

Inhibition of Factor XIIIa in a Canine Model of Coronary Thrombosis: Effect on Reperfusion and Acute Reocclusion After Recombinant Tissue-Type Plasminogen Activator

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The effect of inhibition of factor XIIIa with 2-(1-acetylthio)-5-methylthiazolo[2,3-b]1,3,4-thiadiazolium perchlorate (L-722,151) on coronary thrombolysis and reocclusion was studied in an acute dog model of electrically induced coronary thrombosis. L-722,151 (0.1 mg/kg/min intravenously [IV] or placebo was administered 15 minutes before current initiation (150 μ A) and for the duration of the experiment (270 minutes). Fifteen minutes after thrombus formation, heparin (300 U/kg, IV) was administered, followed 45 minutes later by recombinant tissue-type plasminogen activator (tPA) (10 μ g/kg/min, IV for 90 minutes). Placebo-treated animals thrombosed at 48.9 ± 8.1 minutes (mean \pm SEM) and reperfused in response to tPA at 49.1 ± 9.3 minutes. L-722,151 pretreated animals thrombosed at 44.4 ± 9.7 minutes and reperfused in response to tPA at 16.4 ± 2.8 minutes ($P < .05$ v vehicle). Furthermore, residual thrombus mass was reduced by L-722,151 from 6.9 ± 1.9 mg in placebo-treated animals to 1.7 ± 0.6 mg

($P < .05$ v vehicle). Acute reocclusion occurred in 86% of placebo and in 75% of L-722, 151-treated animals. The incidence of tPA-induced reperfusion in L-722, 151-treated dogs was 100% (8 of 8), whereas only 70% (7 of 10) of placebo-treated dogs reperfused. These results demonstrate that pretreatment with L-722,151 hastens reperfusion time threefold and reduces residual thrombus mass. These effects occurred with no change in systemic blood pressure in response to L-722,151. When L-722,151 was administered 15 minutes after thrombus formation in a separate group of dogs ($n = 5$), no beneficial effect on thrombolysis time or thrombus mass was observed. Thus, the specific factor XIIIa catalyzed crosslinking reaction(s), which may determine(s) resistance to plasmin-mediated fibrin degradation, occur(s) rapidly. Inhibition of this crosslinking by pretreatment with L-722,151 promotes tPA-induced thrombolysis.

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CURRENTLY, intravenous (IV) thrombolytic therapy results in reperfusion in 70% to 75% of patients being treated for acute myocardial infarction, which may be complicated by acute reocclusion in approximately 25% of patients.¹⁻³ However, effective initial thrombolysis does not occur in the remaining 25% to 30% of patients necessitating emergency rescue procedures such as angioplasty or coronary artery bypass grafting. The failure of thrombolytic therapy in some of these patients may be related to the age of the thrombus, as older thrombi have been demonstrated to be more resistant to clot lysis both *in vitro*⁴⁻⁷ and *in vivo*.⁸⁻¹³

The final step in coagulation of blood involves stabilization of fibrin by activated factor XIIIa (FXIIIa).^{14,15} FXIIIa catalyzes several covalent crosslinking reactions, which include formation of fibrin γ -chain dimers,¹⁶ incorporation of a potent plasmin inhibitor, α_2 -antiplasmin, into the α chain of fibrin,^{17,18} and the formation of fibrin α chain polymers.^{19,20} The first two reactions are relatively rapid and are virtually complete within 2 to 5 minutes after the initiation of clot formation. The latter reaction is much slower and may require hours for completion.²¹ These crosslinking reactions result in mechanical stabilization of the thrombus²² and increased resistance to plasmin-mediated degradation.⁴⁻⁷ Incomplete α chain crosslinking of fibrin occurs in normal plasma in which factor XIII concentration is 1 U/mL.²³ However, addition of higher factor XIII concentrations as supplied by purified material or by platelets that contain nearly 50% of blood factor XIII led to a much greater extent of α chain crosslinking.²³ Thus, as clots age, extensive FXIIIa-mediated crosslinking of fibrin occurs, making the clot less susceptible to lysis by endogenously formed plasmin²⁴ or to exogenously administered thrombolytic agents.

The aim of the present investigation is to examine the ability of an inhibitor of FXIIIa to influence recombinant tissue-type plasminogen activator (tPA)-induced clot lysis in an animal model of coronary thrombosis. The major end-points assessed in this model are: (1) the frequency and time

to occurrence of coronary reperfusion and acute reocclusion, and (2) residual thrombus mass after administration of tPA with or without L-722,151 (2-[1-acetylthio]-5-methylthiazolo[2,3-b]1,3,4-thiadiazolium).²⁵ L-722,151 was administered before the initiation of thrombus formation in one group of animals and after thrombus formation in a separate group of dogs.

MATERIALS AND METHODS

Canine coronary artery thrombosis model. Mongrel dogs of either sex (9 to 18 kg) were anesthetized with pentobarbital sodium (30 mg/kg IV), intubated, and ventilated with room air at a tidal volume of 20 mL/kg and a frequency of 12 inflations/min (Harvard respirator, Harvard Apparatus, South Natick, MA). The right femoral artery and vein were exposed, and catheters were inserted for monitoring arterial blood pressure (Statham P23 ID pressure transducer, Gould Inc) and administration of drugs, respectively. The left external jugular vein and the left saphenous vein were also exposed and catheters were inserted for continuous infusion of 5% dextrose in saline (1 mL/kg/h) and infusion of drugs, respectively. A left thoracotomy was performed at the fifth intercostal space and the heart was exposed and suspended in a pericardial cradle. A 2-cm section of the left circumflex (LCX) coronary artery was isolated proximal to the first obtuse marginal branch and instrumented proximal to distal as follows: electromagnetic flow probe (model 501,

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Carolina Medical Electronics, Inc, King, NC) stimulation electrode, adjustable mechanical occluder (Goldblatt clamp) and a silk snare (Fig 1). The stimulation electrode was constructed from a 26-gauge stainless steel hypodermic needle tip attached to a 30-gauge Teflon-insulated silver-coated copper wire. The mechanical occluder was constructed of stainless steel with a stainless steel screw (2-mm diameter), which could be manipulated to control vessel circumference. The length of the Goldblatt clamp was 2.5 mm. Before administration of any experimental drugs, the occluder was sufficiently tightened around the artery to eliminate the reactive hyperemic response without affecting resting LCX coronary blood flow. Care was taken to not traumatize the coronary artery upon placement of the occluder onto the vessel under study.

Continuous recordings of systemic blood pressure and mean and phasic LCX coronary artery blood flow were displayed on a model 7D polygraph (Grass Instrument Co, Quincy, MA). Zero and hyperemic flows were determined by occluding the left circumflex coronary artery distal to the flow probe for 20 seconds with the snare. In this study, thrombosis and thrombolysis were determined with the use of a blood flow probe placed directly onto the coronary artery. Coronary angiography was not performed.

Thirty minutes after surgical preparation, 150 μ A continuous anodal direct current stimulation was initiated and maintained until LCX coronary artery blood flow decreased to and remained at zero. Direct, electrical stimulation was delivered using a Grass constant current unit (model CCUIA), a Grass stimulus isolation unit (model S1U5B), and a Grass stimulator (model S48) connected to the intraluminal coronary artery electrode.²⁶ Sodium heparin was administered 15 minutes post-LCX occlusion in a bolus dose of 300 U/kg IV and every hour thereafter at 100 U/kg IV. No anti-arrhythmic drugs were administered.

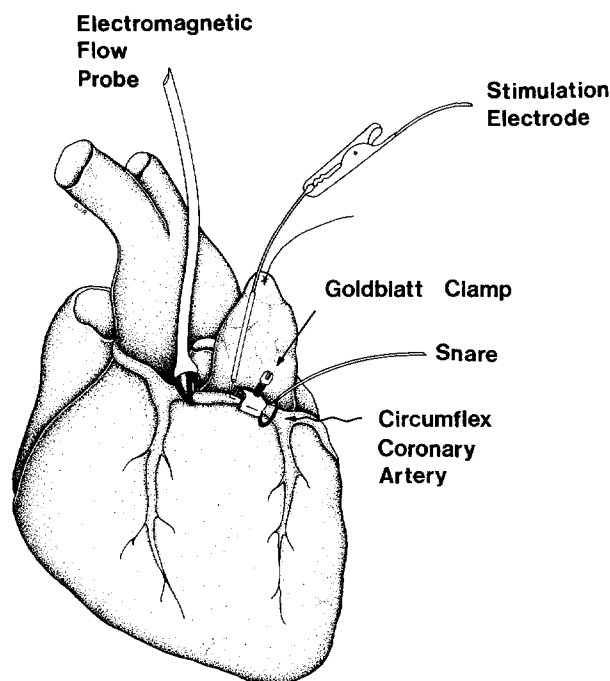


Fig 1. Model of coronary artery thrombosis in the dog. Electrical injury (150 μ A) to the intimal surface of the artery via a needle electrode leads to occlusive thrombus formation. The thrombus is formed in the presence of a flow limiting stenosis with a Goldblatt clamp.

Protocol. Dogs were assigned randomly to one of three groups. In group I ($n = 10$), formulation vehicle or placebo (4% dimethylsulfoxide [DMSO] in phosphate-buffered saline [PBS]) was administered 15 minutes before current was delivered to the circumflex coronary artery and for the duration of the experiment (approximately 270 minutes). One hour after thrombus formation, recombinant tPA (10 μ g/kg/min) was administered IV for 90 minutes. Group II ($n = 8$) consisted of animals receiving L-722,151 (0.1 mg/kg/min, IV) 15 minutes before current initiation and for the duration of experiment. One hour after thrombus formation in group II, tPA was administered at a dose of 10 μ g/kg/min, IV for 90 minutes. Group III animals ($n = 5$) received L-722,151 (0.1 mg/kg/min, IV) 15 minutes after spontaneous electrically induced thrombus formation followed 45 minutes later by tPA at 10 μ g/kg/min, IV for 90 minutes. Thus, all animals in the study received identical doses of heparin and tPA at the same time intervals in the protocol. All animals were observed for 1 hour after termination of tPA to determine the incidence and time to reocclusion. At the end of the 1-hour observation period, experiments were terminated with KCl.

Determination of residual thrombus mass. Just before termination, the circumflex coronary artery was ligated proximal to the blood flow probe and distal to the Goldblatt clamp. The artery was then excised, slit longitudinally, and pinned out on a piece of styrofoam with 26G needles. The thrombus was visualized, blotted with filter paper to remove excess blood, removed from the lumen of the vessel with forceps under a dissecting microscope (10X), and weighed on a milligram balance.

Criteria used to judge the time to thrombosis, reperfusion, and reocclusion. Criteria were developed to judge the time to thrombosis, reperfusion, and reocclusion in the canine model used in this study. Thrombosis was judged complete when coronary blood flow dropped to zero and stayed at zero. Some cycling of blood flow occurs as the thrombus forms; however, only complete, sustained occlusion was used to determine time to thrombosis. Five minutes after complete occlusion, the electrical current to the artery was terminated. tPA-induced reperfusion required that coronary artery blood flow be maintained for at least 5 minutes at a flow rate of at least 50% of control flow. The value used for control blood flow was the coronary blood flow immediately before delivering current to the vessel. Reocclusion was defined as zero blood flow after successful tPA-induced reperfusion. In most cases, cyclical flow reduction (CFRs) occur after tPA-induced reperfusion in this model. Thus, the first CFR that goes to zero flow is used as the temporal endpoint for reocclusion. Some animals exhibit CFRs throughout the 1-hour observation period after termination of tPA, whereas other animals exhibit permanent reocclusion that is devoid of periodic and spontaneous CFRs.

Assay for L-722,151 in plasma. Plasma concentrations of L-722,151 were determined with an in vitro assay based on the direct inhibition of FXIIIa. Purified human FXIII was activated at 0.2 mg/mL by 5 U/mL human thrombin (Sigma, St Louis, MO) for 10 minutes at 25°C in the presence of 100 mmol/L NaCl, 5 mmol/L $\text{Ca}^{2+}\text{Cl}_2$, 1 mmol/L EDTA, 10% (vol/vol) glycerol, and 50 mmol/L tris HCl, pH 7.5. The activation mixture was quenched by hirudin (100 U/mL; Sigma) and diluted 10-fold by buffer containing all components except thrombin. To 160 μ L of this FXIIIa solution was added 5 μ L of plasma samples obtained from the dog during L-722,151 administration, or control (preinfusion) plasma samples to which known concentrations of L-722,151 had been added. Eighty-five microliters of a substrate solution containing 0.33% dimethylated casein (Sigma) and 1.67 mmol/L [¹⁴C] putrescine (5 mCi/mmol; New England Nuclear) was added. After a 20-minute incubation at 37°C, a 20- μ L aliquot was spotted on a Whatman 3-mm filter disc and the covalently bound radioactivity was deter-

mined as previously described.²⁷ A plot of (percent inhibition)¹ versus 1/L-722,151 concentration yields a straight line for standard plasma containing 1 to 100 μmol/L L-722,151, which intercepts the ordinate at 0.1 (corresponding to 100% inhibition at infinite [L-722,151]).

Materials. tPA (Activase®) was purchased commercially from Genentech, Inc (San Francisco, CA). The specific activity of this material was 5.8 × 10⁵ IU/mg. tPA was reconstituted in sterile water supplied by the manufacturer at a concentration of 2 mg/mL. Sodium heparin (1,000 U/mL) was purchased from the Upjohn Co (Kalamazoo, MI). L-722,151 was synthesized at Merck Sharp and Dohme Research Laboratories (West Point, PA).

Statistical analysis. Results are expressed as the mean ± SEM. For analysis of the time to reperfusion, unpaired Student's *t*-test was used. Incidence of reocclusion data was analyzed by Fisher's exact probability test. Hemodynamic analysis was performed with the Mann-Whitney test for pooled samples with equal dispersions.

RESULTS

Animals in group I receiving placebo infusion thrombosed in response to electrolytic injury at 49 ± 8 minutes (Table 1). Thrombolysis occurred in this group at 49 ± 9 minutes after the start of tPA infusion (Fig 2), with acute reocclusion occurring 22 ± 6 minutes after successful reperfusion in 6 of 7 animals. The range of tPA-induced reperfusion times for group I was 25 to 90 minutes. Three animals failed to reperfuse after 90 minutes of tPA infusion. The residual thrombus wet weight in group I was 7 ± 2 mg (Table 1).

Group II animals that received L-722,151 before current initiation and for the duration of the experiment thrombosed at 44 ± 10 minutes, which was not significantly different from group I thrombosis time. However, reperfusion occurred at 16 ± 3 minutes after the start of tPA infusion (Fig 2) with a range of 9 to 32 min (Table 1), a statistically significant (*P* < .05) difference from group I. Acute reocclusion in group II occurred at the same rate and time as group I. However, residual thrombus wet weight was significantly reduced in group II dogs to 2 ± 1 mg. tPA-induced

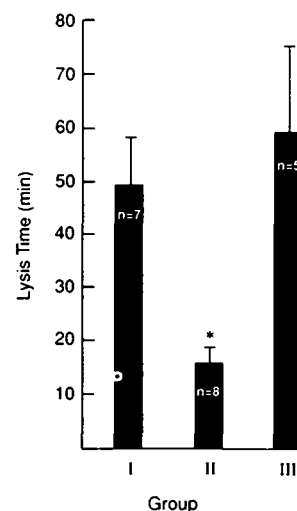


Fig 2. Thrombolysis time in groups I through III. Group I received tPA only (10 μg/kg/min, IV) Group II was pretreated with L-722,151 (0.1 mg/kg/min, IV) before thrombus formation was initiated, followed by tPA. Group III received L-722,151 15 minutes after thrombus formation followed by tPA.

Group I - tPA
Group II - Pre-treatment L-722, 151/tPA
Group III - Post-treatment L-722, 151/tPA

reperfusion occurred in 100% of animals (8 of 8) receiving L-722,151 infusion.

Group III dogs received L-722,151 15 minutes after electrically induced clot formation. The time to reperfusion in these animals was 59 ± 17 minutes after the start of tPA infusion which was very similar to group I dogs (Fig 2). The reocclusion rate and time as well as clot weight and incidence of reperfusion were also similar to group I animals (Table 2). Thus, dogs, not pretreated with L-722, 151 before clot formation failed to reperfuse sooner as had been observed in group II pretreated animals.

A hemodynamic analysis on groups I and II was performed for arterial blood pressure and coronary blood flow (Fig 3). L-722,151 had no significant effect on blood pressure when analyzed at four timepoints in the experiment: control (before current initiation), spontaneous occlusion, tPA-induced reperfusion, and just before termination of the experiment. Coronary blood flow was not affected by L-722,151 at reperfusion; however, at termination there was a slightly greater residual flow for L-722,151-treated animals due to the greater number of vehicle-treated group I animals that exhibited permanent reocclusion (Table 1), and thus zero blood flow.

At an IV infusion rate of 0.1 mg/kg/min, a steady-state concentration of 6 μmol/L L-722,151 was achieved within 30

Table 1. Effect of Pretreatment With L-722, 151 on Coronary Thrombosis, Thrombolysis, and Reocclusion in the Dog Electrolytic Injury Model

	Group I tPA (10 μg/kg/min, IV) + Vehicle	Group II tPA (10 μg/kg/min, IV) + L-722, 151 (0.1 mg/kg/min, IV)
Time to occlusion (min)	48.9 ± 8.1	44.4 ± 9.7
Time to reperfusion (min)*	49.1 ± 9.3	16.4 ± 2.8†
Reocclusion rate (%)‡	6/7 (86%)	6/8 (75%)
Reocclusion time (min)	21.6 ± 6.0	26.2 ± 8.9
Residual thrombus weight (mg)	6.9 ± 1.9	1.7 ± 0.6†
Incidence of reperfusion (%)	7/10 (70%)	8/8 (100%)
N	10	8

*Range (min): group I, 25-90; group II, 9-32.
†Permanent reocclusion: group I, 5 of 6 (83%); group II 3 of 6 (50%).
‡*P* < .05 versus vehicle by one-sided students' *t* test.

Table 2. Effect of L-722, 151 Administered 15 Minutes After Clot Formation on tPA-Induced Thrombolysis in the Dog Electrolytic Injury Model

	Group III tPA (10 μg/kg/min, IV) + L-722, 151 (0.1 mg/kg/min, IV)
Time to occlusion (min)	40.0 ± 7.9
Time to reperfusion (min)	58.5 ± 16.8
Reocclusion rate (%)	3/4 (75%)
Reocclusion time (min)	9.0 ± 3.0
Residual thrombus weight (mg)	5.9 ± 0.9
Incidence of reperfusion (%)	4/5 (80%)
N	5

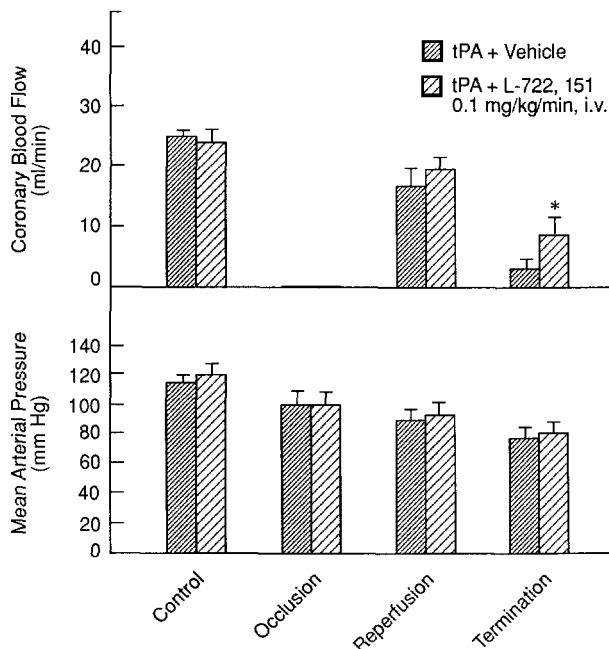


Fig 3. Mean arterial blood pressure and coronary blood flow at control (before drug or initiation of current), at coronary occlusion, at tPA-induced coronary reperfusion, and at the termination of the experiment for dogs in groups I and II. Asterisk indicates that resting coronary blood flow for L-722,151-treated animals is significantly ($P < .05$) higher than placebo-treated animals at the termination of the experiment.

to 60 minutes after the initiation of the infusion (Table 3). This latter result is consistent with a previously determined plasma half-life of 5 to 10 minutes (Bush L., Stern A.M., unpublished observations).

DISCUSSION

The results of this study indicate that pretreatment with L-722,151, an inhibitor of FXIIIa, results in a threefold decrease in thrombolysis time with a concomitant reduction in residual thrombus weight in an in vivo canine model of

Table 3. Plasma Concentrations of L-722, 151

Time After Initiation of IV Infusion* (min)	L-722, 151 Plasma Concentrations ($\mu\text{mol/L}$)	N†
5	1.1 \pm 0.8	2
10	2.4 \pm 0.1	2
15	3.8 \pm 1.9	2
30	4.1 \pm 0.9	2
60	5.9 \pm 0.3	5
90	5.9 \pm 1.0	2
120	7.4 \pm 1.3	5
180	7.7 \pm 1.4	2

*Infusion rate: 0.1 mg/kg/min.

†For five dogs, 1- and 2-hour samples were measured. For two of these dogs determinations at additional times were made. For N = 2 the range is presented, and for N = 5 the standard error is presented.

thrombosis and thrombolysis. This action of L-722,151 is not associated with any change in systemic blood pressure. However, waiting to start L-722, 151 until 15 minutes after thrombus formation completely eliminated the beneficial effect of FXIIIa inhibition on tPA-induced reperfusion time and residual thrombus wet weight. Thus, the specific FXIIIa catalyzed crosslinking reaction(s), which may determine resistance to plasmin-mediated fibrin degradation, occur(s) rapidly in vivo. The full potential of this approach, therefore, may only be realized during prophylactic therapy in a patient population at high risk of thrombosis, such as that encountered in unstable angina or in patients with repeated and predictable episodes of deep vein thrombosis. On the other hand, it may be argued that patients with unstable angina, treated with an inhibitor of FXIIIa to prevent crosslinking that go on to infarct, may spontaneously lyse in response to their own endogenous tPA or may be more responsive to exogenously administered thrombolytic agents. It does not appear that FXIIIa inhibition prevents reocclusion, which suggests that incorporation of an anti-platelet agent into a thrombolytic protocol may be necessary as well. It would be of interest to combine an FXIIIa inhibitor with an anti-platelet agent to determine if lysis time as well as acute reocclusion could be decreased.

The results from this in vivo study do not address the exact mechanism by which an inhibitor of FXIIIa is decreasing thrombolysis time. As mentioned above, this action could be due to an inhibition of fibrin γ chain dimer formation, fibrin α chain polymer formation, or the incorporation of α_2 antiplasmin into the α chain of fibrin. In vitro at 5 $\mu\text{mol/L}$ L-722,151 γ chain dimer formation is inhibited less than 20%, while α chain polymer formation is 100% inhibited and α_2 antiplasmin incorporation is 75% to 80% inhibited.²⁵ In vivo studies (Bush L., Stern A.M., unpublished observations) in a canine model of thrombosis at the same infusion rate of L-722,151 used in these studies demonstrated complete inhibition of α fibrin chain polymer formation with no appreciable inhibition of γ chain dimer formation. α_2 antiplasmin incorporation was not determined. These results indicate that γ chain dimer formation is not significantly inhibited by L-722,151 at concentrations achieved in vivo (2 to 10 $\mu\text{mol/L}$) and that the enhancements in reperfusion rates result from either the inhibition of α polymer formation or α_2 antiplasmin incorporation into the clot. However, our present data is insufficient to determine the relative contribution of each of these mechanisms in shortening lysis times. The extremely narrow temporal window would argue in favor of the kinetically more rapid reaction, α_2 antiplasmin incorporation, being the most significant mechanism for enhancing lysis rates in vivo. However, if clot penetration of L-722,151 becomes in part limiting this kinetic argument becomes less cogent. In addition, although in in vitro static systems the α_2 antiplasmin incorporation reaction appears to be the most important,²⁸ in vivo, especially for arterial clots, the mechanical stability generated by α chain polymers²² may become an important factor not realized in vitro.

Regardless of the exact mechanism by which the inhibition of FXIIIa leads to enhanced clot lysis, our studies demon-

strate that FXIIIa is a potentially important target for therapeutic control of thrombosis. The FXIIIa inhibitor used in this study, L-722,151, is a pharmacologic tool and not a candidate for clinical development. However, L-722,151 may serve as a prototype for the development of safe and efficacious FXIIIa inhibitors.

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