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Polyethylene Glycol–Derivatized Cysteine-Substitution Variants of Recombinant Staphylokinase for Single-Bolus Treatment of Acute Myocardial Infarction

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Background—Thrombolytic therapy of acute myocardial infarction (AMI) is evolving toward bolus administration. Derivatization of proteins with polyethylene glycol (PEG) may reduce their clearance.

Methods and Results—A staphylokinase (SakSTAR) variant with 12 amino acid substitutions to reduce its antigenicity, SakSTAR (K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, K135R), and with Ser in position 3 mutated into Cys (code SY161), was derivatized with maleimide-PEG with M_r of 5000 (P5), 10 000 (P10), or 20 000 (P20). The PEGylated variants recognized only one third of the antibodies elicited with wild-type SakSTAR in AMI patients. In experimental animals, plasma clearances were reduced 2.5- to 5-fold with P5, 5- to 20-fold with P10, and 20-fold with P20, and bolus injection induced pulmonary plasma clot lysis at doses inversely related to their clearance. Intravenous bolus injection of 5 mg of the P5, P10, or P20 variants in AMI patients was associated with plasma half-lives ($t_{1/2\alpha}$) of 13, 30, and 120 minutes and clearances of 75, 43, and 8 mL/min, respectively, compared with 3 minutes and 360 mL/min for SakSTAR. Injection of 5 mg P5 variant restored TIMI-3 flow within 60 minutes in 14 of 18 AMI patients (78%, 95% CI 55% to 91%) and of 2.5 mg in 7 of 11 patients (63%, 95% CI 35% to 85%), both in the absence of fibrinogen degradation. The immunogenicity of the variants was significantly ($P < 0.002$) reduced.

Conclusions—The staphylokinase variant SY161-P5, derivatized with one linear polyethylene glycol molecule of M_r 5000, is a promising fibrin-selective agent for single-bolus coronary thrombolysis. (*Circulation*. 2000;102:1766-1772.)

Key Words: ■ myocardial infarction ■ thrombolysis ■ fibrinogen

Thrombolysis has become routine therapy for acute myocardial infarction (AMI), given worldwide to >750 000 patients per year.¹ Recent efforts to improve treatment have focussed on derivatives of tissue-type plasminogen activator (t-PA) that can be administered by bolus injection, including reteplase,² a domain deletion variant given as a double bolus, and TNK-tPA,³ generated by site-directed mutagenesis, given as a single bolus.

Staphylokinase (SakSTAR), a 136–amino acid profibrinolytic bacterial protein, is at least equipotent to t-PA for coronary artery recanalization, significantly more fibrin-selective and obtainable in high yield by cytosolic expression in *Escherichia coli*.⁴ However, it has a relatively short plasma half-life,⁵ and its administration induces neutralizing antibody formation in a majority of patients.^{6,7}

A comprehensive analysis of staphylokinase variants by sequential additive site directed mutagenesis identified several variants with intact specific activities and fibrin-selective thrombolytic potency, which recognized only one third of the antibodies elicited by treatment with wild-type staphyloki-

nase.⁸ SakSTAR (K35A, E65Q, K74R, D82A, S84A, T90A, E99D, T101S, E108A, K109A, K130T, K135R) (code SY155) with a thrombolytic potency comparable to that of wild-type SakSTAR in patients with peripheral arterial occlusion, was selected for further clinical development. Variants of wild-type SakSTAR are identified by the substituted amino acids in single letter symbols followed by their position number in the mature staphylokinase sequence (136 amino acids) and by the substituting amino acids in single-letter symbols. However, this component was cleared from plasma at a rate similar to that of wild-type SakSTAR.

The plasma clearance and the immunogenicity of heterologous proteins may be reduced by derivatization with polyethylene glycol (PEG).^{9,10} Usually, PEG molecules are covalently attached to proteins by lysine residues. Staphylokinase contains as many as 20 lysine residues, some of which are essential for its activity, but it does not contain cysteine. Therefore, PEGylated SakSTAR was produced by site-directed substitution of selected amino acids with cysteine and derivatization with linear PEG molecules contain-

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ing thiol-specific functional groups, yielding homogeneous end products with reduced clearances.¹¹

In the present study, Ser in position 3 of staphylokinase was mutagenized to Cys, which was specifically PEGylated with maleimide-PEG with M_r 5000 to 20 000. In vitro and in vivo evaluation indicated that monoPEGylation prolongs the circulatory half-life of staphylokinase while maintaining its thrombolytic potency when bolus injected at a reduced dose in patients with AMI.

Methods

Reagents and Methods

The template vector for mutagenesis, *pMEX602sakB* (ie, *pMEX.Sak-STAR*), and the recombinant DNA reagents and methods used have been described elsewhere.^{8,11,12}

Construction of Expression Plasmids

The variant SakSTAR(S3C, K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, K135R, (code SY161) was constructed by PCR, using as template the previously described⁸ *pMEX*-derivative encoding SY155. To introduce the cysteine in position 3, the fragments were amplified by cycling 30 times (5 seconds at 94°C, 5 seconds at 50°C, 10 seconds at 72°C) with the mutagenic primer LY459, 5'-CCTCATATGTCAAG-TTGTTTCGACAAAGGA (the modified bases are underlined) and the external 3' end primer 819D, 5'-CAGGCTGAAAATCTTC-TCTCATCCGCC. The products were purified, digested with *HindIII*, and cloned into the *Stul-HindIII* vector fragment of *pMEX-STAR*. Variant SY160 was constructed, which was identical to SY161 except for a Gln instead of an Arg substitution in position 74, starting from the *pMEX*-derivative encoding SY151.¹⁰

Expression and Preparative Purification of SakSTAR Variants

Eighteen-liter cultures (in 1- to 2-L batches) of SY161 and SY160 were grown for 20 hours in terrific broth medium¹³ supplemented with 100 µg/mL ampicillin and induced with 200 µmol/L IPTG during the last 3 hours. The cells were pelleted, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer, pH 6.0, disrupted by sonication, and cleared by centrifugation.

The compounds were purified on a 10×7 cm column of SP-Sepharose, equilibrated with 0.01 mol/L phosphate buffer, pH between 5.5 and 6.0, and eluted with a 1 mol/L NaCl gradient (3 column volumes). Fractions containing SakSTAR were pooled, solid NaCl was added to 2.5 mol/L, and the material was applied to a 10×20 cm column of phenyl-Sepharose, followed by stepwise elution with 0.01 mol/L phosphate buffer, pH between 5.5 and 6.0. After desalting on a 10×45 cm column of Sephadex G25, concentration on a 5×10 cm column of SP-Sepharose by elution with 1.0 mol/L NaCl, and gel filtration on a sterilized 6×60 cm column of Superdex 75 equilibrated with PBS buffer, pH 7.5, SakSTAR-containing fractions were pooled for derivatization with polyethylene glycol.

Chemical Cross-Linking of SakSTAR Cysteine Mutants With Polyethylene Glycol

The cysteine side chain was targeted with activated linear polyethylene glycol molecules with a M_r of 5 kDa, 10 kDa, or 20 kDa. Mal-PEG (Shearwater Polymers Europe) carries a maleimide group that reacts specifically with thiol groups under mild conditions. Derivatization was achieved by reduction with dithiothreitol, desalting on Sephadex G25, and incubating the molecule (100 µmol/L) with 3× Mal-PEG in 10 mmol/L phosphate buffer, pH 7.9, at room temperature. After reaction for 60 minutes, the mixture was desalted on Sephadex G25, concentrated on SP-Sephadex, and gel-filtered on Superdex G75. The PEGylated SakSTAR variant containing frac-

tions were pooled and adjusted to 1 mg/mL, and the material was sterilized by filtration through a 0.22-µm Millipore filter.

Analytical Methods

The methodology for in vitro evaluation, pharmacokinetics, and thrombolytic potency in hamsters has been described in detail elsewhere.^{5,14–16}

Thrombolytic and Immunogenic Properties of PEGylated SakSTAR Variants in Patients

Patients were studied after giving informed consent, and the protocol was approved by the Human Studies Committee of the University of Leuven, Belgium. Thirty-five patients with AMI (age ≤75 years), examined within 6 hours and with ST-segment elevation (≥1 mm in at least 2 limb or ≥2 mm in at least 2 precordial leads) were included. Exclusion criteria were as previously described.^{5–7}

SY161 carrying a single molecule of P5, P10, or P20 was given as a 5-mg IV bolus over a period of 3 minutes, in groups of 3 patients each (9 patients total). Plasma samples were drawn for pharmacokinetic analysis at 1, 10, 20, 30, 45, 60, 90, 120, 180, and 240 minutes, and Sak-related antigen was determined by ELISA.¹⁵ Thereafter, an additional 9 patients were treated with SY161-P5 and 6 patients with SY160-P5. Finally, 11 patients were treated with an intravenous bolus of 2.5 mg of the P5 variants.

After 60 minutes (and secondarily at 24 hours), a coronary angiogram was obtained to determine patency of the infarct-related artery, graded as described by the Thrombolysis in Myocardial Infarction (TIMI) study group, which constituted the primary end point. PTCA at the operator's discretion was allowed after the angiogram for the primary end point. Aspirin (160 mg or 320 mg) was given on admission and daily thereafter. Heparin (Novo Nordisk) was administered from entry as a 5000-IU bolus followed by a 1000-IU/h infusion, adjusted at 6-hour intervals to maintain the aPTT within 55 to 75 seconds. Plasma samples were collected immediately before and at different time points after injection of PEG-Sak and analyzed as described elsewhere.¹⁵

Absorption of Antibodies Elicited With PEGylated SakSTAR Variants

Plasma samples obtained 2 to 4 weeks after treatment with staphylokinase-neutralizing activity >5 µg/mL were used and compared with a plasma pool of 40 patients with AMI, obtained after treatment with SakSTAR.

The plasma samples were serially diluted (50- to 400-fold) for the construction of individual calibration curves for antibody binding to each of the SakSTAR variants, absorbed for 10 minutes with 250 nmol/L of the SakSTAR variants, and residual binding to immobilized SakSTAR or SakSTAR variants was determined by biospecific interaction analysis as described elsewhere.¹⁵ Residual binding was expressed in percent of unabsorbed plasma, by use of the individual calibration curves.

Statistical Analysis

Data are expressed as mean values±SEM or as a median (15 to 85 percentile ranges). Significance levels were determined by paired or unpaired Student's *t* test or by Mann-Whitney rank sum test, as appropriate. Fisher's exact test was carried out when indicated in the text. Two-tailed *P*<0.05 was considered to indicate statistical significance. Confidence intervals were determined by the Statexact software (Version 3.01, Cytel Software Corp).

Results

Characterization of PEGylated SakSTAR Variants

The specific activities of the PEGylated variants purified to homogeneity from culture volumes of 18 L (Table 1) were at least as high as that of wild-type SakSTAR. The endotoxin content was <1 EU/mg. SDS gel electrophoresis of a 10-µg sample revealed single main components with apparent M_r of

TABLE 1. Characterization of Polyethylene-Glycol Derivatized Cysteine-Substituted SakSTAR Variants

Compound	Code	Protein, mg	Specific Activity, kU/mg	Endotoxin, EU/mg	Fibrinolytic Potency in Human Plasma, C ₅₀ in $\mu\text{g/mL}$	Antibody Absorption, %
SakSTAR	SakSTAR	2400	150	2.9	0.23	95
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY155	76	180	0.3	0.21	36
SakSTAR(S3C-P5,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY161-P5	130	140	<0.1	0.19	35
SakSTAR(S3C-P10,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY161-P10	130	160	<0.1	0.22	38
SakSTAR(S3C-P20,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY161-P20	310	160	<0.1	0.39	44
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY151	630	170	0.1	0.24	32
SakSTAR(S3C-P5,K35A,E65Q,K74Q,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	SY160-P5	180	150	0.1	0.25	33

30 000, 36 000, and 50 000 for P5, P10, and P20 derivatives, respectively (Figure 1). In addition, small amounts were observed of unPEGylated monomers (M_r 16 000).

The preparations were sterile on 3-day testing and did not provoke acute reaction, nor reduced weight, after intravenous bolus injection of 3 mg/kg body wt in mice (not shown).

Biological Properties of PEGylated SakSTAR Variants

Dose- and time-dependent lysis of ¹²⁵I-fibrin-labeled human plasma clots submerged in human plasma were obtained with equieffective concentrations (causing 50% clot lysis in 2 hours; C₅₀) ranging from 0.19 to 0.39 $\mu\text{g/mL}$ for the PEGylated SakSTAR variants, as compared with a C₅₀ value of 0.23 $\mu\text{g/mL}$ for wild-type SakSTAR (Table 1). At these concentrations, no significant fibrinogen degradation occurred (not shown).

PEGylated compounds remained fully active for up to ≥ 5 days at 37°C. SDS-PAGE after 3 days at 37°C revealed that the covalent bonds linking the maleimide-PEG molecule(s) to the cysteine in the staphylokinase moiety remained intact (data not shown).

Pharmacokinetic Properties in Hamsters and Rabbits and in Patients With AMI

The disposition of staphylokinase-related antigen from blood after bolus injection of 100 $\mu\text{g/kg}$ of the SakSTAR variants in hamsters or rabbits occurred with initial plasma half-lives ($t_{1/2\alpha}$) and plasma clearances (Clp), summarized in Table 2. The disposition rate of staphylokinase-related antigen from blood after bolus injection of 5 mg of the PEGylated

SakSTAR variants in patients with AMI yielded initial plasma half-lives ($t_{1/2\alpha}$) and the plasma clearances (Clp) summarized in Table 2.

The clearances of PEGylated variants were inversely proportional to the molecular weight of the PEG molecules, with an average reduction of 2.5- to 5-fold with P5, 5- to 20-fold with P10, and 20-fold with P20, relative to wild-type SakSTAR.

Thrombolytic Properties of PEGylated SakSTAR Variants in Hamster Pulmonary Embolism Model

SakSTAR and all PEGylated variants induced dose-related clot lysis in a hamster pulmonary embolism model (Figure 2). SY161-P5 had a 2.5-fold lower C₅₀ value than wild-type SakSTAR, SY161-P10 had a 3-fold lower C₅₀ value, and SY161-P20 had a 5-fold lower C₅₀ value. Fibrinogen and α_2 -antiplasmin levels did not decrease after bolus administration of any of the agents (not shown). The thrombolytic potency of SY160-P5 was indistinguishable from that of SY161-P5 (not shown).

Thrombolytic and Immunogenic Properties in Patients With AMI

Table 3 summarizes results of 12 patients treated with 5 mg SY161-P5 and of 6 patients treated with 5 mg SY160-P5. Fourteen of the 18 patients had TIMI grade 3 flow at 60 minutes that persisted at 90 minutes (78%, 95% CI 55% to 91%). After the qualifying 60-minute angiogram was obtained, PTCA was performed in 4 patients with a TIMI flow ≤ 2 . Of the 14 patients who had TIMI grade 3 flow at 60 minutes, 13 had a corrected TIMI frame count (CTFC) of ≤ 30 (20 ± 6 , mean \pm SEM), corresponding to a normal flow, whereas the other patient had a CTFC of 46, which corresponds to TIMI grade 2 flow. The mean percent stenosis in this group was 65 ± 5 (mean \pm SEM) at 60 minutes and 58 ± 5 at 24 hours ($P=0.09$). Eight of these patients underwent PTCA or stent placement after 24 hours for severe stenosis. Ejection fraction, obtained echocardiographically during hospital stay, was 64 ± 12 for all patients (68 ± 10 versus 57 ± 13

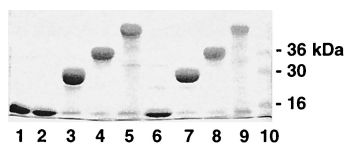


Figure 1. 10% SDS-PAGE gel under nonreducing conditions: Lane 1: SY155; lane 2: SY161-P5; lane 3: SY161-P10; lane 4: SY161-P20; lane 5: molecular weight calibration mixture.

TABLE 2. Pharmacokinetic Parameters of PEGylated SakSTAR Variants After Bolus Injection in Hamsters, Rabbits and Patients With AMI

Compound	Hamsters (n=4)		Rabbits (n=4)		Patients with AMI			
	$t_{1/2 \alpha^*}$, min	Clp, mL/min	$t_{1/2 \alpha^*}$, min	Clp, mL/min	n	Dose, mg	$t_{1/2 \alpha^*}$, min	Clp, mL/min
SakSTAR	2.8	2.2±0.2	3.0	13±0.1	12	20	3.0	360
SY155	2.5	1.6±0.2	4.0	12±0.7
SY161-P5	4.0	0.4±0.0	7.0	5.0±0.7	11	5.0	13±1.5	70±8.6
SY161-P10	9.0	0.1±0.0	7.0	2.6±0.7	3	5.0	30±2.9	43±5.0
SY161-P20	24	0.1±0.0	6.0	0.6±0.1	3	5.0	120±8.8	7.6±0.9
SY151	3.0	0.6±0.1	2.5	19±2.0
SY160-P5	3.0	1.1±0.1	6.0	6.9±0.8	6	5.0	13±2.0	77±11

Data represent mean±SEM of groups of 4 hamsters or rabbits given 100 µg/kg of compound or of n AMI patients given 5 mg PEGylated or 20 mg wild-type staphylokinase.

for patients with TIMI 3 or TIMI ≤2 flow, respectively, $P=0.10$), indicating a preserved left ventricular function. Treatment-related complications consisted of Mallory-Weiss bleeding in one patient who also received abciximab during PTCA at 60 minutes and a large puncture site hematoma in another patient requiring blood transfusion. No hemodynamic problems or allergic reactions were observed.

Table 3 also summarizes results of an additional 11 patients treated with an intravenous bolus of 2.5 mg of the P5 variants, resulting in TIMI grade 3 flow at 60 minutes in 7 patients (63%, 95% CI 35% to 85%). PTCA was performed at 60 minutes in the patients with TIMI flow 0 and in 3 patients at 24 hours because of severe stenosis.

Table 4 summarizes results of hemostasis analyses in the patients given a 5-mg or a 2.5-mg bolus of SY161-P5 or SY160-P5, as compared with a group of 12 patients given 30 mg SakSTAR over 30 minutes. Fibrinogen, plasminogen, and α_2 -antiplasmin at 240 minutes remained essentially unchanged, reflecting the high fibrin-selectivity of these agents. Median D-dimer levels increased from 190 ng/mL at baseline to 4300 and 3200 ng/mL respectively, indicating significant in vivo fibrin digestion. Antibody-related SY160-P5- or SY161-P5-neutralizing activity were low at baseline but

thereafter increased to reach median values at 3 to 4 weeks of 11 µg SakSTAR variant neutralized per milliliter of plasma in patients treated with 5 mg (Table 4), as compared with 21 µg SakSTAR neutralized per milliliter in 10 patients treated with a 30-minute infusion of 30 mg wild-type SakSTAR ($P=0.002$). With 2.5 mg of PEGylated variant, neutralizing activity increased to a median value of 3 µg SakSTAR variant neutralized per milliliter of plasma ($P=0.0013$ versus wild-type SakSTAR) (Table 4).

Absorption With SakSTAR Variants of Antibodies elicited in patients by treatment with SakSTAR variants

Overt immunization (neutralizing activity at 3 to 4 weeks of ≥ 5 µg compound per mL plasma) was observed in 10 of 15 patients treated with 5 mg and in 2 of 7 patients treated with 2.5 mg PEGylated SakSTAR variants (Table 4). Wild-type SakSTAR absorbed >95% of the antibodies binding to insolubilized SakSTAR, SY151, or SY155, respectively, irrespective of the compound used for treatment, indicating that no neoantigens were introduced by the substitution mutagenesis. SakSTAR, SY151, and SY155, respectively, absorbed in excess of 95% of the antibodies binding to insolubilized SakSTAR, SY151, or SY155, respectively, from plasma of patients treated with SakSTAR, SY160-P5, or SY161-P5, respectively (data not shown), which supports the validity of the absorption method used. From pooled plasma of 40 patients treated with SakSTAR, SY155 absorbed 36%, and SY151 bound 32%, which confirms the progressive elimination of B-lymphocyte epitopes by the sequential additive mutagenesis.

Discussion

The present study reports on the preclinical and initial clinical evaluation of polyethylene glycol-derivatized (PEGylated) variants of recombinant staphylokinase for single-bolus treatment of AMI. Introduction of cysteine in the SakSTAR molecule and derivatization with thiol-directed PEG derivatives allowed specific derivatization yielding homogeneous derivatives, whereas substitution in the NH_2 -terminal region, which is removed during process-

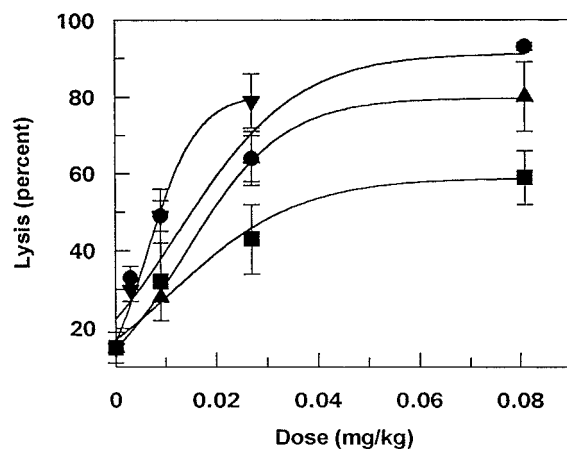


Figure 2. Dose-response curves after bolus injection of PEGylated SakSTAR variants in hamster pulmonary embolism model. ■, SY155; ▲, SY161-P5; ●, SY161-P10; ▼, SY161-P20

TABLE 3. Characteristics and Outcomes of Patients With AMI After Single-Bolus Injection of 5 or 2.5 mg SakSTAR Variant Derivatized With Polyethylene Glycol 5000

Patient ID	Compound	Sex	Age, y	Localization of AMI (by ECG)	Duration Symptoms to Medication (h)	Infarct-Related Vessel	TIMI Flow, 60 min	CTFC, 60 min	Coronary Angiography			
									% Stenosis, 60 min	TIMI flow, 24 h	Intervention	Complications
5-mg Dose												
VRJ	SY161-P5	M	54	Anterior	3.5	LAD	3	24	87	3	Y	...
SH	SY161-P5	M	65	Inferior	1.5	RCA	3	15	74	3	Y	...
HL	SY161-P5	M	70	Lateral	3.5	CX	3	26	42	3	Y	...
TK	SY161-P5	M	68	Inferior	2.5	RCA	2	44	78	3	Y*	...
DF	SY161-P5	M	46	Anterior	2.0	LAD	3	14	75	3	Y	...
MK	SY161-P5	M	42	Anterior	3.5	LAD	3	ND	54	3	N	...
AW	SY161-P5	M	39	Anterior	1.5	LAD	3	15	46	3	N	...
JJ	SY161-P5	M	67	Inferior	1.5	RCA	3	10	72	3	Y	...
GL	SY161-P5	M	75	Inf-Post	2.5	Bypass	0	100	100	3	Y*	Mallory-Weiss
MUA	SY161-P5	M	75	Anterior	3.0	LAD	2	25	86	3	Y*	...
SJ	SY161-P5	M	66	Inferior	3.5	RCA	3	30	51	3	N	...
VM	SY161-P5	M	49	Inferior	3.5	RCA	3	17	81	3	Y	...
VR	SY160-P5	M	62	Inf-Post	3.5	CX	3	24	72	3	Y	...
GL	SY160-P5	F	53	Inferior	4.0	RCA	3	27	81	3	N	...
PJ	SY160-P5	M	72	Lateral	3.0	CX	3	46	84	3	N	...
MA	SY160-P5	M	74	Inferior	5.0	RCA	2	70	86	3	Y*	...
VE	SY160-P5	M	74	Inferior	4.5	CX	3	15	63	3	Y	...
CH	SY160-P5	F	67	Inf-Post	4.0	CX	3	9	81	3	N	Severe PS bleeding
Mean±SEM			62±2.8		3.1±0.2			30±6.0	73±4.0			
TIMI 3 flow							14/18 78% (CI 55–91)					
2.5-mg Dose												
AF	SY161-P5	M	74	Inf-Post	4.5	RCA	0	100	100	...	Y	...
LL	SY161-P5	M	51	Inf-Lat	4.0	RCA	0	100	100	3	Y	...
VJ	SY160-P5	M	61	Ant-Sept	1.0	LAD	3	24	44	3	N	...
VCR	SY160-P5	M	62	Inferior	2.0	CX	3	28	73	3	Y	...
BA	SY160-P5	M	53	Ant-Sept	1.5	LAD	3	33	67	3	Y	...
HC	SY160-P5	V	65	Inferior	3.0	RCA	3	15	80	3	N	...
VRH	SY160-P5	M	59	Ant-Lat	2.5	LAD	3	26	50	3	N	...
RM	SY160-P5	V	59	Ant-Sept	2.0	LAD	3	26	77	3	N	...
VROJ	SY160-P5	M	56	Inf-Lat	1.0	RCA	0	100	83	...	Y	...
VMF	SY160-P5	M	46	Ant-Sept	3.0	LAD	0	100	100	...	Y	...
AM	SY160-P5	M	47	Inferior	3.0	RCA	3	ND	ND	3	Y	...
Mean±SEM			57±2.3		2.5±0.3			55±12	77±6.0			
TIMI 3 flow							7/11 63% (CI 35–85)					

EF indicates ejection fraction; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; ND, not determined; PS, puncture site; RCA, right coronary artery; RV, right ventricular; TIMI, thrombolysis in myocardial infarction.

*Intervention performed after the 60-minute angiogram.

ing of SakSTAR, yielded derivatives with high thrombolytic potency.

The selected cysteine-substitution variant SY161 that was derivatized with maleimide-PEG (Mal-PEG) with molecular

weights of 5000 (P5), 10 000 (P10), or 20 000 (P20) was produced in sufficiently large amounts and conditioned for the evaluation of their pharmacokinetics and thrombolytic potencies in hamsters and of their thrombolytic potency/immunogenicity

TABLE 4. Hemostasis Variables Before and After Bolus Injection of 5 or 2.5 mg PEGylated SakSTAR Variants in Patients With AMI

	Fibrinogen, g/L		Plasminogen, %		α_2 -Antiplasmin, %		D-Dimer, ng/mL		Neutralizing Activity, μ g/mL	
	Before	240 min	Before	240 min	Before	240 min	Before	Peak	Before	Peak
SakSTAR (n=12)*										
Mean \pm SEM	3.3 \pm 0.5	2.9 \pm 0.4	89 \pm 1.0	78 \pm 9	73 \pm 6	44 \pm 8				
Median (15–85 percentiles)							500 (250–760)	5100 (1700–23 000)	0.4 (0.0–0.8)	21 (10–110)
5 mg-Dose										
VRJ	2.8	2.8	89	85	79	66	680	25 000	0.0	12
SH	2.8	2.5	94	84	92	71	240	11 000	0.0	59
HL	2.4	2.2	79	74	71	66	390	11 000	0.0	9.6
TK	3.1	2.5	83	73	81	70	3600	14 000	0.9	4.2
DF	1.7	1.6	87	83	86	82	130	460	0.3	...
MK	2.8	3.1	110	110	94	100	96	4300	0.0	1.9
AW	2.1	2.0	100	100	97	92	58	200	0.3	0.0
JJ	3.0	2.5	86	78	100	64	120	2100	0.5	11
GL	4.1	3.2	100	83	110	76	120	12 000	0.0	30
MA	1.9	1.5	83	68	88	71	1000	4300	0.0	...
SG	2.2	1.7	92	70	94	76	430	6400	0.0	6.4
VM	2.2	1.7	98	81	99	82	390	2000	0.0	3.6
VR	2.4	2.2	85	84	96	80	35	1400	0.6	29
GL	2.9	3.9	70	100	85	110	260	4000	0.3	11
PJ	2.1	2.0	97	99	98	90	1400	19 000	0.0	3.6
MA	2.1	2.4	66	65	72	77	92	2300	0.0	11
VE	2.0	1.9	85	92	89	89	130	4700	0.0	66
CH	2.8	3.1	91	110	99	100	100	710	0.3	...
Mean \pm SEM	2.5 \pm 0.1	2.4 \pm 0.2	89 \pm 3.0	85 \pm 3.0	90 \pm 2.0	82 \pm 3.0				
Median (15–85 percentiles)							190 (94–820)	4300 (600–13 000)	0.0 (0.0–0.4)	11 (3.6–30)
2.5-mg Dose										
AF	2.5	2.2	93	87	92	78	31	1900	0.0	...
LL	2.6	2.8	74	82	84	87	130	15 000	...	0.0
VJ		1.7		68		81		750	...	4.6
VCR	2.1	2.3	85	81	84	72	190	3600
BA	1.8	1.8	98	98	100	100	170	1700	0.1	9.6
HC	2.9	2.2	87	71	110	90	280	13 000	1.2	1.2
VRH	2.7	2.9	82	79	88	86	80	3600	0.0	0.6
RM	1.9	1.9	96	100	90	100	200	1000	0.6	9.7
VROJ	2.8	2.3	90	67	87	62	220	6400	0.0	...
VMF	1.9	2.0	100	57	100	98	190	3200	0.0	2.8
AM	2.9	2.9	95	92	92	91	100	1500	0.1	
Mean \pm SEM	2.4 \pm 0.1	2.3 \pm 0.1	90 \pm 2.6	80 \pm 4.1	93 \pm 2.6	86 \pm 3.6				
Median (15–85 percentiles)							180 (87–210)	3200 (1300–9700)	0.0 (0.0–0.6)	3 (0.5–9.6)

*30-min infusion of 30 mg wild-type SakSTAR (unpublished).

ratios in patients with AMI. PEGylated SY161 had an intact apparent specific activity, maintained fibrinolytic potency and fibrin-selectivity in a human plasma milieu, and had a markedly reduced reactivity with anti-SakSTAR antibodies in pooled immunized patient plasma. The clearances of the PEGylated SY161 derivatives in hamsters, rabbits, and in patients with AMI

were reduced inversely proportionally to the molecular weight of the PEG molecules.

Intravenous administration of SY161-P5 as a 5-mg bolus in 12 patients and of SY160-P5 in 6 patients with AMI resulted in complete recanalization (TIMI grade 3 flow) in 14 patients (78%), and partial recanalization (TIMI grade 2 flow) in 2,

without hemodynamic or allergic reactions and without measurable systemic plasminogen activation. A bolus dose of 2.5 mg still produced TIMI grade 3 flow at 60 minutes in 7 of 11 patients with AMI (63%).

After administration of variant SakSTAR, neutralizing antibody titers increased after 3 to 4 weeks to a median level that was significantly lower than after administration of wild-type SakSTAR. The antibodies induced by treatment with the SakSTAR variants were completely absorbed by SakSTAR, indicating that immunization was not due to neo-epitopes generated by the amino acid substitutions but to residual epitopes in the variants. However, although there was a significant reduction in immunogenicity, the residual immunization remained too high to warrant repeated use of the drug.

In summary, on the basis of the present study, SakSTAR (S3C-P5, K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K09A, K130T, K135R) (code SY161-P5) has been selected for clinical development toward fibrin-selective thrombolytic therapy by single intravenous bolus injection in patients with AMI.

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