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Thrombin inhibitory and clot-specific fibrinolytic activities of the urokinase variant, M23 (rscu-PA-40 kDa/Hir)

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Abstract

The recombinant bifunctional urokinase variant, M23 (rscu-PA-40 kDA/Hir), comprising the kringle and protease domain of single-chain urokinase-type plasminogen activator and a C-terminal fragment of hirudin in one single-chain molecule, was evaluated for its thrombin-inhibitory and fibrinolytic properties in vitro and in vivo. M23 inhibited thrombin-activated coagulation of human blood and thrombin-induced aggregation of human platelet rich plasma in a concentration-dependent manner. The ADP-induced aggregation of human platelet rich plasma was not influenced by M23. In contrast, recombinant single-chain urokinase-type plasminogen activator (saruplase) inhibited neither blood coagulation nor platelet rich plasma aggregation. M23 and saruplase both lysed radiolabelled human thrombi immersed in human plasma (Chandler Loop system) with equal potency. However, there was a significantly lower systemic generation of plasmin (measured as consumption of α_2-antiplasmin) by M23 compared to saruplase. In anaesthetized non-heparinized rabbits, experimental femoral artery thrombosis was treated with intravenous bolus injections of M23 or saruplase (6 mg/kg, each). Thrombolytic restoration of arterial blood perfusion was significantly higher in M23- than in saruplase-treated rabbits. Plasma fibrinogen concentrations were decreased markedly in saruplase-treated animals, but remained at significantly higher levels in M23-treated rabbits. In conclusion, the bifunctional molecule, M23, showed thrombin inhibitory and fibrinolytic properties in human in vitro systems and exerted superior thrombolytic effects to saruplase in rabbit femoral artery thrombosis. In vitro and in vivo data indicate that the fibrinolytic activity of M23 is highly clot-specific.

Keywords: Urokinase variant; Fibrinolysis; Thrombin inhibition; Platelet aggregation; Clot specificity; (Rabbit)

1. Introduction

Thrombolytic therapy of acute myocardial infarction has been shown to restore coronary patency, to improve ventricular function and to reduce mortality (GISSI Study Group, 1987; Shammas et al., 1993). The benefit from thrombolytic therapy, however, is impaired by incomplete reperfusion of thrombotic coronary occlusions and by coronary re-occlusions in a substantial number of patients (Reiner and Wasserman, 1994). Residual fibrin-bound thrombin appears to be the main causative factor for rethrombosis (Rapaport, 1991). The multiple effects of thrombin on the hemostatic system – which include conversion of fibrinogen to fibrin, activation of factors V, VIII and XIII, and stimulation of platelet aggregation – explain its pivotal role for on-going thrombosis during thrombolysis. When incorporated into a fibrin clot, thrombin remains functionally active (Francis et al., 1983; Fenton, 1988) and stimulates feedback activation of the clotting system (Kumar et al., 1994). Furthermore, blood thrombin levels increase during thrombolytic therapy and induce a procoagulant response (Gulba et al., 1991; Gram et al., 1993). Anti-thrombin agents have thus been recommended as a conjunctive therapy in order to improve arterial thrombolysis (Zoldhelyi et al., 1992).

None of the currently available fibrinolytic agents inhibits thrombin. We have constructed a bifunctional molecule, M23 (rscu-PA-40 kDA/Hir), which combines fibrinolytic and thrombin-inhibitory activities. M23 comprises the protease domain and the kringle structure of the fibrinolytic unglycosylated single chain urokinase-type plasminogen activator (saruplase) and, as an additional

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thrombin-inhibiting structure, the C-terminal part of hirudin fused via a linker sequence to the protease domain of saruplase. The expected fibrinolytic and thrombin-inhibiting properties of M23 were studied in in vitro systems using human blood or plasma and in an in vivo model of arterial thrombosis in rabbits. Furthermore, it was hoped that the hirudin part of M23 would retain an affinity for clot-bound thrombin and target M23 to a thrombus; this would enhance the clot specificity of its fibrinolytic action and reduce systemic plasminogen activation. Thus, special attention was given to the possible thrombus-restricted specificity of plasminogen activation by M23, which was evaluated by comparison to the fibrin-specific thrombolytic agent, saruplase (Hanbücken et al., 1987).

2. Material and methods

2.1. Test compounds

2.1.1. Production of M23 (rscu-PA-40 kDa / Hir)

The C-terminal region of hirudin, comprising residues 53–65 of the hirudin sequence, was fused via a 14-residue linker sequence to a C-terminal segment of rscu-PA (recombinant single-chain urokinase-type plasminogen activator, saruplase) consisting of residues Ser^{47} to Leu^{411} of saruplase (Fig. 1). This combined thrombin inhibitory and fibrinolytic activity in one molecule. The expression plasmid for M23 (rscu-PA-40 kDA/Hir) was constructed by modifying the expression vector pBF160 (Brigelius-Flohé et al., 1992) which originally carried a synthetic gene encoding rscu-PA under the control of a synthetic trp promoter. That region of the gene encoding residues 1–46 of rscu-PA was removed by digestion of pBF160 with *Nde*I and *Nco*I, and a synthetic DNA linker comprising a ribosomal binding site and a transcriptional start codon was inserted. The resulting plasmid, pSJ41, was digested with *Bam*HI and *Hin*dIII and a fragment encoding the C-terminal of the uPA moiety, the 14-amino acid linker sequence and the 13-amino acid-long fragment of hirudin was inserted. The resultant expression plasmid was designated pSJ95. pSJ95 was checked by extensive restriction analyses and sequencing of the synthetic gene. A detailed description of the plasmid construction is given in the patent publication DE43 23 754 C 1 (Steffens et al., 1994). Recombinant expression in *E. coli* JM 103 carrying pSJ95 and purification of M23 (rscu-PA-40 kDa/Hir) was performed as described before for rscu-PA (Brigelius-Flohé et al., 1992).

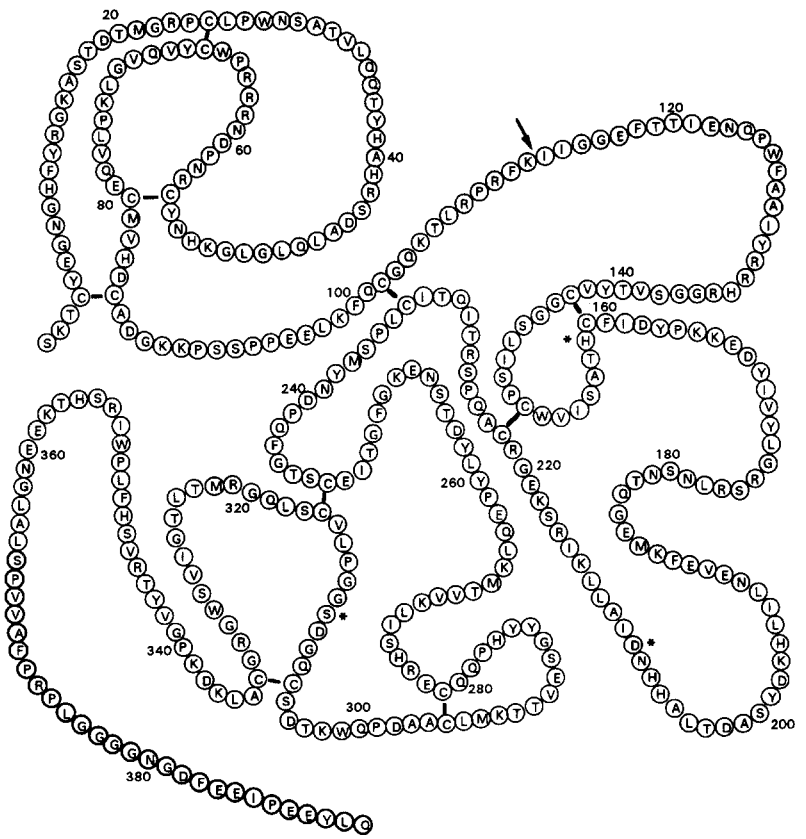

Fig. 1. Schematic representation of the primary structure of M23 (rscu-PA-40 kDa/Hir). The residues are represented by their single letter symbols and black bars indicate disulphide bonds. The active site residues His^{158}, Asp^{209} and Ser^{310} are indicated with an asterisk and the arrow indicates the cleavage site for conversion to two-chain M23. The thrombin inhibitory domain begins with residue 366.

2.1.2. Saruplase (rscu-PA)

Saruplase (recombinant unglycosylated full-length single-chain urokinase-type plasminogen activator), as well as M23, were produced in, and purified from, genetically transformed *E. coli* bacteria as described previously (Holmes et al., 1985).

The molecular weight is 44 kDa for M23 and 46 kDa for saruplase. The concentrations and dosages of M23 and saruplase are given on a weight basis (μg/ml and mg/kg). As the difference in molecular weight is only small, the molar concentrations and dosages of M23 and saruplase are very similar.

2.2. Thrombin-induced coagulation of human plasma

Nine parts of human blood, freshly drawn from a male volunteer, were mixed with one part of sodium citrate (3.8%) and centrifuged for 10 min at $2000 \times g$. The plasma was frozen (-20°C) immediately after centrifugation. For testing, plasma was diluted 1/10 with veronal acetate buffer (Boehringer Mannheim/Germany). 200 μl of the diluted plasma and 50 μl of a solution of M23 or saruplase or saline (vehicle) were placed into a cuvette of the coagulometer (Biomatic 2000 two-channel aggregometer, Sarstedt, Nümbrecht/Germany) and incubated at 37°C for 1 min. Then 50 μl of a thrombin solution (Fibrinogen-a-reagent, Boehringer Mannheim/Germany) was added and the time to formation of a clot was measured. If clotting was not finished within 300 s as in the presence of high concentrations of M23, the coagulation test was stopped. The time to thrombin-induced coagulation (thrombin clotting time) is given in [s].

2.2.1. Statistical evaluation

There were 6–8 single experiments for each concentration of M23 or saruplase or for saline in the presence of eight different thrombin concentrations, ranging from 0.25 to 32 NIH-U/ml. A regression analysis for the clotting time induced by 1 NIH-U/ml thrombin in the presence of different concentrations of M23 was used to calculate the concentration (and 95% confidence limits) of M23 that induced a three-fold prolongation of the normal thrombin time (TT $\times$ 3).

2.3. Aggregation of human platelet rich plasma

Freshly drawn venous blood from three voluntary donors was used. Nine parts of the blood were mixed with one part of sodium citrate (3.8%). Platelet rich plasma was prepared by centrifugation of the citrated blood at $200 \times g$ for 15 min; platelet poor plasma was separated by further centrifugation at $1200 \times g$ for 15 min. A 12-channel turbidimetric Born-Michal aggregometer was calibrated with platelet poor plasma for 100% light transmission and with platelet rich plasma for 0% light transmission.

Platelet rich plasma, 500 μl, was incubated with 25 μl of a given concentration of M23 or saruplase or appropriate blank (vehicle) for 2 min at 37°C under constant stirring. Aggregation was then induced by addition of 25 μl of thrombin (0.5 of 1.0 NIH/ml) or adenosine diphosphate (ADP; Boehringer Mannheim/Germany) (1.0 or 10 μM). Aggregation was measured as the increase in light transmission and was calculated as:

$$\text{aggregation} = \left(\frac{\%\text{transmission platelet rich plasma + thrombin (or ADP)}}{\%\text{transmission platelet poor plasma}} \right) \times 100\,[\%]$$

2.3.1. Statistical evaluation

To test the effects on thrombin-induced aggregation, three platelet rich plasma preparations from each of the three donors were used for each experimental group ($n = 9$). Group size in ADP-induced aggregation experiments was $n = 6$ (2 platelet rich plasma preparations from each of the three donors). For thrombin-induced aggregation, percentage inhibition by M23 versus the control group (saline as vehicle) was calculated. The regression analysis for the concentration-dependent inhibition by M23 was used to calculate the concentration producing 50% inhibition of thrombin-induced aggregation (IC_{50} value with 95% confidence limits).

2.4. In vitro fibrinolysis of human whole blood thrombi

Freshly drawn blood from nine volunteer donors was used. The formation of thrombi, and the subsequent lysis of these thrombi, was performed in tubular loops rotating on a tilted turning table (modified Chandler Loop, as described by Hanbücken et al. (1987)). Human fibrinogen (Kabi Vitrum, Munich/Germany) was labelled with Bolton-Hunter reagent (Amersham International, Amersham/UK) and 2 ml of citrated whole human blood was mixed with ^{125}I-labelled fibrinogen (final concentration 100 000 cpm/ml) in a polyethylene tube. After recalcification, the two ends of the tube were joined to form a loop. The loop was placed on a rotating cylinder and rotated at 12 rpm. After 30 min the thrombus that had been formed in the loop was poured out, washed with saline, and dried on gauze. When its ^{125}I content had been determined in a γ-counter, the thrombus was transferred into a new polyethylene tube filled with autologous citrated plasma. Different concentrations of M23 or saruplase, or vehicle (saline) were added, and the tube was rotated as described above for 180 min. At 0 (baseline), 30, 60 and 180 min, 200 μl plasma samples were taken to determine the plasma radioactivity. Thrombolysis was measured by the increase in plasma radioactivity during the experiment. Plasma radioactivity was calculated as a percent of the initial total

^{125}I content of the thrombus before the start of lysis (baseline value).

From the plasma samples taken at the various time points (see above), 100 μl aliquots were used for determination of plasma α_2-antiplasmin. For each determination, two plasma samples were pooled.

α_2-Antiplasmin was determined according to the assay described by Saito (1988) using the Berichrom α_2-antiplasmin test kit (Behring, Marburg/Germany). Briefly, plasmin is added in excess to citrated plasma. α_2-Antiplasmin of the plasma sample and plasmin form an inactive complex. The activity of the residual free plasmin is measured by its reaction with the chromogenic plasmin substrate, D-norvalyl-cyclohexylalanyl-lysyl-*p*-nitroanilide. This activity is negatively correlated to the α_2-antiplasmin concentration and is read off from a calibration curve.

2.4.1. Statistical evaluation

6–8 single experiments per group were performed. Regression analysis for the extent of lysis at 180 min was used to calculate the concentrations of M23 and saruplase that induced lysis to an extent of 50% (EC_{50} values, incl. 95% confidence limits). In a similar manner, EC_{50} values were calculated for a 50% reduction of plasma α_2-antiplasmin levels.

2.5. Rabbit femoral artery thrombolysis

Male SPF-White New Zealand rabbits, weighing 1.7–2.0 kg (from a commercial breeder) were used. Anesthesia was initiated with thiopentone (25 mg/kg) given into a marginal ear vein. After tracheotomy, the spontaneously breathing animals then inhaled a 60/40 vol.% mixture of N_2O/carbogen; the flow was set to 1.6 l/min to provide a constant positive airway pressure ventilation during deep inspirations also. A minimum positive end-expiratory pressure of about 3 cm H_2O was built up to inhibit the collapse of alveoli. Reflexes were suppressed with propionylpromazine (Combelen, Bayer, Leverkusen/Germany) that was given as a 0.2 mg/kg i.v. bolus followed by an i.v. infusion of 0.2 mg/kg × h through the ear vein. The animals were placed on a heated operation desk in a supine position. Polyethylene catheters were inserted into the left jugular vein for intravenous applications, and into the left carotid artery for measurement of arterial blood pressure. The carotid artery catheter was connected to a Statham P23Gb pressure transducer (Hellige, Freiburg/Germany).

For induction of thrombosis, a femoral artery was isolated distally from the inguinal ligament. An electromagnetic flow probe (lumen 1.0 mm, type A; Hellige, Freiburg/Germany) was placed around the femoral artery 1.5 cm distally to the superficial epigastric artery to measure femoral artery blood flow. Two snares, 1 cm apart, were loosely tied around the femoral artery, with the branch of the superficial epigastric artery just between the two snares. The proximal snare was just distal to the branch of the deep femoral artery. The superficial epigastric artery was cannulated with a PP10-polyethylene catheter. To induce thrombus formation, the snares were occluded and thrombin (10 NIH-U in 10 μl; diluted from fibrinogen-a-reagent ≅ human thrombin, Boehringer Mannheim/Germany) and 25 μl of ^{125}I-human fibrinogen (human fibrinogen, Kabi Vitrum, Munich/Germany, labelled with Bolton-Hunter reagent, Amersham International, Amersham/UK; ~200000 cpm) were injected into the isolated segment of the femoral artery via the cannulated side branch. After 30 min the snares were released and the formation of an occlusive thrombus was verified by the indication of zero flow by the electromagnetic flow probe. The blood flow signal was amplified and shown on a recorder (Philips, Eindhoven/Netherlands). A NaI-crystal scintillation γ-detector (Harshaw/Netherlands) was placed as near as possible (≤ 1.5 cm) over the radiolabelled thrombus. The radioactivity values were acquired using the Accu Spec/NaI Plus-Master Board (Canberra-Packard, Frankfurt/Germany).

After an equilibration period of 30 min, baseline values for femoral artery blood flow, mean arterial blood pressure, and heart rate were recorded at 5 min intervals. An arterial blood sample was drawn to determine the baseline level of plasma fibrinogen (determined by the Clauss method). The two snares around the femoral artery were then ligated and the thrombus was induced as described above. Sixty min after thrombus induction (≅ time 0 min), 6 mg/kg M23 or saruplase was injected as an intravenous bolus. The femoral artery blood flow was recorded at 5 min intervals for 90 min after application of these boli. Additionally, the time to the appearance of the first flow signal after the start of the treatment regimen was determined. The ^{125}I-fibrin radioactivity of the thrombus was measured before the start of the treatment (baseline) and then at 5 min intervals until the end of the experiment, 90 min after the start of treatment. At the end of the experiment, the residual thrombus was removed, and the background radioactivity of the remaining preparation was measured with the detector in the same position as during the experiment. This background radioactivity was subtracted from all measured values obtained during the experiment. Blood fibrinogen levels were determined at 60 and 90 min after the start of the intravenous application.

2.5.1. Statistical evaluation

Femoral artery blood flow and residual thrombus ^{125}I-fibrin radioactivity were calculated as percentages of the baseline values. The total reperfusion blood flow was calculated as the area under the blood flow over time curve (AUC-reperfusion blood flow).

Six single experiments per group were performed. Inter-group differences were evaluated by means of the

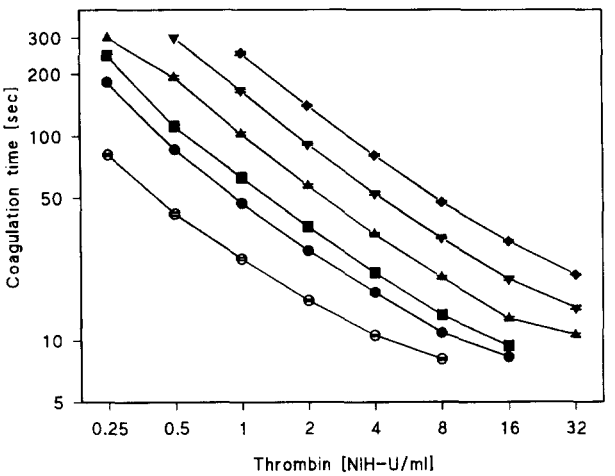

Fig. 2. Inhibition of thrombin-induced clotting of human blood, shown as prolongation of coagulation time, by different concentrations of M23 (○ vehicle solution; ● 5 μg/ml; ■ 10 μg/ml; △ 20 μg/ml; ▽ 40 μg/ml; ◆ 80 μg/ml). Data as means ± S.E.M. of $n = 6–8$ single experiments.

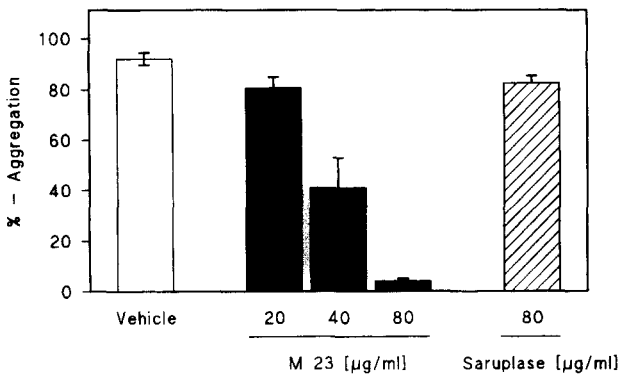

Fig. 3. Aggregatory response of human platelet rich plasma to 0.5 NIH-U/ml thrombin in the presence of vehicle (saline), different concentrations of M23, or of saruplase. Data as means ± S.E.M. of $n = 9$ single experiments.

two-tailed unpaired t test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Inhibition of thrombin-induced coagulation of human plasma

Thrombin induced a concentration-dependent (0.25–32 NIH-U/ml) activation of coagulation of human plasma; clotting times were reduced from 80 s to less than 10 s. M23 inhibited the effect of thrombin in a concentration-dependent manner between 5–80 μg/ml, and shifted the thrombin concentration-effect curve to the right (Fig. 2). M23 prolonged the clotting time in the presence of 1 NIH-U/ml thrombin by a factor of 3 (TT × 3) at a mean concentration of 10.1 (8.9–11.4) μg/ml (Table 1). The effects of thrombin on coagulation time of human plasma were not influenced at all by saruplase up to a concentration of 80 μg/ml (data not shown).

3.2. Inhibition of thrombin-induced aggregation of human platelet rich plasma

In the presence of saline (as vehicle), 0.5 NIH-U/ml thrombin induced an aggregatory response of 92 ± 2% in human platelet rich plasma. M23 (20–80 μg/ml) inhibited thrombin-induced human platelet rich plasma aggregation in a concentration-dependent manner. After incubation with 80 μg/ml M23, aggregation was reduced to 4 ± 1%, whereas it was still 82 ± 3% after incubation with 80 μg/ml saruplase (Fig. 3). M23 inhibited thrombin-induced human PRP aggregation by 50% at a mean concentration of 37.2 (31.4–43.8) μg/ml (Table 1). Aggregation of human platelet rich plasma by means of another stimulant, adenosine diphosphate (ADP; 10 μM), which induced an aggregatory response of 76 ± 3%, was virtually unaffected by 40 and 80 μg/ml M23 (Fig. 4). Thus, the anti-aggregatory effect of M23 is specific for platelet activation by thrombin.

3.3. In vitro fibrinolysis of human whole blood thrombi

In the Chandler Loop system, M23 and saruplase exerted similar time- and concentration-dependent lysis of ^{125}I-fibrin-labelled thrombi made from human whole blood.

Table 1
In vitro efficacy values of M23 and saruplase

Substance	Coagulation	Thrombin-stimulated aggregation platelet rich plasma	Fibrinolysis	Decrease of α_2-antiplasmin
	TT × 3	IC_{50}	EC_{50}	EC_{50}
M23	10.1 (8.9–11.4)	37.2 (31.4–43.8)	0.5 (0.3–1.4)	1.4 (1.1–2.0)
Saruplase	No effect	> 80	0.4 (0.3–0.5)	0.6 (0.5–0.8)

All values are given in [μg/ml] as mean concentrations incl. 95% confidence limits. For further explanation see Materials and methods.

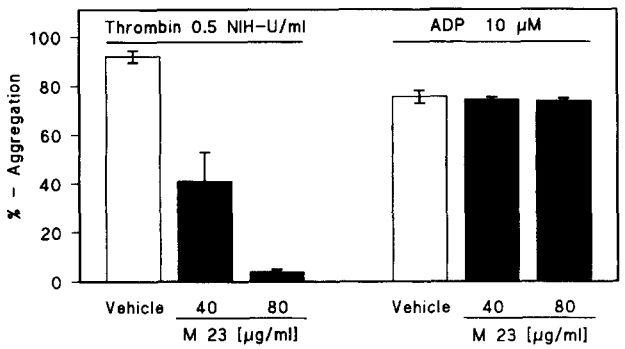

Fig. 4. Comparison of the effects of M23 on thrombin-induced (left side), and ADP-induced (right side) human platelet rich plasma aggregation. Data as means ± S.E.M. of $n = 6–9$ single experiments.

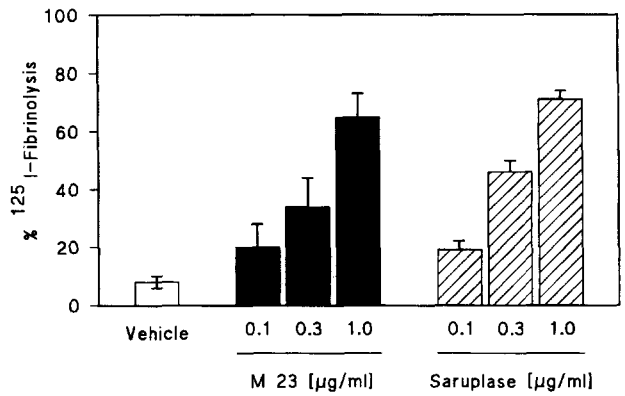

Fig. 5. Extents of lysis of incorporated ^{125}I-fibrin in human thrombi in the Chandler Loop system 180 min after addition of vehicle (saline), or different concentrations of M23, or of saruplase. Data as means ± S.E.M. of $n = 6–8$ single experiments.

The spontaneous extent of lysis at 180 min, calculated from the release of incorporated ^{125}I-fibrin into the surrounding plasma milieu, was 8 ± 2% in vehicle (saline)-treated samples. In the presence of 0.1–1.0 μg/ml M23, or saruplase, the extents of lysis ranged from 20 ± 8% to 65 ± 8%, and from 19 ± 3% to 71 ± 3%, respectively (Fig. 5). Increasing either concentration to 3 μg/ml did not further augment the lytic effects (data not shown). Mean concentrations of 0.5 (0.3–1.4) μg/ml M23 and 0.4 (0.3–0.5) μg/ml saruplase were needed to achieve 50% lysis (Table 1).

Incubation with 0.1–3.0 μg/ml M23 or saruplase led to time- and concentration-dependent decreases of plasma

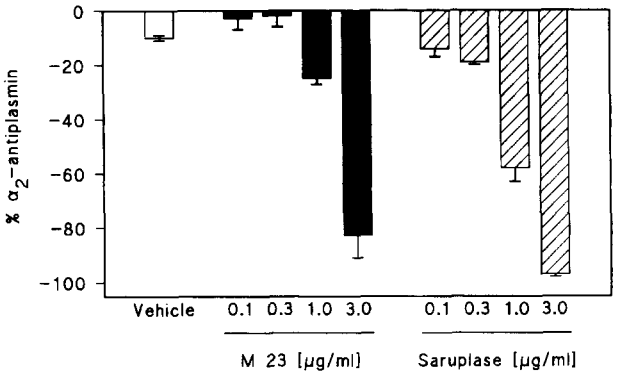

Fig. 6. Changes in plasma α_2-antiplasmin concentrations in the Chandler Loop system 180 min after addition of vehicle (saline), or different concentrations of M23, or of saruplase. Data as means ± S.E.M. of $n = 6–8$ single experiments.

α_2-antiplasmin levels with maximum reductions of 83 ± 8% and 97 ± 1% (Fig. 6). Saruplase proved to be significantly more potent than M23 to reduce plasma α_2-antiplasmin: M23 reduced α_2-antiplasmin by 50% only at a mean concentration of 1.4 (1.1–2.0) μg/ml, which is significantly higher than the respective value of 0.6 (0.5–0.8) μg/ml for saruplase (Table 1).

3.4. Rabbit femoral artery thrombolysis

There were no significant differences between the two groups randomized to receive an intravenous bolus injection of either M23 or saruplase with respect to their baseline values for mean arterial blood pressure, heart rate, femoral artery blood flow or ^{125}I-fibrin radioactivity (Table 2).

The intravenous bolus injections of saruplase and M23 (6 mg/kg each) lysed femoral artery thrombi in non-heparinized rabbits as shown by the time-dependent decreases of ^{125}I-fibrin that had been incorporated into these thrombi. The time course of fibrinolysis was similar for the two agents. The fibrinolytic effect was more pronounced with M23 (44 ± 12%) than with saruplase (22 ± 5%), but this difference just did not reach statistical significance (Fig. 7).

Reperfusion of thrombotically occluded femoral arteries was achieved in all six rabbits treated with M23, and none of the re-canalized arteries re-occluded during the observation period of 90 min. In saruplase-treated rabbits, five of

Table 2
Baseline values for mean arterial blood pressure, heart rate, femoral artery blood flow and ^{125}I-fibrin radioactivity in the rabbit femoral artery thrombosis model

Treatment group	Mean arterial blood pressure mm Hg	Heart rate n/min	Femoral artery blood flow ml/min	Incorporated ^{125}I-fibrin radioactivity cpm
M23	84 ± 6	331 ± 8	9.8 ± 0.7	11 008 ± 2516
Saruplase	94 ± 6	296 ± 3	9.6 ± 1.0	10 100 ± 2689

Data as means ± S.E.M. of $n = 6$ single experiments.

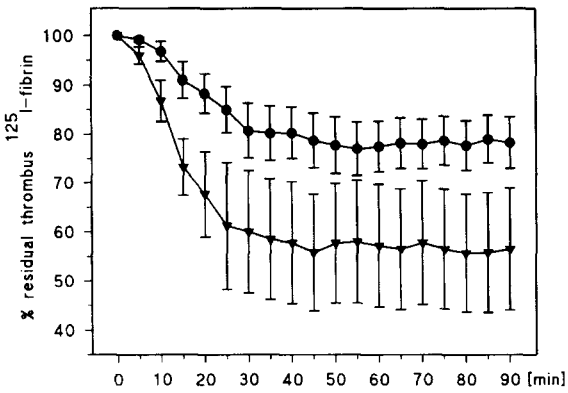

Fig. 7. Decrease of ^{125}I-fibrin content in femoral artery thrombi of rabbits after i.v. bolus ($t = 0$ min) injections of 6 mg/kg M23 (▼), or saruplase (●). Data as means ± S.E.M. of $n = 6$ single experiments.

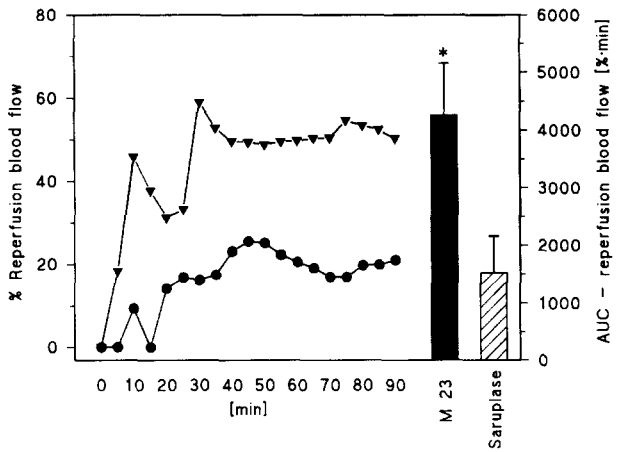

Fig. 8. Time course of mean reperfusion blood flow (plotted against the left y axis) in thrombotically occluded femoral arteries in rabbits after i.v. bolus injections of 6 mg/kg M23 (▼), or 6 mg/kg saruplase (●). The bar graphs (plotted against the right y axis) present the total area of the blood flow over time curve (as means ± S.E.M.) for M23-treated and saruplase-treated rabbits ($n = 6$). The asterisk indicates a significant difference ($P < 0.05$) between the treated groups.

six arteries were re-perfused and there was one re-occlusion (Table 3). The time between intravenous bolus injection and re-perfusion was 9 ± 3 min for M23, which was not significantly shorter than the reperfusion time of 26 ± 12 min in saruplase-treated animals.

At all time points, the re-perfusion blood flow was higher in M23-treated rabbits than in saruplase-treated rabbits. The total reperfusion blood flow over the complete

Table 3
Time to reperfusion and reperfusion/reocclusion rates in rabbit femoral artery thrombosis

Treatment group	Time to reperfusion	Incidence of	
		reperfusion	reocclusion
M23	9 ± 3 min	6/6	0/6
Saruplase	26 ± 12 min	5/6	1/5

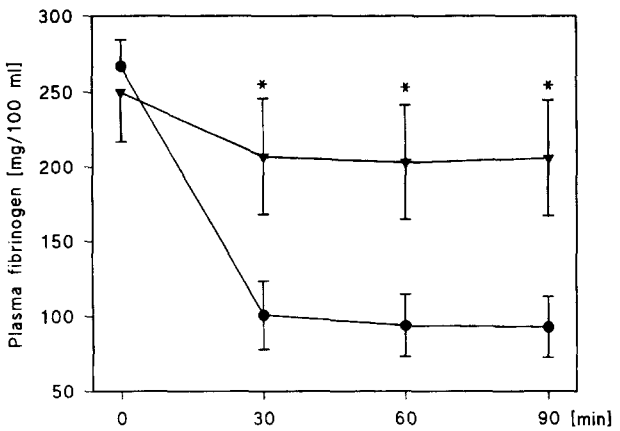

Fig. 9. Plasma fibrinogen levels before and after treatment with 6 mg/kg (i.v. bolus) M23 (▼) or saruplase (●) in rabbit femoral artery thrombosis. Data as means ± S.E.M. of $n = 6$ single experiments. Asterisks indicate significant differences ($P < 0.05$) between the treated groups.

experimental period of 90 min was significantly higher in rabbits treated with bolus injections of M23 than in animals treated with saruplase (Fig. 8).

Previous experience with this experimental model has shown that injection of saline (as a vehicle control) neither restored blood flow (Schneider, 1991) nor dissolved incorporated ^{125}I-fibrin (unpublished data).

Pre-treatment plasma fibrinogen levels did not differ significantly between the two groups (250 ± 33 vs. 267 ± 17 mg/100 ml). Treatment with bolus-injected saruplase resulted in a pronounced reduction of plasma fibrinogen by almost 70%. In contrast, there was only a modest decrease of plasma fibrinogen concentrations (by 20%) after injection of M23. Thus, there were significantly higher plasma fibrinogen concentrations in the rabbits given M23 than in those which received saruplase (Fig. 9).

4. Discussion

The thrombin inhibitory activity of the bifunctional molecule, M23, which was designed to combine fibrinolytic and thrombin inhibitory properties, was shown as a concentration-dependent prolongation of thrombin clotting time in human plasma. Thrombin inhibitory activity was further demonstrated by the potential of M23 to inhibit thrombin-induced aggregation in human platelet rich plasma. This inhibition was proved to be specific for thrombin, because aggregation induced by another stimulant, ADP, was not inhibited by M23. In contrast to M23, the fibrinolytic agent, saruplase, did not affect either thrombin-stimulated coagulation or platelet aggregation. It has to be considered that, in platelet rich plasma, thrombin stimulation induces both aggregation and coagulation. Thus, the effect of M23 on the thrombin-induced platelet

aggregation cannot be interpreted unequivocally as inhibition of thrombin-induced aggregation; it is presumably due to an effect on coagulation or on coagulation and aggregation.

The fibrinolytic potencies of M23 and saruplase were assessed by their abilities to cause lysis of radiolabelled human thrombi in the Chandler Loop system. The almost identical EC_{50} values for M23 and saruplase indicate that M23 retained the full fibrinolytic potency of its parent molecule, saruplase. Obviously, the attachment of elements from the hirudin sequence as a thrombin inhibitory moiety does not interfere with the fibrinolytic capacity of M23.

Based on these in vitro data, M23 has a higher fibrinolytic than thrombin inhibitory potency. The concentrations needed for lysis of human thrombi in the Chandler Loop system were much lower than the concentrations that inhibited thrombin-induced coagulation or platelet aggregation (0.5 vs. 10.1 or 37.2 μg/ml). Thus, it had to be shown whether the two components of M23 in fact worked together after in vivo administration. If the two activities were cooperative, the combination of fibrinolytic and thrombin inhibitory properties in M23 should result in a superior lytic potency in arterial thrombolysis compared to a purely fibrinolytic agent. There is much evidence from experimental studies that thrombin inhibition increases the effects of arterial thrombolysis. Anti-thrombin agents increase the rate of arterial re-canalization, shorten the time to reperfusion, augment the height of reperfusion blood flow, and prevent, or reduce the rate of, re-occlusion (Zoldhelyi et al., 1992; Schneider, 1991; Haskel et al., 1991; Sitko et al., 1992; Yao et al., 1992).

We therefore investigated the effect of M23 in an arterial thrombolysis model in rabbits. A previous study with this experimental model demonstrated dose-dependent lytic effects of saruplase, which could be enhanced by heparin and the direct thrombin inhibitor, argatroban (Schneider, 1991). An intermediate dose of 6 mg/kg saruplase, arrived at from this earlier study, was used, and its effects were compared to the effects of the same dose of M23. In order to evaluate a possible advantage of M23 over saruplase due to its additional thrombin inhibitory activity, the animals were not anti-coagulated. We found an enhanced dissolution of ^{125}I-fibrin radioactivity of the thrombi and an accelerated reperfusion in M23-treated rabbits. Reperfusion rates were submaximal and re-occlusion rates were low in saruplase-treated rabbits. In M23-treated animals, however, all thrombotically occluded femoral arteries were re-canalized, and there was no re-occlusion at all. In addition to these differences, M23 treatment yielded a significantly higher reperfusion blood flow than saruplase treatment. Enhancement of reperfusion blood flow after thrombolysis with saruplase or tissue type plasminogen activator by conjunctive antithrombotic treatment has been described in a similar manner (Schneider, 1991; Sitko et al., 1992). Thus, these results suggest that M23, at least in the rabbit model, exerts a higher thrombolytic efficacy than saruplase, and that this superiority is attributable to the additional thrombin-inhibitory activity.

Another aspect of combining fibrinolytic and thrombin inhibitory moieties in M23 is to target this agent to a thrombus via its affinity to clot-bound thrombin. In a comparable way, affinity for immobilized thrombin was reported for a covalent complex of streptokinase to recombinant hirudin (Phaneuf et al., 1994). Here we demonstrate a highly clot-specific lysis by the bifunctional molecule, M23, that may be attributable to the thrombin affinity of its thrombin inhibitory moiety in both a human in vitro system and an experimental animal in vivo model. In vitro, the lower systemic plasminogen activation of M23 compared to saruplase was shown by the significantly higher concentrations of M23 required to reduce the levels of plasma α_2-antiplasmin; reduction of plasma α_2-antiplasmin is a sensitive measure of systemic plasmin generation. In the in vivo model, the high clot-specific activity of M23 was supported by the finding of only minor reductions of circulating fibrinogen concentrations in comparison to the strong depletion observed in saruplase-treated rabbits. These results again suggest that plasminogen activation by M23 is mainly restricted to the site of the thrombus.

It may be surprising that this clot-specific fibrinolytic activity was found at concentrations about one order of magnitude lower than those needed for prolongation of thrombin clotting time. The affinity to the thrombin anion-binding exosite may account for the thrombus selectivity, whereas much higher concentrations of M23 are necessary to inhibit the catalytic activity of thrombin.

The interesting pharmacological profile of M23 will have to be confirmed in other experimental animal species and models. It is tempting to speculate that the clot specificity of M23, and the low systemic lytic effect resulting therefrom, can reduce the risk of bleeding, which is a major complication of thrombolytic therapy. Further thrombosis/thrombolysis experiments in dogs support the idea that M23 bears a low risk of bleeding (manuscript in preparation). In conclusion, the bifunctional urokinase variant, M23, combines fibrinolytic and thrombin-inhibitory properties. Thrombin inhibition by M23 is shown in vitro as inhibition of thrombin-induced coagulation of human plasma and inhibition of thrombin-induced aggregation of human platelet rich plasma. In an arterial thrombosis model in the rabbit, M23 exerts very efficient thrombolysis. In vitro lysis of human thrombi, and arterial thrombolysis in rabbits demonstrate a high clot specificity of M23 with only minor systemic lytic effects.

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