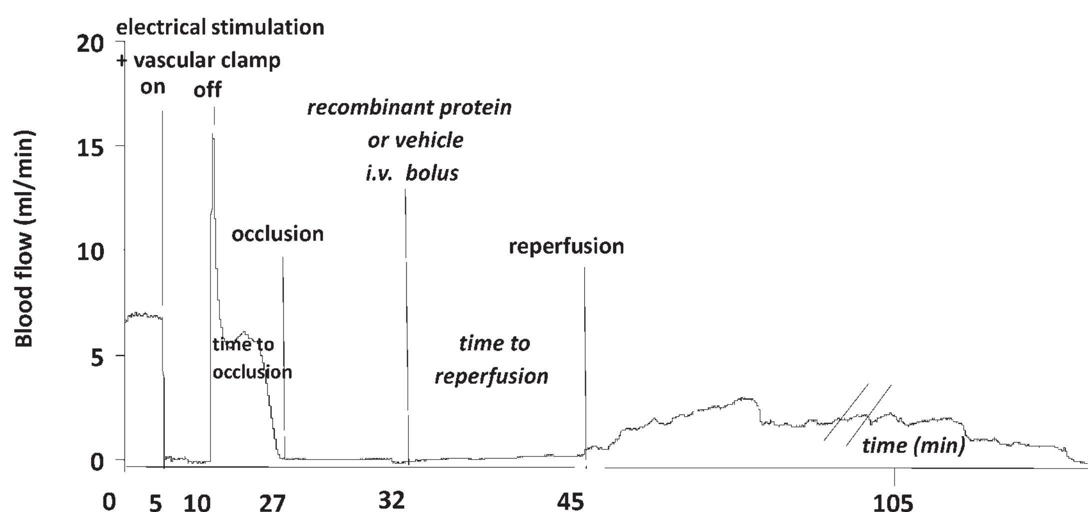

Fig. 1. Experimental protocol shown as a record of blood flow in a selected animal. Five minutes after occlusion, the recombinant proteins were administered by an intravenous (*iv*) bolus injection. The patency status of the carotid artery was monitored continuously for 75 min following the protein administration

Arterial thrombosis model

The rats were anesthetized by intraperitoneal injection of pentobarbital (Vetbutal, Biowet, Poland, 45 mg/kg) and then fixed on the operating table. Thrombosis was induced secondarily by electrical stimulation and injury of the endothelium as previously described [11, 23, 24]. The left common carotid artery was then separated from the surrounding tissue along the length of at least 20 mm. A hook-shaped stainless steel electrode was inserted into the left carotid artery. A tiny piece of parafilm "M" (5 mm × 20 mm) was placed under the electrode to provide electrical isolation, and the hook electrode was placed directly into the artery.

The second electrode was inserted subcutaneously in the abdominal region. Both of the electrodes were connected to a circuit with a constant current generator. A Doppler flow probe (1 mm-diameter, Hugo Sachs Elektronik – Harvard Apparatus GmbH, Germany) was placed in contact with the exposed artery downstream of the electrode and connected to a blood flowmeter (The HSE-TRANSONIC Transit Time Flowmeter, Germany). Blood flow was monitored continuously during the entire study.

The experimental protocol is shown as a record of the blood flow in a selected animal in Figure 1. Thrombosis was induced by electrical stimulation (2 mA/5 min), and a hemostatic clamp delivered this stimulation to the outer surface of the left common carotid artery. The hemostatic clamp was placed between the electrode and the Doppler flow probe, and the extent of thrombotic occlusion was expressed as a decrease in the carotid blood flow. Five minutes after the formation of an occlusive thrombus, the animals received the recombinant protein *via* a bolus injection (1.5 or 3.0 mg/kg) or VEH (3.0 ml/kg) in the femoral vein.

The carotid patency status was monitored continuously for 75 min after the injections were administered. The patency status from the beginning of the injection of the recombinant protein until the end of the observation period was schematically represented for each animal (Fig. 2). The carotid patency status was expressed according to one of the following classifications: 1) persistent occlusion (PO), no reflow; 2) cyclic reflow (CR), cyclic reflow and reocclusion after initial reflow; 3) persistent patency (PP), persistent flow without reocclusion after the initial reflow.

At the end of the study (75 min after the recombinant protein administration), blood samples were taken for the hemostatic analysis, and the thrombus was stored at room temperature for 24 h and weighed.

Template bleeding time

At the end of the experiment but prior to the removal of the arterial thrombus, the template bleeding time (BT) was measured according to Dejana et al. [8].

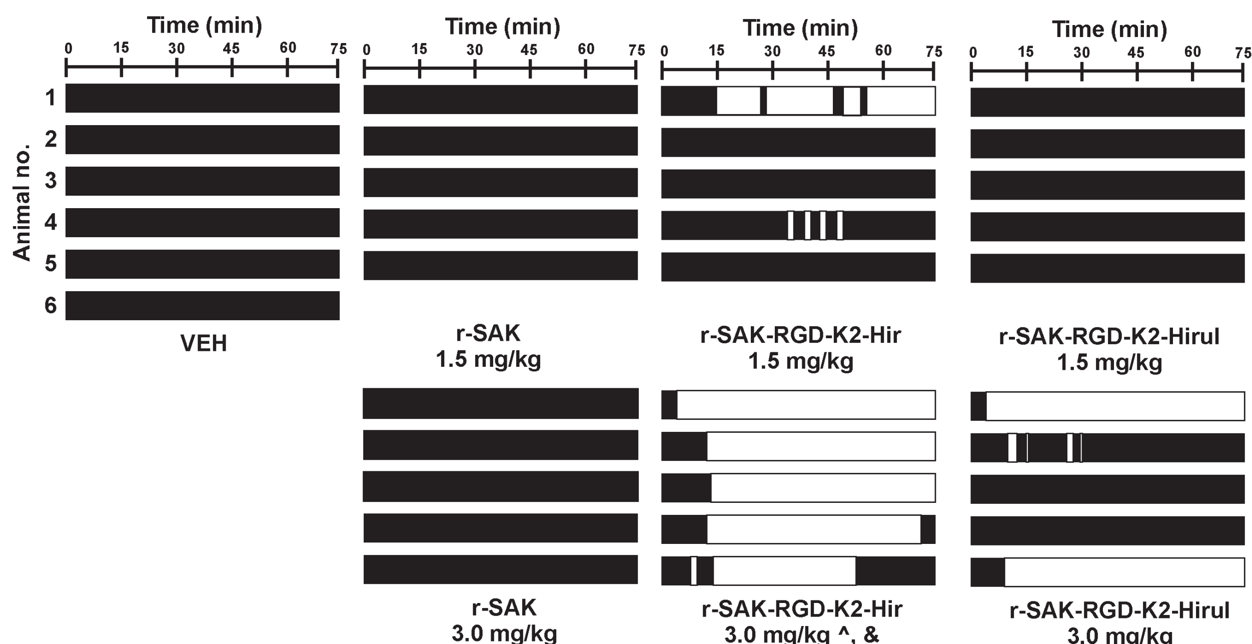

Fig. 2. Schematic representation of the patency status of the carotid artery in individual animals for 75 min following the injection of the recombinant proteins. The open and solid portions of each bar indicate the patency and occlusion, respectively. ^ $p < 0.05$ *vs.* VEH, & $p < 0.05$ *vs.* r-SAK (Kruskal-Wallis test)

A standardized device was applied longitudinally to the dorsal portion of the tail between 6 and 9 cm from the tip, and care was taken to avoid large veins. Immediately following the injury, the tail was placed into a cylinder that contained isotonic saline at 37°C, and the bleeding time was measured from the moment that the tail was surgically cut until the bleeding completely stopped (i.e., a lack of bleeding for < 30 s).

Hemostatic parameters

The blood samples were drawn from the right ventricle of the heart before the arterial thrombus was removed. The blood was mixed with 3.13% sodium citrate (Sigma-Aldrich, Poznań, Poland) at a ratio of 9:1 and was centrifuged for 20 min at 3,500 × g at 4°C. The activated partial thromboplastin time (APTT), prothrombin time (PT), and the levels of fibrinogen and $\alpha2$ plasmin inhibitor were determined by performing routine laboratory assays using the coagulometer Coag Chrom 3003 (Bio-ksel, Grudziądz, Poland) and standard laboratory reagents (Bio-ksel, Grudziądz, Poland).

Hemorrhage analysis

The surgical wound sites were inspected for gross hemorrhage; they were graded 0 if no hemorrhage was seen, 1) if a small amount of hemorrhage or serosanguinous fluid was seen, and 2) if large amounts of blood were present in the surgical wounds [37].

Statistical analysis

The Kruskal-Wallis nonparametric analysis of variance was performed on the ranks of the ordered variables of the carotid arterial patency. A comparison among the groups for the reperfusion and reocclusion rates was performed using Fisher's exact test. Dunnett's multiple comparison tests were used to evaluate the duration of patency, whereas all of the other parameters were compared between the groups by means of the Mann-Whitney test. The data are shown as the mean ± SEM. In calculating the thrombus weight, the lack of thrombus was defined as 0.0 mg, and p values of less than 0.05 were considered to be statistically significant. The Kruskal-Wallis test followed by the Wilcoxon rank-sum test was used to analyze the hemorrhage scores.

Tab. 1. Effect of the recombinant proteins on the patency status of the carotid artery. The data are shown as the mean ± SEM of 5–6 animals. ** $p < 0.01$, *** $p < 0.001$ *vs.* VEH, ## $p < 0.01$, ### $p < 0.001$ *vs.* r-SAK (Mann-Whitney test), ^ $p < 0.05$ *vs.* VEH, & $p < 0.05$ *vs.* r-SAK (Kruskal-Wallis test) for the patency status. ♦♦ $p < 0.01$ *vs.* VEH, $p < 0.01$ *vs.* r-SAK (Fisher's exact test) for the reperfusion rate

	Dose (mg/kg)	Carotid blood flow (ml/min)		Patency status of the carotid artery			Reperfusion rate	Reocclusion rate
		Initial	Final	PO	CR	PP		
VEH	–	6.6 ± 1.1	0.1 ± 0.1	6	0	0	0/6	–
r-SAK	1.5	7.0 ± 0.5	0.1 ± 0.1	5	0	0	0/5	–
r-SAK	3.0	6.4 ± 0.4	0.1 ± 0.1	5	0	0	0/5	–
SAK-RGD-K_2-Hir	1.5	6.7 ± 0.7	$0.9 \pm 0.9^{**,\ \#\#}$	3	2	0	2/5	2/2
SAK-RGD-K_2-Hir	3.0	6.4 ± 0.2	$1.2 \pm 0.7^{***,\ \#\#\#,\ \wedge,\ \&}$	0	2	3	5/5 ♦♦,••	2/5
SAK-RGD-K_2-Hirul	1.5	6.5 ± 0.3	0.1 ± 0.1	5	0	0	0/5	–
SAK-RGD-K_2-Hirul	3.0	6.3 ± 0.4	$0.7 \pm 0.4^{***,\ \#\#\#}$	2	1	2	3/5	1/3

Results

The patency status of the carotid artery following recombinant protein administration

Blood flow was monitored continuously from the beginning of the study (initial blood flow – IBF) until its end (final blood flow – FBF). The carotid blood flow began to fall several minutes after the end of the electrical stimulation and stabilized at the zero level within approximately 10 ± 0.5 min, which represents the time to occlusion (Fig. 1). To evaluate the thrombolytic activity of the recombinant proteins, we only used the animals whose carotid flow remained stable and at the zero level without recovery for at least 5 min following the initial thrombus formation. The IBF was comparable in all of the study groups. In the groups that were treated with r-SAK (1.5 or 3.0 mg/kg) and SAK-RGD-K2-Hirul (1.5 mg/kg), we did not observe any flow at the end of the experiment. The FBF following the administration of SAK-RGD-K2-Hir (1.5 or 3.0 mg/kg) and SAK-RGD-K2-Hirul (3.0 mg/kg) was restored, and it was significantly lower than that of the VEH and r-SAK groups ($p < 0.01$; $p < 0.001$) (Tab. 1).

The patency status of the carotid artery, the reperfusion rate and the reocclusion rate are summarized in Table 1. The patency statuses of individual animals are also represented schematically in Figure 2. All of the animals that were given VEH showed a persistent occlusion. r-SAK caused a persistent occlusion at both doses (1.5 and 3.0 mg/kg). SAK-RGD-K2-Hir caused a cyclic reflow in 2 out of 5 animals at both of the doses (1.5 and 3.0 mg/kg), it only caused persistent patency at the higher dose (in 3 out of 5 animals). SAK-RGD-K2-Hirul caused a persistent occlusion in all of the animals at a dose of 1.5 mg/kg, but at a higher dose, cyclic reflow was observed in 1 out of 5 animals, and persistent patency was observed in 2 out of 5 animals. Therefore, the patency status of the group that was treated with 3.0 mg/kg of SAK-RGD-K2-Hir exhibited a significant improvement when compared to the VEH and r-SAK groups ($p < 0.05$, Kruskal-Wallis test).

The reperfusion rate (which was defined as the ratio of the animals that attained reperfusion to all of the animals in the group) was the highest (5 out of 5 animals) following the administration of SAK-RGD-K2-Hir (3.0 mg/kg). The injection of SAK-RGD-K2-Hir (1.5 mg/kg) or SAK-RGD-K2-Hirul (3.0 mg/kg) caused a reperfusion rate of 2 out of 5 or 3 out of 5 animals, respectively. We did not observe the reperfusion following the r-SAK (1.5 or 3.0 mg/kg) and SAK-RGD-K2-Hirul (1.5 mg/kg) injections.

The reocclusion rate (which was defined as the ratio of the animals that attained reocclusion to the animals that attained reperfusion) in the animals that were treated with SAK-RGD-K2-Hir at both doses (1.5 and 3.0 mg/kg) was 2 out of 2 and 2 out of 5 animals, respectively. The lowest reocclusion rate (1 out of 3 animals) was observed following the SAK-RGD-K2-Hirul (3.0 mg/kg) administration.

The time to reperfusion (which was defined as the time from the recombinant protein administration to the moment at which an increase in the blood flow was observed) is shown in Figure 3. The longest time

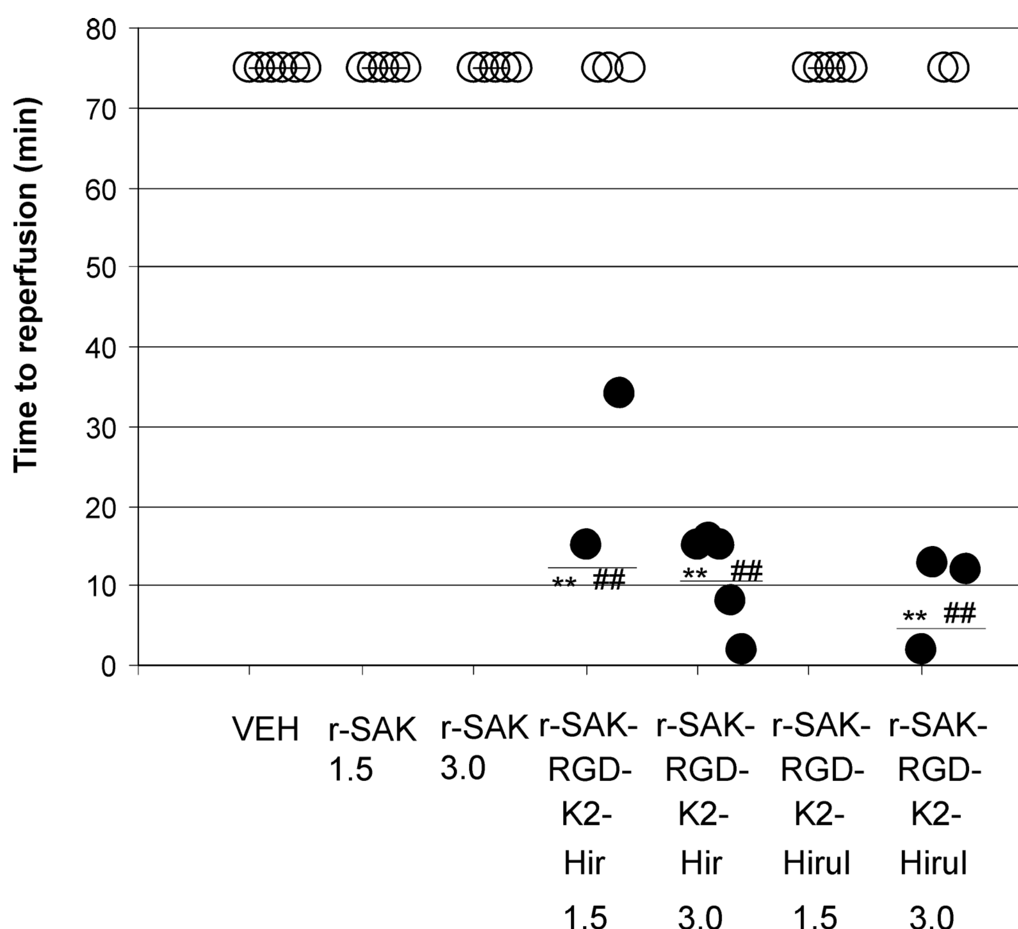

Fig. 3. Effect of recombinant proteins on the time to reperfusion in the rat model of arterial thrombosis. The open and closed circles represent the time to reperfusion in each animal. The bar indicates the median value of the time to reperfusion in each group. The open circles represent the animals that failed to attain reperfusion by 75 min after the injection of the recombinant proteins. ** $p < 0.01$ *vs.* VEH, ## $p < 0.01$ *vs.* r-SAK (Mann-Whitney test)

to reperfusion was observed in the groups that were treated with SAK-RGD-K2-Hir at both doses (approximately 12 min), and it was significantly shorter when compared to the VEH and r-SAK groups ($p < 0.01$). SAK-RGD-K2-Hirul only significantly shortened the time to reperfusion when compared to the VEH and r-SAK groups when it was administered at a dose of 3.0 mg/kg (5 min *vs.* 75 min, $p < 0.01$).

Duration of the carotid artery patency

The duration of the carotid artery patency for 75 min following the injection of the recombinant proteins is shown in Figure 4. The lowest duration of carotid patency was noted following the SAK-RGD-K2-Hir (1.5 mg/kg) administration. Following the injection of SAK-RGD-K2-Hir (3.0 mg/kg), a significant rise in the duration of the carotid patency when compared to the VEH and r-SAK groups ($p < 0.001$) was observed. SAK-RGD-K2-Hirul (3.0 mg/kg) also increased the duration of the carotid patency, but this increase was not significant.

Thrombus weight

As shown in Figure 5, a significant reduction in the arterial thrombus weight was observed following the injection of both of the doses of SAK-RGD-K2-Hir and the 3.0 mg/kg dose of SAK-RGD-K2-Hirul. The highest decrease in the arterial thrombus weight was observed in the animals that were treated with SAK-RGD-K2-Hir at a dose of 3.0 mg/kg (0.2 ± 0.04 mg *vs.* 1.1 ± 0.2 mg; $p < 0.001$ and 0.7 ± 0.07 mg; $p < 0.01$ for VEH and r-SAK, respectively). After the ad-

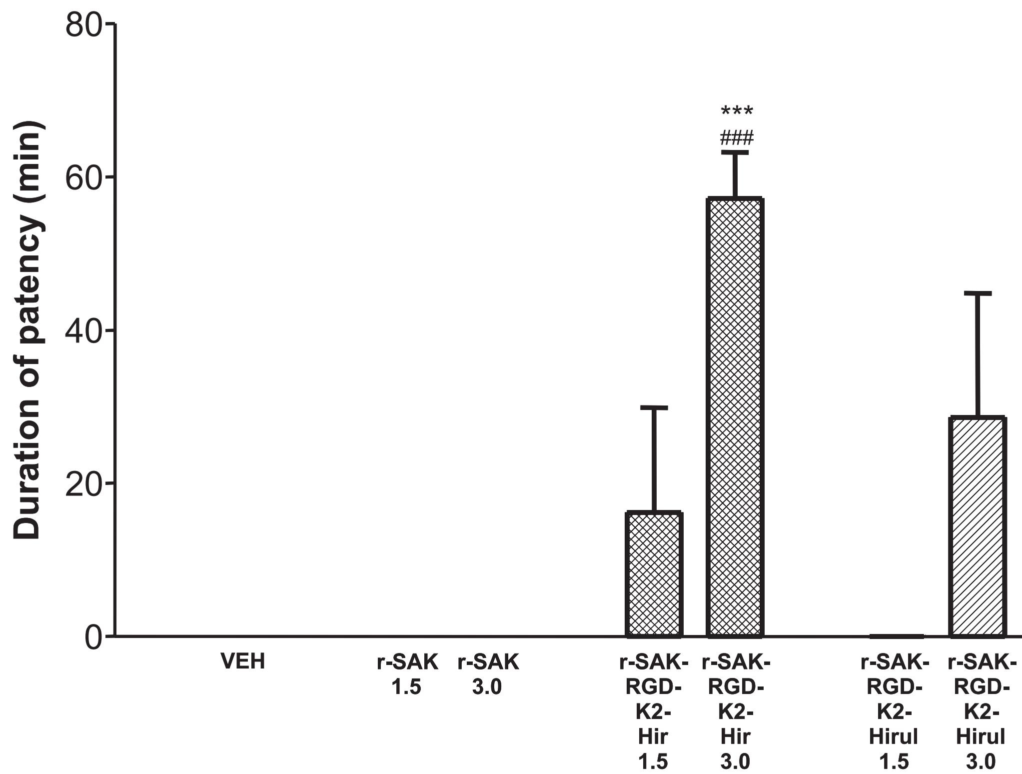

Fig. 4. Duration of carotid patency for 75 min after the injection of the recombinant proteins. The data are shown as the mean ± SEM of 5–6 animals. *** $p < 0.001$ *vs.* VEH, ### $p < 0.001$ *vs.* r-SAK (Dunnett multiple comparison test)

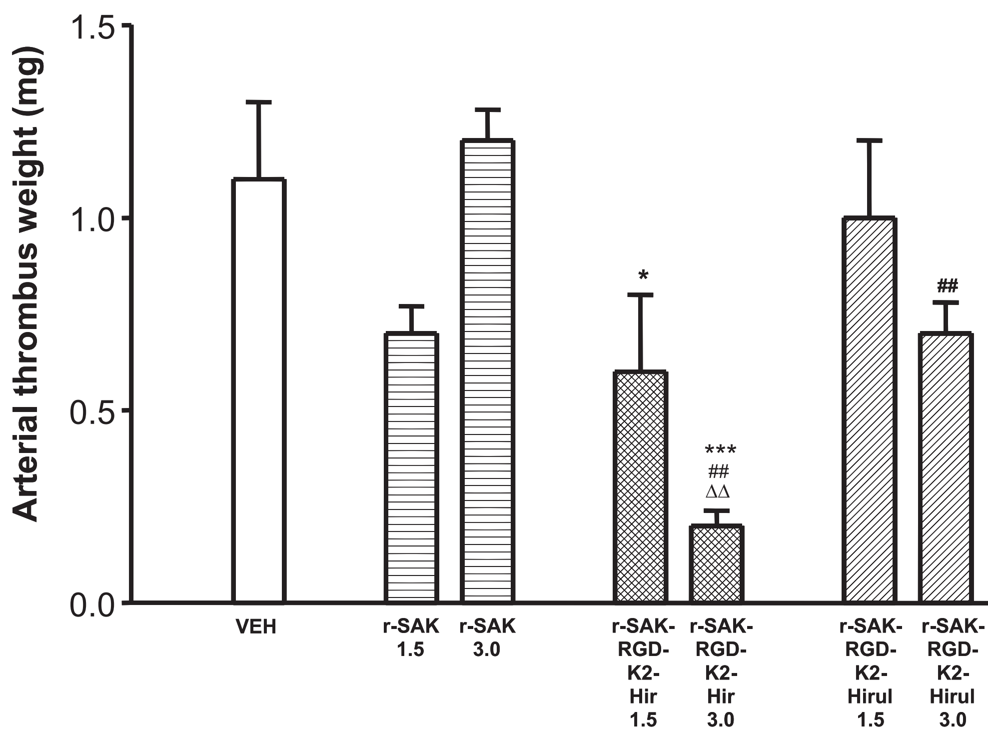

Fig. 5. Thrombolytic activities of the recombinant proteins determined by the decreased arterial thrombus. The data are shown as the mean ± SEM of 5–6 animals. * $p < 0.05$, *** $p < 0.001$ *vs.* VEH; ## $p < 0.01$ *vs.* r-SAK, ΔΔ $p < 0.01$ *vs.* SAK-RGD-K2-Hirul (Mann-Whitney test)

Tab. 2. Influence of the recombinant proteins on the template bleeding time (measured in the animals with a bleeding time < 5 min) and the hemostatic parameters in the arterial thrombosis model. The data are shown as the mean ± SEM of 5–6 animals. * $p < 0.05$, ** $p < 0.01$ *vs.* VEH; # $p < 0.05$ *vs.* r-SAK (Mann-Whitney test)

	Dose (mg/kg)	BT (s)	Number of animals with BT > 5 min	APTT (s)	PT (s)	Fibrinogen (g/l)	α2 plasmin inhibitor (%)
VEH	–	112 ± 10	0	27 ± 2	17 ± 1	2.5 ± 0.3	113 ± 10
r-SAK	1.5	107 ± 14	0	27 ± 1	17 ± 1	2.7 ± 0.2	109 ± 21
r-SAK	3.0	173 ± 21	0	24 ± 1	18 ± 2	2.3 ± 0.2	116 ± 14
SAK-RGD-K_2-Hir	1.5	152 > 5 min	4	35 4 no clot	18 ± 1	2.6 ± 0.2	128 ± 20
SAK-RGD-K_2-Hir	3.0	> 5 min	5	no clot	23 ± 2*, #	2.1 ± 0.2	144 ± 1
SAK-RGD-K_2-Hirul	1.5	> 5 min	5	no clot	23 ± 3*, #	2.8 ± 0.1	98 ± 22
SAK-RGD-K_2-Hirul	3.0	> 5 min	5	no clot	23 ± 2**, #	2.2 ± 0.1	119 ± 15

ministration of SAK-RGD-K2-Hirul (3.0 mg/kg), we observed a significant reduction in the arterial thrombus weight in comparison to the r-SAK-treated group ($p < 0.01$). A significant difference between SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul (3.0 mg/kg; $p < 0.01$) was observed. There were no differences in the thrombus weight for the groups that were treated with r-SAK as compared to those that were treated with VEH.

Bleeding time and hemostatic parameters

In the arterial thrombosis model, the BT was longer than 5 min in all of the animals that received either dose of SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul (1.5 or 3.0 mg/kg). The administration r-SAK at a dose of 1.5 mg/kg did not influence the BT, but at a higher dose, the tendency of a prolonged BT was observed (Tab. 2).

The APTT was longer than 180 s in the groups that received either dose of SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul. The PT was markedly prolonged in the groups of animals that were treated with SAK-RGD-K2-Hir (only at a dose of 3.0 mg/kg) or by either dose of SAK-RGD-K2-Hirul when compared to VEH and r-SAK ($p < 0.05$).

There were no differences in the fibrinogen levels between all of the groups (Tab. 2). There were also no changes in the α2 plasmin inhibitor levels in the groups that were treated with either dose of SAK-RGD-K2-Hir, SAK-RGD-K2-Hirul or r-SAK (1.5 or 3.0 mg/kg).

Tab. 3. Summary of hemorrhage data. Grading system: 0 = no hemorrhage; 1 = small amount of bleeding in surgical wound sites; 2 = large amount of blood in surgical wounds and/or sufficient blood loss. * $p < 0.05$ (Kruskal-Wallis test)

	Dose (mg/kg)	Wound hemorrhage	
		N animals	the degree of bleeding
VEH	–	6 – –	0 1 2
r-SAK	1.5	5 – –	0 1 2
r-SAK	3.0	5 – –	0 1 2
SAK-RGD-K2-Hir	1.5	4 1 –	0 1 2
SAK-RGD-K2-Hir*	3.0	1 2 2	0 1 2
SAK-RGD-K2-Hirul	1.5	5 – –	0 1 2
SAK-RGD-K2-Hirul*	3.0	1 2 2	0 1 2

Hemorrhage

All data regarding hemorrhaging are summarized in Table 3. Tissue hemorrhage was observed after the injection of SAK-RGD-K2-Hir at both doses (1.5 and 3.0 mg/kg). The low dose only caused a small hemorrhage (1 animal), but the high dose caused severe wound-site hemorrhages (grade 2) in two animals. After the injection of SAK-RGD-K2-Hirul (3.0 mg/kg), we observed a hemorrhage in 4 out of 5 experimental rats. We did not observe any hemorrhages following the injection of r-SAK.

Discussion

Our study was designed to determine the thrombolytic effect of the newly synthesized recombinant protein r-SAK-RGD-K2-Hirul in the arterial thrombosis model in rats. SAK-RGD-K2-Hirul contains the following domains: the plasminogen activator staphylokinase (SAK), the K2 domain of t-PA for fibrin binding, the antiplatelet RGD sequence (Arg-Gly-Asp) and the hirulog antithrombotic agent.

We have previously shown that the intravenous bolus injection of SAK-RGD-K2-Hir (which is a protein that contains hirudin) causes strong thrombolytic activity in the arterial and venous thrombosis model in rats [24]. Others have also shown that recombinant molecules that contain hirudin cause potential thrombolytic effects in rabbit and baboon arterial and venous thrombosis models [3, 17, 31].

Hirulog is a highly specific peptide that blocks the catalytic site and anion-binding exosite of thrombin. It inactivates clot-bound thrombin and prevents thrombin-induced platelet aggregation [16]. In the canine model of coronary artery thrombosis, hirulog has been shown to increase the incidence of reperfusion, decrease the incidence of reocclusion and increase the time-to-reocclusion [15, 36]. The intravenous injection of hirulog in the rat venous thrombosis model caused a dose-dependent reduction in thrombus weight at a dose of 0.1 mg/kg/min, which essentially provided the complete interruption of venous thrombus formation [19]. In the baboon model of arterial thrombosis, hirulog displayed a dose-dependent antithrombotic activity and reduction in platelet-dependent thrombus formation [13]. In a porcine model of arterial thrombus, hirulog has been shown to drive development of recurrent platelet-dependent thrombosis following deep injury and high-grade stenosis [20, 22].

Until now, only a few reports have mentioned the use of hirulog to construct new thrombin-specific inhibitors, such as the hirulog-like peptide (HLP). This peptide was designed based on the sequences of hirulog-1, hirudin and the thrombin receptor. HLP effectively reduced neointima formation or restenosis in rat, rabbit and minipig vascular injury models and inhibited the expression of the tissue factors TGF-β and platelet-derived growth factor [6, 26, 27, 34]. However, there are no data regarding the incorporation of hirulog into recombinant proteins that display antiplatelet and thrombolytic properties.

In the present study, we demonstrated a new approach regarding the structure and composition of recombinant proteins that contain direct thrombin inhibitors. We hypothesized that the smaller size of hirulog would improve the contact of the recombinant molecules with thrombin at the site of the thrombus. Our previous *in vitro* study showed that SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul are potent and fast-acting clot-dissolving agents that contain antithrombin and antiplatelet properties [14, 25].

In our model of thrombosis, the thrombolytic effects of both of the proteins were dose-dependent. SAK-RGD-K2-Hirul displayed a shorter time to reperfusion (approximately 5 min) at a dose of 3.0 mg/kg *vs.* 12 min that were measured for SAK-RGD-K2-Hir. SAK-RGD-K2-Hirul (3.0 mg/kg) also restored the patency of occluded arteries in 2 out of 5 animals, and it demonstrated the highest final blood flow (Tab. 1 and Fig. 2). Similarly, SAK-RGD-K2-Hir (3.0 mg/kg) restored the patency of occluded arteries in 3 out of 5 animals, while reocclusion occurred in 2 animals. SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul significantly reduced the arterial thrombus weight in comparison to r-SAK ($p < 0.05$). In summary, the intravenous bolus infusion of SAK-RGD-K2-Hirul did not compare favorably with SAK-RGD-K2-Hir in the lysis of an arterial thrombus and the restoration of persistent carotid patency.

We confirmed the high fibrin affinities of SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul that were demonstrated in a previous *in vitro* study [14]. The administration of either dose of SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul (1.5 or 3.0 mg/kg) did not induce a systemic lytic state in any of the animals,

which was shown by the unchanged levels of fibrinogen and the α2 plasmin inhibitor.

Recombinant proteins that contained hirulog or hirudin caused dose-dependent hemorrhagic complications. We observed a significant prolongation of the PT and the APTT in the groups that received both SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul. This was most evident in the animals that were infused with the proteins at a dose of 3.0 mg/kg (Tab. 2). Because the prolongation of the hemostatic parameters did not occur following the r-SAK administration, it is reasonable to assume that the significant prolongation of these parameters was due to the antithrombin activity of the recombinant proteins.

The thrombus that is produced in the carotid artery is primarily platelet-rich, and this has been previously demonstrated [5, 34]. During the pharmacologic lysis of a thrombus, plasmin- and thrombin-induced platelet activation occurs [2, 10, 12]. To inhibit the platelet activation, we incorporated an antiplatelet RGD sequence (Arg-Gly-Asp) into the recombinant proteins to block the GPIIb/IIIa receptor. The strong thrombolytic activity and the prevention of reocclusion in our arterial thrombosis model were observed following the administration of SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul when compared to that of r-SAK. Moreover, in an *in vitro* study, we observed the anti-aggregative activity of the chimeric proteins, while r-SAK alone failed to inhibit platelet aggregation [14].

The presence of hirulog and hirudin in proteins appears to offer additional advantages for the inhibition of platelet activity in our model of arterial thrombosis. Similarly, other researchers have shown that direct thrombin inhibitors display antiplatelet activity in rats [9]. The inhibition of coagulation and platelet aggregation is expected to increase the tail bleeding time in the recombinant protein-treated rats. Indeed, we observed a significant prolongation of the bleeding time (BT) (most animals bled for over 5 min) following the injection of SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul. We noticed bleeding from tissues at the time of thrombus isolation from the carotid artery and during blood sample collection. This effect was dose-dependent, and it was the most visible when the higher doses of both of the chimeric proteins were administered. However, there was no bleeding that led to the death of the animals (Tab. 3).

In conclusion, our findings show the thrombolytic activity of an intravenous bolus injection of the novel thrombolytic agent SAK-RGD-K2-Hirul in rats. This protein compares favorably with r-SAK in rat arterial thrombolysis, but it does not compare favorably with SAK-RGD-K2-Hir. These results provide the rationale for further testing of the thrombolytic efficacy of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir in different animal models of thrombosis as well as for establishing the associated risks of bleeding complications.

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Conflict of interest:
No conflicts of interest are declared.

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