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# Synergy of *in vitro* Thrombolytic Action of Combinations of Recombinant Staphylokinase and Single-Chain Urokinase-Type Plasminogen Activator

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**Abstract**—Kinetics of lysis of human plasma clots immersed in plasma were studied *in vitro* at 37°C under the influence of recombinant staphylokinase, single-chain urokinase-type plasminogen activator (scu-PA), and their simultaneous and consecutive combinations. Staphylokinase and scu-PA caused concentration- and time-dependent lysis of the clots; 32 nM staphylokinase and 75 nM scu-PA separately caused 50% lysis in 4 h. At these equally effective concentrations staphylokinase in 4 h induced a significantly lesser exhaustion of the plasma plasminogen, $\alpha_2$-antiplasmin, and fibrinogen than scu-PA. Combinations of staphylokinase (<30 nM) and scu-PA (<75 nM) rendered synergic thrombolytic action on the clots. The synergy of thrombolytic action was more pronounced on the simultaneous addition of the two agents than on their consecutive addition, scu-PA 30 min after staphylokinase. In 4 h after the addition, staphylokinase (25 nM) or scu-PA (15 nM) induced 24% and 2% lysis, respectively, whereas the simultaneous and consecutive combination of the same concentrations of these agents induced 58% and 50% lysis, respectively. The simultaneous combination of 15 nM staphylokinase and 15 nM scu-PA resulted in maximal 3.8-fold increase in the thrombolytic effect as compared to the expected total effect of the individual agents. Synergic combinations of the two agents caused lesser exhaustion of plasma plasminogen, $\alpha_2$-antiplasmin, and fibrinogen as compared with the expected total effect of these agents used separately. Thus, simultaneous and consecutive combinations of staphylokinase and scu-PA in a relatively narrow range of their concentrations possessed synergistic fibrin-selective thrombolytic action on the plasma clot *in vitro*.

*Key words*: staphylokinase, single- and two-chain urokinase-type plasminogen activators, combined effect, thrombolysis, synergy, fibrinogenolysis

Plasminogen activators used in therapy of thromboembolic diseases convert endogenous plasminogen to plasmin, which directly dissolves the fibrin of a thrombus. Streptokinase (SK) and two-chain urokinase-type plasminogen activator (tcu-PA), which have no affinity for fibrin, activate circulating and fibrin-bound plasminogens at similar rates [1, 2]. The systemic activation of plasminogen (plasma concentration of 1.5-2.0 μM) into plasmin results in exhaustion of the physiological plasmin inhibitor $\alpha_2$-antiplasmin (plasma concentration 1 μM) [1, 3]. The excess of broad-specificity plasmin [4] can destroy some plasma proteins including fibrinogen and factors V and VIII and cause hemorrhagic complications [1, 2]. Fibrin-specific plasminogen activators, such as tissue plasminogen activator (t-PA) and to a lesser degree a single-chain urokinase-type plasminogen activator (scu-PA) activate plasminogen on the surface of fibrin [5-7]. However, therapeutic high doses of these fibrin-specific agents induce systemic activation of plasminogen. Introduction of streptokinase or t-PA, which are more often used in treatment of acute myocardial infarction, results in a practically complete or 50%, respectively, exhaustion of fibrinogen, and the full opening of the artery is obtained only in 50-57% of cases [8, 9]. The hope for increase in the efficiency of thrombolysis is associated with recently elaborated plasminogen activators that have the greater specificity for fibrin or improved pharmacokinetic properties. The new generation of plas-

*Abbreviations*: Pg) plasminogen; Pm) plasmin; STA) staphylokinase; SK) streptokinase; Pg–STA and Pm–STA) equimolar complexes plasminogen–staphylokinase and plasmin–staphylokinase, respectively; Pg–SK and Pm–SK) equimolar complexes plasminogen–streptokinase and plasmin–streptokinase, respectively; scu-PA and tcu-PA) single-chain and two-chain urokinase-type plasminogen activators, respectively; $\alpha_2$-AP) $\alpha_2$-antiplasmin; PAI-1) inhibitor of plasminogen activators; t-PA) tissue plasminogen activator.

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minogen activators includes mutants of tissue plasminogen activator, such as rt-PA, TNK-t-PA, and lanoteplase, and also plasminogen activator from the saliva of vampire bat (DSPA) and recombinant staphylokinase (STA) [1, 2, 9-11]. In experiments on animals staphylokinase was shown to be a promising thrombolytic agent [2, 12]. Similarly to streptokinase, staphylokinase indirectly activates plasminogen. It forms with plasminogen an inactive stoichiometric complex Pg–STA, which is converted to the Pm–STA complex capable of activating the excess plasminogen [13]. Staphylokinase has no affinity for fibrin. The mechanism of fibrin-selectivity of its action is due to the difference in the rates of inhibition of the Pm–STA complex with $\alpha_2$-antiplasmin in plasma ($k_i$ = $2.7 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$) and on the fibrin surface ($k_i$ = $2.4 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$) [14]. Recent clinical testing on patients with thromboembolic diseases has shown a high thrombolytic potential of staphylokinase alongside with low level of systemic effects [13, 15, 16]. However, as an alien protein, it causes the production of staphylokinase-neutralizing antibodies [13, 17].

Because an ideal thrombolytic agent is absent, it is necessary to optimize the ratios of risk/benefit and cost/benefit. The use of synergic combinations of different plasminogen activators is one of the approaches to increase the efficiency of thrombolysis. Synergy that was first detected *in vitro* between t-PA and scu-PA [18] was afterwards confirmed by experiments on animals and by therapy of patients with acute myocardial infarction [19, 20]. Due to synergy of two agents, their total dose required for recanalization of the coronary artery could be two-threefold decreased, and this also decreased the level of systemic effects [21]. Later tcu-PA [22] and streptokinase [23] were shown to potentiate the thrombolytic effect of scu-PA.

Thrombolytic effects of combinations of staphylokinase with other agents have not been studied. Because mechanisms of fibrin-specific effects of staphylokinase and scu-PA are different, combinations of these agents were expected to be synergic. This work was designed to study the potential synergy of staphylokinase and scu-PA and assess the fibrin-specificity of the combined effect of both agents.

## MATERIALS AND METHODS

The reagents used were as follows: Lys-plasminogen, human fibrinogen, and human thrombin (Enterprise for Diagnostic and Pharmaceutical Preparations, Minsk, Belarus); streptokinase (47 kD, 6300 IU/mg dry weight) from Reyon Pharmaceutical Co. Ltd. (Korea); solution of highly purified recombinant staphylokinase (SAK-STAR, 15.5 kD, 0.98 mg/ml) from Center for Molecular and Vascular Biology (University of Leuven, Belgium); recombinant urokinase-type single-chain plasminogen activator (50 kD, 100,000 IU/mg protein) from the Laboratory of Gene Engineering, Institute of Experimental Cardiology, Russian Cardiology Center, Ministry of Health (Moscow, Russia); ancistron from venom of the *Agkistrodon halys* snake (Tekhnologiya-Standart, Russia); pancreatic trypsin inhibitor (Gedeon Richter, Hungary); H,D-Val-Leu-Lys-pNA dihydrochloride (S-2251) (Sigma, USA); lyophilized standard human blood plasma (Diagnostico Stago, France); pool of human blood plasma (Hematology Research Center, Ministry of Health, Moscow, Russia).

The main buffer solution was 0.05 M Tris-HCl buffer (pH 7.4) with 0.15 M NaCl (buffer A) which in some experiments also contained other components.

**Plasmin was prepared** by activation of 0.2 μM Lys-plasminogen with a catalytic concentration of streptokinase ([Pg]/[SK] = 100 : 1 (M/M)) in buffer A supplemented with 20% glycerol (v/v) and 0.01% Tween 80 at 37°C. Completeness of the zymogen conversion to the enzyme was determined by the maximum amidase activity of samples taken. The resulting plasmin solution was divided into aliquots and kept at –20°C.

**The amidase activity** of plasmin and stoichiometric complexes Pm–STA, Pm–SK, and Pg–SK was determined by the initial rate of hydrolysis of 0.6 mM S-2251 in buffer A at 25°C, taking the molar coefficient of *p*-nitroaniline absorption at 405 nm and pH 7.4 for 10,000 $M^{-1} \cdot cm^{-1}$. The final enzyme concentration in the cuvette was 10-100 nM. The specific amidase activity was expressed as the change in absorption of the substrate solution at 405 nm in 1 min caused by 1 mg of the enzyme.

**Thrombolytic and side effects** of staphylokinase, scu-PA, and their simultaneous and consecutive combinations were studied *in vitro* based on determination of the rate of lysis of the solid-phase plasma clot and of decrease in the levels of plasminogen, fibrinogen, and $\alpha_2$-antiplasmin in the plasma above the clot under the influence of individual agents or their combinations. For assessment of these effects columns of the plasma gel were formed in standard tubes ($d$ = 9.5 mm) by addition of 20 μl of thrombin solution (40 units/ml) to 0.8 ml of human plasma. The tubes were shaken and left upright for 1.5-2 h at 25°C. To the resulting plasma clots, 0.45 ml of human plasma was added and the tubes were incubated at 37°C for 15 min.

*Fibrinolysis* was initiated by addition of 50 μl of staphylokinase, scu-PA, or their mixture (on simultaneous combination). In the case of consecutive combination, scu-PA was added 30 min after the addition of staphylokinase. Effects of the individual agents were determined at the plasma concentrations of staphylokinase from 10 to 100 nM and those of scu-PA from 15 to 150 nM. In combinations, the concentrations of the agents varied in the range of 10-50 nM (staphylokinase) and 15-100 nM (scu-PA). The fibrinolysis kinetics were

followed by decrease with time in the height of the gel column (mm) at 37°C using a cathetometer [24]. Each experiment was thrice repeated.

To assess the side effects of various concentrations of the individual agents and their simultaneous or consecutive combinations, residual concentrations of plasminogen, fibrinogen, and $\alpha_2$-antiplasmin were determined in the plasma samples taken during the fibrinolysis.

*Determination of plasminogen concentration in plasma.* The method is based on addition to plasma of excess streptokinase, which stoichiometrically binds the total plasma plasminogen with production of an activator complex Pg–SK. The amidase activity of the Pg–SK complex is proportional to plasminogen concentration in the plasma, because under experimental conditions inhibitors of plasma fail to affect the activity of the complex.

The plasma specimens under study were diluted sixfold in buffer A. Into a well of a 96-well plate 50 μl of the diluted plasma and 50 μl of streptokinase solution in buffer A (5000 IU/ml) were introduced (into the control well instead of streptokinase 50 μl of buffer A was added), incubated for 4 min at 25°C, and then 50 μl of 2 mM S-2251 was added. After 2 min the substrate hydrolysis was stopped by addition of 50 μl of 50% acetic acid, and the absorption of the specimen was determined compared to the control at 405 nm using an Anthos 2020 microplate reader (Austria). The experiment was thrice performed with each specimen. The content of plasminogen in the specimen was determined by calibration curve obtained with solutions of purified plasminogen (0.1-3.2 μM).

*Determination of $\alpha_2$-antiplasmin in plasma.* The method is based on addition to the plasma of excess plasmin, which stoichiometrically binds the total plasma $\alpha_2$-antiplasmin with production of inactive enzyme–inhibitor complex, with a subsequent determination of activity of the residual plasmin, which is inversely proportional to the inhibitor concentration in plasma.

Specimens of the plasma under study were eightfold diluted in buffer A that contained 0.12 M methyl amine and 0.015 M sodium azide. Into a well of the plate 50 μl of the diluted plasma, 50 μl of 0.2 μM plasmin in buffer A containing 20% glycerol (v/v), and 0.01% Tween 80 were added (into the control well instead of plasmin 50 μl of the same buffer was added), the mixture was incubated for 2 min at 25°C and supplemented with 50 μl of 2 mM S-2251. After 4 min the substrate hydrolysis was stopped by addition of 50 μl of 50% acetic acid, and the specimen absorption was determined compared to the control at 405 nm. The experiment was thrice performed with each specimen. The content (in %) of $\alpha_2$-antiplasmin in the plasma was determined by the calibration curve obtained for different dilutions of standard plasma with known content of $\alpha_2$-antiplasmin.

*Determination of fibrinogen concentration in plasma.* The method is based on determination of the rate of plasma fibrinogen conversion to fibrin under the influence of ancistron. The rate of increase in turbidity is proportional to concentration of fibrinogen in the plasma.

Plasma specimens under study were fivefold diluted in buffer A containing pancreatic trypsin inhibitor (0.01 mg/ml). Into a well of the plate 250 μl of the diluted plasma was introduced, then 50 μl of ancistron solution (0.18 unit/ml) was added, and increase in turbidity of the solution was determined at 405 nm every 2 min with an Antos 2020 microplate reader. The rate of fibrin production was determined as the tangent of the slope angle of the linear region of the curve, and the fibrinogen concentration in the specimen was calculated using the calibration curve obtained for solutions of purified human fibrinogen (0.2-5.0 mg/ml). The experiment was thrice performed with each specimen.

## RESULTS

Figure 1 shows the kinetics of lysis of plasma clots under the influence of staphylokinase (a) and scu-PA (b). No spontaneous lysis of the clots was recorded during 6 h of observation, whereas the concentration- and time-dependent fibrinolysis occurred in the presence of both agents. Figure 1 presents dependences of the clot lysis degree for 2 and 4 h on concentrations of staphylokinase (c) and scu-PA (d). The concentrations which caused the 50% lysis in 2 and 4 h were 70 and 32 nM staphylokinase, respectively, and 135 and 75 nM scu-PA, respectively.

Figure 2 presents curves of decrease in the plasma fibrinogen level during lysis of clots under the influence of varied concentrations of staphylokinase (a) and scu-PA (b). From these data the dependences of the residual fibrinogen in 2 and 4 h of the lysis on the concentration of staphylokinase (c) and scu-PA (d) were obtained (Fig. 2). Obviously, during the clot lysis scu-PA destroyed fibrinogen in the surrounding plasma stronger than staphylokinase. At equally effective concentrations of these agents, in 4 h the plasma fibrinogen level was decreased 54% in the presence of scu-PA and 27% in the presence of staphylokinase.

The combined thrombolytic effects of staphylokinase and scu-PA were studied on both their simultaneous and consecutive additions into the plasma surrounding clot. Figure 3a presents kinetic curves of the clot lysis initiated by the simultaneous and consecutive combinations of 15 nM staphylokinase and 15 nM scu-PA. On the simultaneous addition of the two agents (curve *5*) the lag-phase was shortened and the rate of clot lysis was significantly increased. The fibrinolytic effect of this combination observed in 4 h after the introduction of the agents was 3.8-fold higher than the expected effect (curve *3*) resulting by summation of the effects of the individual agents (curves *1* and *2*). The potentiating

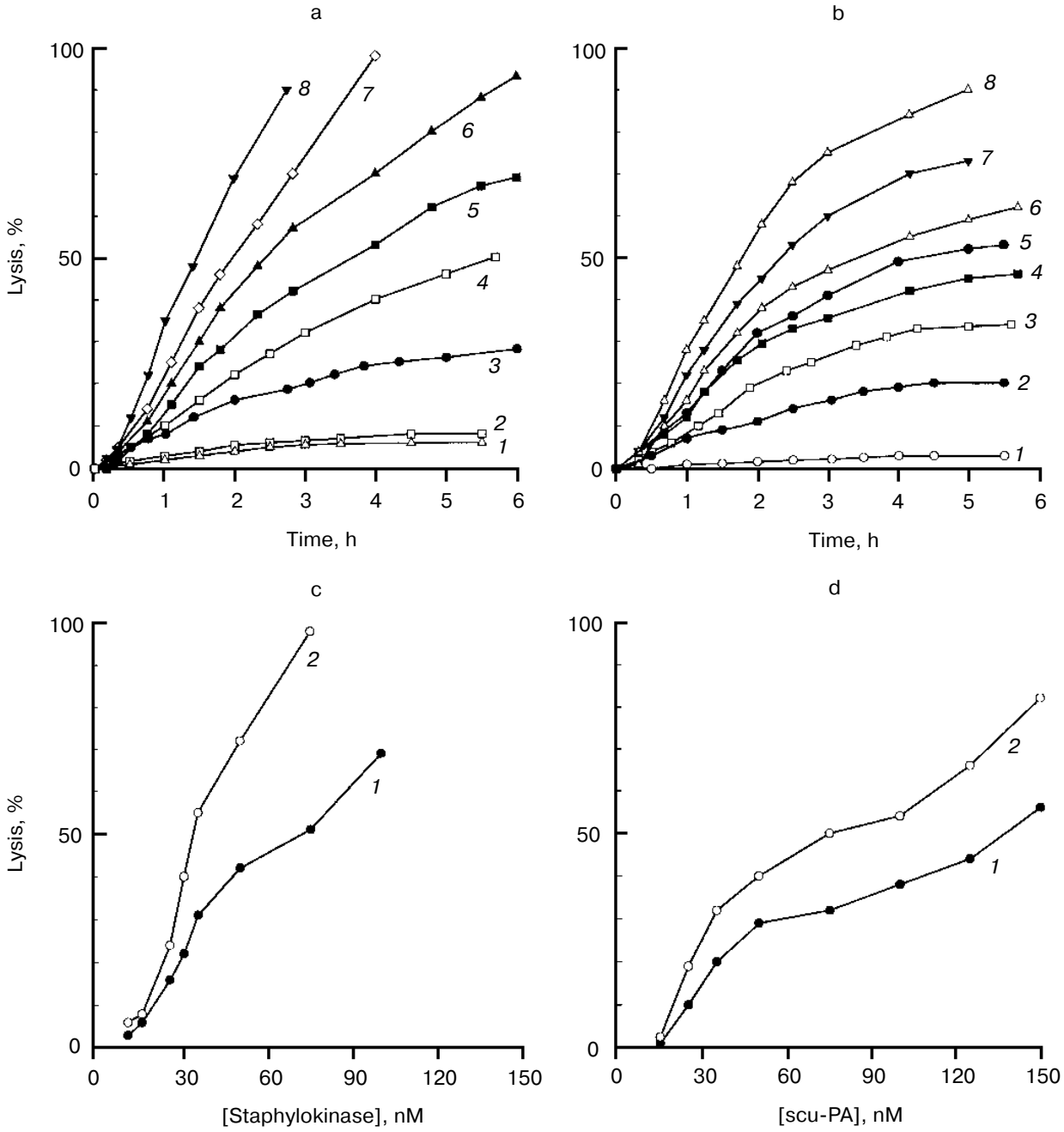



**Fig. 1.** Kinetics of lysis of plasma clots immersed in plasma under the influence of staphylokinase (a) and scu-PA (b). Staphylokinase concentrations were 10 (*1*), 15 (*2*), 25 (*3*), 30 (*4*), 35 (*5*), 50 (*6*), 75 (*7*), and 100 nM (*8*); scu-PA concentrations were 15 (*1*), 25 (*2*), 35 (*3*), 50 (*4*), 75 (*5*), 100 (*6*), 125 (*7*), and 150 nM (*8*). c, d) Dependences of the clot lysis degree on the concentrations of staphylokinase (c) and scu-PA (d) after 2 (*1*) and 4 h (*2*) ($p < 0.001$).

effect of fibrinolysis was somewhat lower on introduction of scu-PA 30 min after staphylokinase (curve *4*) than in the case of their simultaneous introduction. Both combinations of staphylokinase and scu-PA caused less destruction of the plasma fibrinogen than was expected based on additivity of their separate effects (Fig. 3b). However, the synergic fibrinolytic effect of the consecutive combination of the two agents was accompanied by a significantly lower degradation of fibrinogen.

Fibrinolytic effects of simultaneous and consecutive combinations of staphylokinase and scu-PA were studied at different concentrations of both agents at molar ratios of STA/scu-PA varied from 1 : 10 to 3.3 : 1. The interaction

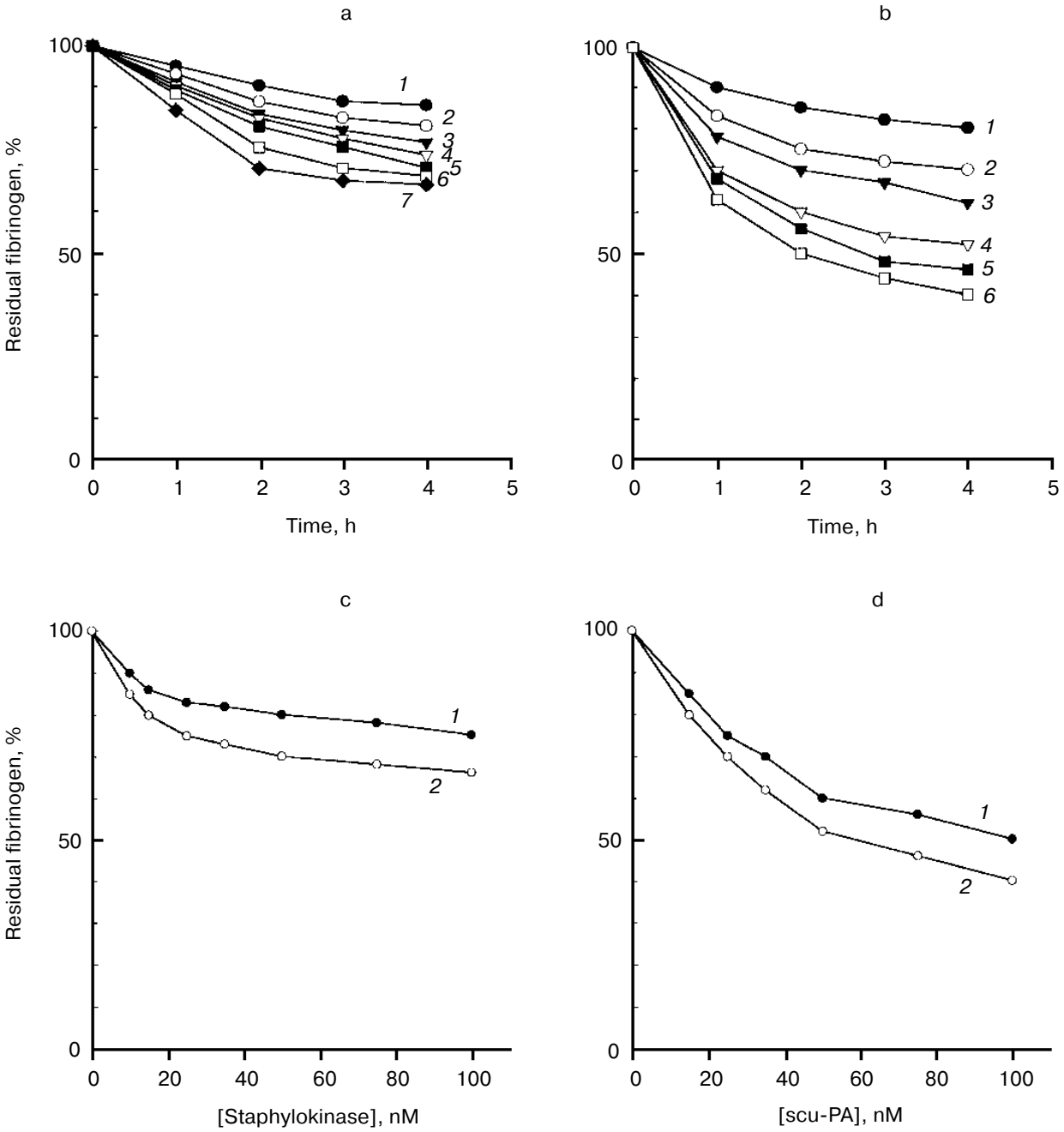



**Fig. 2.** Kinetics of decrease in the plasma fibrinogen concentration during lysis of the plasma clots under the influence of staphylokinase (a) and scu-PA (b). Concentrations of staphylokinase were 10 (*1*), 15 (*2*), 25 (*3*), 35 (*4*), 50 (*5*), 75 (*6*), and 100 nM (*7*); concentrations of scu-PA were 15 (*1*), 25 (*2*), 35 (*3*), 50 (*4*), 75 (*5*), and 100 nM (*6*). c, d) Residual concentrations of fibrinogen after 2 (*1*) and 4 h (*2*) depending on the concentration of staphylokinase (c) and scu-PA (d) ($p < 0.001$).

between staphylokinase and scu-PA during the clot lysis was analyzed using an algebraic fractional method [25]:

$$A/A_e + B/B_e = C, \quad (1)$$

where $A_e$ and $B_e$ are equally effective doses of staphylokinase and scu-PA which separately induced the same fibrinolytic effect in 4 h; $A$ and $B$ are concentrations of these agents in the combination which induced the same fibrinolytic effect in 4 h. If $C < 1$ the combination is synergic, $C = 1$ with additivity, and $C > 1$ with antagonism of the two agents.

Synergic fibrinolysis of different efficiency (the lysis degree in 4 h was increased 1.3-3.8-fold compared to

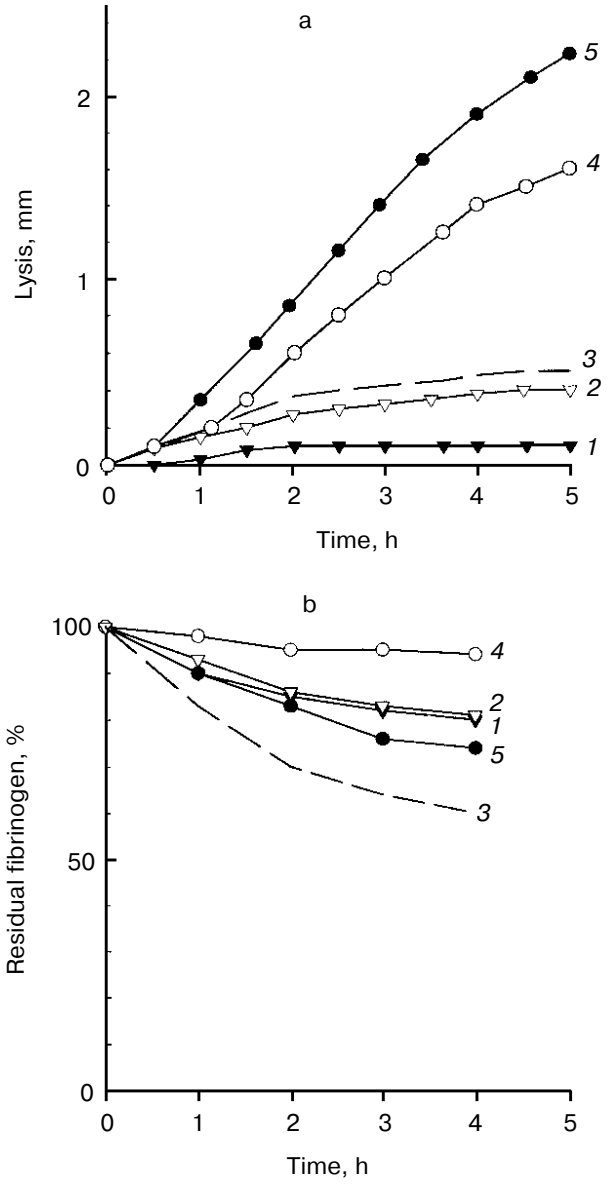



**Fig. 3.** Kinetics of lysis of the plasma clots (a) and decrease in the fibrinogen level in the surrounding plasma (b) under the influence of 15 nM scu-PA (*1*), 15 nM staphylokinase (*2*), and their consecutive (*4*) and simultaneous (*5*) combination. The broken lines (*3*) show the expected total effects of the same concentrations of these agents ($p < 0.001$).

expected values) was found for the combinations in which the staphylokinase concentration was not higher than 30 nM and that of scu-PA was not higher than 75 nM, and the STA/scu-PA ratio was changed in the range from 1 : 2.5 to 1.7 : 1. The sum of algebraic fractions (*C*) for these combinations was 0.5-0.85 and 0.7-0.95 at the simultaneous and consecutive introduction of the agents, respectively. At the above-mentioned ratios of STA/scu-PA the simultaneous combinations of staphylokinase and scu-PA induced the more pronounced synergic fibrinolytic effect than the consecutive combinations.

All synergic combinations of staphylokinase and scu-PA resulted in the lower activation of plasminogen, destruction of fibrinogen, and exhaustion of $\alpha_2$-antiplasmin in the surrounding plasma than expected based on summation of the effects of concentrations of the individual agents used in the combinations. And the consecutive combinations resulted in lesser exhaustion of the plasma proteins. With increase in the scu-PA content in the combination and in the total concentration of both agents, the synergy of thrombolytic effects of the combinations decreased and the systemic effects increased, although remained lower than the expected values (table).

## DISCUSSION

The synergy of thrombolytic effects of scu-PA combinations with other plasminogen activators seems to be due to activation by t-PA, tcu-PA, and streptokinase of Glu-plasminogen molecules bound to the intrachain lysines of intact fibrin and, as a result, the initiation on its surface of generation of COOH-terminal lysine residues. scu-PA selectively activates molecules of Glu-plasminogen bound to these new sites [7, 21, 26]. This hypothesis is supported by the finding that the pretreatment of plasma clots with plasmin increased the fibrinolytic effect of scu-PA [21]. A thrombin-activatable fibrinolysis inhibitor (TAFI), known as procarboxypeptidase B, has been recently discovered and is now being intensively studied. The proenzyme is converted with thrombin into active enzyme TAFIa during the blood clotting. TAFIa inhibits fibrinolysis eliminating COOH-terminal lysine residues from the degrading clot and, thus, decreases the cofactor function of fibrin in the activation of Glu-plasminogen with tissue plasminogen activator. At relatively high concentrations TAFIa can directly inhibit plasmin [27-29]. Despite the inhibitory effect of TAFIa, combinations of scu-PA with t-PA or other plasminogen activators provide for increased lysis of the plasma clots. In particular, this can be due to the recently found ability of plasmin to regulate the activity of TAFIa [30]. Plasmin can partially activate TAFI, but the TAFIa activity is unstable at 37°C. Proteolysis of TAFIa with plasmin results in its inactivation with production of a fragment with molecular weight of 44.3 kD. A similar fragment of TAFIa was recorded during lysis of the plasma clots with t-PA and in the plasma of patients with myocardial infarction treated with t-PA [30]. It would be suggested that on the combined effect of plasminogen activators, triggers of fibrinolysis (t-PA, tcu-PA, and streptokinase) activate molecules of Glu-plasminogen bound to intrachain lysines of intact fibrin, and TAFIa should not influence this first phase of fibrinolysis. The plasmin generated concurrently degrades fibrin and TAFIa that results in inactivation of

Lysis degree of plasma clots and residual levels of fibrinogen, plasminogen, and $\alpha_2$-antiplasmin in the surrounding plasma in 4 h after the simultaneous and consecutive* (values in parentheses) addition of staphylokinase and scu-PA

| Effect, % | | Combinations of STA + scu-PA, nM | | |
|---|---|---|---|---|
| | | 15 + 15 | 15 + 25 | 15 + 35 |
| Lysis degree | observed | 38 ± 4 (28 ± 3) | 53 ± 3 (35 ± 3.5) | 70 ± 3.1 (49 ± 3.2) |
| | expected | 10 | 28 | 42 |
| Residual fibrinogen | observed | 78 ± 2 (94 ± 2.7) | 70 ± 2.1 (85 ± 2.2) | 65 ± 2.3 (82 ± 2.6) |
| | expected | 60 | 50 | 45 |
| Residual plasminogen | observed | 60 ± 2 (68 ± 2.5) | 55 ± 2.2 (63 ± 2.4) | 35 ± 2 (45 ± 2.2) |
| | expected | 48 | 35 | 15 |
| Residual $\alpha_2$-antiplasmin | observed | 45 ± 3 (55 ± 2.7) | 28 ± 2.5 (35 ± 3) | 20 ± 2.8 (30 ± 3) |
| | expected | 35 | 15 | 10 |

Note: Residual concentrations of the proteins are expressed in % of their initial levels in the plasma. The expected effects are obtained by summation of the effects of concentrations of the individual agents used in the combinations.

* scu-PA was introduced 30 min after treatment of the clots with staphylokinase ($p < 0.001$).

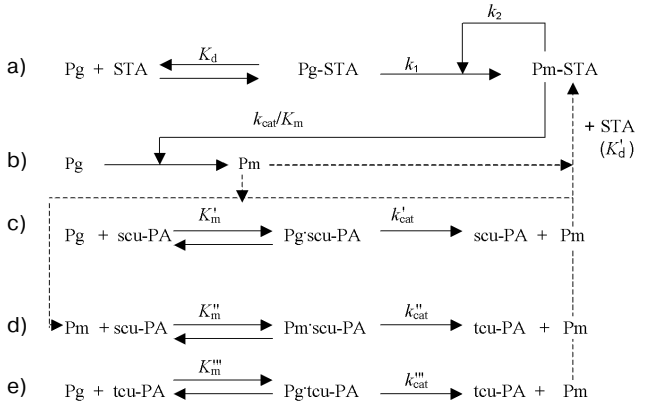
TAFIa and increase in the concentration of Glu-plasminogen on the clot whose activation with the triggers or scu-PA is the second, faster phase of fibrinolysis. Because mechanisms of plasminogen activation and fibrin-specificity of action of staphylokinase and scu-PA are different, we studied the ability of staphylokinase to potentiate the fibrinolytic effect of scu-PA on simultaneous and consecutive combinations of the two agents.

Staphylokinase and scu-PA initiated the concentration- and time-dependent lysis of the plasma clots: 32 nM staphylokinase and 75 nM scu-PA provided for 50% lysis in 4 h. At these equally effective concentrations staphylokinase caused insignificant activation of the plasma fibrinolytic system and fibrinogenolysis, whereas scu-PA more markedly decreased the plasma levels of plasminogen, $\alpha_2$-antiplasmin, and fibrinogen. Low concentrations of staphylokinase potentiated the fibrinolytic effect of scu-PA. The shortening of the lag-phase specific for both individual agents and increase in the lysis rate were concurrently observed. The simultaneous combination of 15 nM staphylokinase and 15 nM scu-PA resulted in the maximal 3.8-fold increase in the thrombolytic effect as compared to the total effect of the same concentrations of these agents used separately. The algebraic fractional analysis of the dose–effect curves has shown that the simultaneous introduction of staphylokinase and scu-PA caused the higher synergic effect than the consecutive introduction. Thus, 25 nM staphylokinase and 15 nM scu-PA added separately caused (in 4 h) 24% and 2% lysis, respectively, whereas their simultaneous and consecutive combinations resulted in 58% and 50% lysis, respectively. Staphylokinase and scu-PA were synergic in a relatively narrow range of concentrations. The concentration range for display of synergy of the thrombolytic effects was also limited in the case of scu-PA combinations with other plasminogen activators [18, 20, 22, 23]. With increase in the scu-PA concentration in the combination and in the total concentration of the two agents the synergy decreased, and the combinations containing >30 nM staphylokinase and >75 nM scu-PA rendered additive effects on the clot. Simultaneous and consecutive synergic combinations of staphylokinase and scu-PA were fibrin-selective, because of fibrinogenolysis caused by them was lower than the expected total effect of the corresponding concentrations of these agents used separately.

On the combined effect of these agents staphylokinase initiates the first phase of lysis of the intact clot that results in a partial degradation of fibrin. Depending on conditions, plasminogen can have three different conformations: α, β, and γ [31]. It was suggested that plasminogen in the circulation should have a closed α-conforma-

tion, plasminogen bound to intact fibrin should have a half-closed β-conformation, and plasminogen bound to the partially degraded fibrin should have a fully open γ-conformation [29, 31]. Different sensitivity to activation of these plasminogen conformations partially explains the mechanism of fibrin-selectivity of scu-PA and staphylokinase. The activity of scu-PA itself is only 0.4% of the tcu-PA activity, but it is sharply increased with regard to plasminogen bound to the partially degraded fibrin [21, 32]. In other words, scu-PA recognizes not the α- and β-, but the γ-conformation of plasminogen. And just this explains the fibrin-selectivity of the scu-PA action. The fibrin-selectivity also depends on its activation with plasmin in tcu-PA on the fibrin surface [7]. The main mechanism of fibrin-selectivity of the staphylokinase action is associated with the difference in the rates of inhibition of the Pm–STA complex in the circulation and on the fibrin surface [14]. The higher affinity of staphylokinase for plasmin and the γ-conformation of plasminogen than for its α- and β-conformations is an additional mechanism [13, 33].

Based on the current data on mechanisms of scu-PA and staphylokinase action, the synergy of their effects can be explained as follows. Staphylokinase (15.5 kD) diffuses into the surface layer of the clot (100-150 μm) and binds to molecules of Glu-plasminogen ($K_d$ = 7.4 μM) which are bound to intrachain lysines of intact fibrin [33]. Initial trace concentrations of the active complex Pm–STA produced from the Pg–STA complex (a) ($k_1$ = $4 \cdot 10^{-7}$ $sec^{-1}$ [34]) trigger the chain of reactions on the fibrin surface:




The Pm–STA complex catalyzes both its own generation from the inactive Pg–STA complex (a) ($k_2$ = $1.3 \cdot 10^{-2}$ $\mu M^{-1} \cdot sec^{-1}$ [34]) and the conversion of excess plasminogen to plasmin (b) ($k_{cat}/K_m$ = 0.27 $\mu M^{-1} \cdot sec^{-1}$ [35]). The plasmin produced specifically cleaves peptide bonds formed by carboxyl groups of lysine. As a result, on the surface of partially degraded fibrin COOH-terminal residues of lysine are generated in increasing amounts. On binding to these sites, Glu-plasminogen takes the open γ-conformation, which is a preferable substrate for scu-PA [29, 32]. Thus, staphylokinase causes the accumulation of Glu-plasminogen on the fibrin surface and, thus, accelerates its activation under the influence of scu-PA (c). Plasmin produced on activation of plasminogen under the influence of Pm-STA (b) and scu-PA (c) converts scu-PA to tcu-PA (d) ($k''_{cat}/K''_m$ = 0.046 $\mu M^{-1} \cdot sec^{-1}$) which activates plasminogen more efficiently (e) ($k'''_{cat}/K'''_m$ = 0.1 $\mu M^{-1} \cdot sec^{-1}$) than scu-PA (c) ($k'_{cat}/K'_m$ = 0.001 $\mu M^{-1} \cdot sec^{-1}$) [36]. The reactions (b)-(e) result in increase in the concentration of plasmin that has a positive inverse effect. On one hand, the concentration of the catalytically more effective tcu-PA gradually increases, and, on the other hand, the plasmin produced binds free staphylokinase molecules from the plasma because their binding to plasmin is stronger ($K'_d$ = 0.05 μM) than to plasminogen ($K_d$ = 7.4 μM) [33]. Incidentally an active complex Pm–STA is immediately produced without the slow stage of activation of the Pg–STA complex. The increase in the Pm–STA concentration additionally accelerates the reactions (a)-(e). Such a complementary effect of low concentrations of staphylokinase and scu-PA significantly increases the rate of the clot lysis. The lower synergy on the consecutive combination of the two agents seems to be due to the delayed (30 min later) switching of the reactions (c)-(e) which produce tcu-PA and increase the concentration of plasmin. Increase in the concentration of scu-PA in the combination leads to gradual decrease in synergy effect up to the additive effect of two agents on the clot that seems to be associated with a rapid increase in the concentration of non-fibrin-specific tcu-PA produced.

The plasma contains 1 μM $\alpha_2$-antiplasmin and 0.4 nM PAI-1 (the specific inhibitor of tcu-PA and scu-PA) [3]. The Pm–STA complex is rapidly neutralized with $\alpha_2$-antiplasmin in the plasma and 100-fold more slowly on the fibrin surface [14]. Therefore, the fibrinolytic effect of staphylokinase is associated with a low level of fibrinogenolysis. Concentrations of scu-PA that cause a measurable lysis (>15 nM) are higher than plasma concentrations of PAI-1. During fibrinolysis, scu-PA in the plasma can be partially converted into tcu-PA, and the latter activates the plasma plasminogen because of the low content of PAI-1. This is the explanation for the higher level of fibrinogenolysis caused with scu-PA as compared to staphylokinase.

On the combined introduction of the two agents into the plasma surrounding the clot, staphylokinase initiates increase in the concentration of scu-PA bound to Glu-plasminogen molecules on the clot surface. On the simultaneous addition of combinations with low content of scu-PA this binding is likely to noticeably decrease the plasma concentration of scu-PA, that entails the decrease in the rates of tcu-PA generation and activation of plasminogen in the plasma, and consequently, the rate of fibrinogenolysis. On the consecutive addition of scu-PA and staphylokinase, the effect of scu-PA and tcu-PA on the

plasma plasminogen starts later (after 30 min), and therefore, fibrinogenolysis observed after 2 or 4 h is lower than in the case of simultaneous addition.

Synergic combinations of staphylokinase and scu-PA *in vitro* caused the noticeable decrease (although less than expected) in the levels of plasminogen, $\alpha_2$-antiplasmin, and fibrinogen in a small volume of the plasma surrounding the clot. It seems that the systemic activation of fibrinolysis should be negligibly low *in vivo* because of rapid clearance of staphylokinase and scu-PA from the circulation. Further studies are promising for assessment of synergy and fibrin-selectivity of the *in vivo* thrombolytic effect of staphylokinase and scu-PA combinations. The synergy of action of thrombolytic agents is very important for practice. The use of synergic combinations of staphylokinase and scu-PA would significantly decrease the necessary total dose of the expensive scu-PA and antigenic staphylokinase, and this would decrease the systemic effects, the cost, and the risk of thrombolytic therapy.

Thus, the findings have shown the potentiating effect of staphylokinase on the *in vitro* lysis of the plasma clots under the action of scu-PA. Simultaneous combinations of low concentrations of both agents caused a stronger increase in the thrombolytic effect than their consecutive combinations. Both combinations of staphylokinase and scu-PA displayed the fibrin-selective synergic thrombolytic effect, but the consecutive addition of the agents was accompanied by a lower fibrinogenolysis.

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