The novel synthetic serine protease inhibitor CU-2010 dose-dependently reduces postoperative blood loss and improves postischemic recovery after cardiac surgery in a canine model

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Background: Serine protease inhibitors such as aprotinin reduce perioperative blood loss and may improve post-pump cardiac performance owing to their anti-inflammatory properties. After the “aprotinin era,” we investigated the efficacy of the novel synthetic serine protease inhibitors CU-2010 with improved coagulatory and anti-inflammatory profile on blood loss and reperfusion injury in a canine model.

Methods: Thirty-six dogs were divided into 6 groups: control, aprotinin (n = 8; Hammersmith scheme), and CU-2010 (0.5, 0.83, 1.25, and 1.66 mg/kg). All animals underwent 90 minutes of cardiopulmonary bypass with 60 minutes of hypothermic cardioplegic arrest. End points were blood loss during the first 2 hours after application of protamine, as well as recovery of myocardial contractility (slope of the end-systolic pressure–volume relationship, coronary blood flow, and vascular reactivity).

Results: CU-2010 dose-dependently reduced blood loss to a degree comparable with that of aprotinin at lower doses and even further improved at higher doses (control/aprotinin/CU-2010 in increasing doses: 142 ± 13, 66 ± 17, 95 ± 16, 57 ± 17, 46 ± 3, and 13 ± 4 mL; *P* < .05). Whereas aprotinin did not influence myocardial function, CU-2010 improved the recovery of end-systolic pressure–volume relationship (control 60 ± 6 mg/kg vs aprotinin 73 ± 7 mg/kg vs CU-2010 1.66 mg/kg; 102% ± 8%; *P* < .05). Coronary blood flow (52 ± 4 vs 88 ± 7 vs 96 ± 7; *P* < .05) and response to acetylcholine (44% ± 6% vs 77% ± 7% vs 81% ± 6%; *P* < .05) were improved by both aprotinin and CU-2010.

Conclusions: The novel serine protease inhibitor CU-2010 significantly reduced blood loss after cardiac surgery comparable with aprotinin. Furthermore, an additionally improved anti-inflammatory profile led to a significantly improved postischemic recovery of myocardial and endothelial function. (*J Thorac Cardiovasc Surg* 2010;139:732–40)

In cardiac surgery, the use of cardiopulmonary bypass (CPB) triggers activation of the coagulation system and evokes systemic inflammatory responses, which may result in excessive bleeding and other complications in the perioperative and postoperative periods.¹ Despite administration of large amounts of heparin, contact with the artificial surfaces of the heart–lung machine results in generation of thrombin² and activation of plasmin.

Prophylactic antifibrinolytic therapies during cardiac surgery are now widely accepted as a strategy to attenuate bleeding complications.^{3,4} The most thoroughly evaluated antifibrinolytic agents are aprotinin, an active-site serine protease inhibitor isolated from bovine lung, and tranexamic acid, a synthetic lysine analog that specifically blocks the lysine binding site in plasminogen and prevents its conversion to plasmin as well as access to fibrin.³ Many studies and meta-analyses have proven that both drugs reduce bleeding tendency and allogeneic blood requirement in cardiac^{4–6} and noncardiac⁷ surgery.

The kallikrein–kinin system is an endogenous metabolic cascade, the triggering of which results in the release of vasoactive kinins (bradykinin-related peptides). The pharmacologically active kinins, which are often considered as either proinflammatory or cardioprotective, are implicated in many physiologic and pathologic processes. It was shown that plasma kallikrein inhibitors, in particular aprotinin, reduce inflammatory response and ischemia/reperfusion injury in various models.⁸

The nonspecific serine protease inhibitor aprotinin had been used to reduce the perioperative blood loss and the need for blood transfusion after CPB. Aprotinin is produced

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Abbreviations and Acronyms

ACT	= activated clotting time
aPTT	= partial thromboplastin time
CPB	= cardiopulmonary bypass
dP/dt	= left ventricular pressure development
ESPVR	= end-systolic pressure–volume relationship
MCF	= maximum clot strength
PRSW	= preload recruitable stroke work
PT	= prothrombin time

from bovine tissue; therefore, the possibility of an allergic reaction must be considered whenever this drug is used. The safety of aprotinin has recently been questioned because of suspected excess mortality^{9,10} and impaired renal function,^{11,12} which, despite some controversy,^{13–15} finally led to suspension of worldwide marketing.¹⁶

With aprotinin's multiple interactions indicating a possible positive effect of broad-spectrum serine protease inhibition during cardiac or major surgery, a targeted modulation of protease activity by synthetic peptidomimetic inhibitors was considered a plausible approach to more effective therapeutics. Beyond aprotinin's primary targets, plasmin and plasma kallikrein, the inhibition of procoagulant enzymes is of interest because the activation of thrombin plays a central role in CPB-related coagulopathy, and attenuation of enzyme activity upstream in the coagulation pathways might mitigate such activation. As a result, a small synthetic molecule with an adapted selectivity profile and equal antifibrinolytic efficacy compared with aprotinin or tranexamic acid and an improved safety profile is desirable.

CU-2010 (Curacyte Discovery, Leipzig, Germany) is a novel, small synthetic compound (molecular weight, 700 Da) with peptide-like characteristics that allow substrate-like binding to the active site of serine proteases (see Katz and associates¹⁷ for a review of this class of compounds). CU-2010 shows rapid clearance after intravenous infusion, with an elimination half-life of approximately 20 minutes in rats and dogs. We¹⁸ previously showed that CU-2010 effectively reduced postoperative blood loss either applied according to the Hammersmith scheme or as a simple continuous infusion. This effect was comparable with that of aprotinin.

The aim of the present study was to establish a dose–response relation in a clinically relevant model (ie, CPB with cardioplegic arrest and reperfusion). A further aim of the present study was to investigate whether CU-2010 is able to improve the recovery of myocardial and coronary vascular function after cardioplegic arrest and reperfusion.

MATERIAL AND METHODS

Animals

Thirty-six dogs (foxhounds) weighing 22 to 35 kg were used in this experiment. All animals received humane care in compliance with the "Prin-

ciples of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The experiments were approved by the Ethical Committee of the Land Baden-Württemberg for Animal Experimentation.

Surgical Preparation and CPB

The dogs were premedicated with propionylpromazine and anesthetized with a bolus of pentobarbital (15 mg/kg initial bolus and then 0.5 mg · kg⁻¹ · h⁻¹ intravenously), paralyzed with pancuronium bromide (0.1 mg/kg as a bolus and then 0.2 mg · kg⁻¹ · h⁻¹ intravenously), and endotracheally intubated. The dogs were ventilated with a mixture of room air and oxygen (inspired oxygen fraction = 60%) at a frequency of 12 to 15 breaths/min and a tidal volume starting at 15 mL · kg⁻¹ · min⁻¹. The settings were adjusted by maintaining arterial partial carbon dioxide pressure levels between 35 and 40 mm Hg. The femoral artery and vein were cannulated for recording mean arterial pressure and taking blood samples for the analysis of blood gases, electrolytes and pH, and parameters of blood coagulation. Intravenous volume substitution was carried out with Ringer solution to maintain hemodynamic stability. According to the values of potassium, bicarbonate, and base excess, substitution included administration of potassium chloride and sodium bicarbonate (8.4%). Neither catecholamines nor other hormonal or pressor substances were administered. Rectal temperature and standard peripheral electrocardiogram were monitored continuously.

After left anterolateral thoracotomy in the fourth intercostal space, pericardiotomy and isolation of the great vessels were performed. After systemic anticoagulation with sodium heparin (Liquemin, 300 U/kg; Hoffmann-La Roche, Basel, Switzerland), the left subclavian artery was cannulated for arterial perfusion. The venous cannula was placed in the right atrium. The extracorporeal circuit consisted of a heat exchanger, a venous reservoir, a roller pump, and a membrane oxygenator primed with Ringer lactate solution (1000 mL) supplemented with heparin (150 U/kg) and 20 mL sodium bicarbonate (8.4%). Hypothermic CPB was performed for 90 minutes at a lowest temperature of 28 °C. Pump flow rate was adjusted to keep cardiac output constant at preoperative levels. Alpha-stat management was applied. Hematocrit values were in the physiologic range in all animals (28%–32%) at the beginning of the experiments, decreased to a level of 24% owing to hemodilution during CPB, and recovered spontaneously on reperfusion in all animals. There were no differences among the groups at any time point (data not shown). After initiation of CPB, the hearts were arrested with 1000 mL crystalloid cardioplegia (Custodiol HTK solution; Odyssey Pharmaceuticals, Inc, Florham Park, NJ). After 60 minutes, the aortic crossclamp was released and the reperfusion phase was initiated. After weaning from CPB, heparin was antagonized by intravenous protamine (300 U/kg) over 10 minutes and the animals were monitored for 120 minutes.

Experimental Groups

Thirty-six dogs were divided into 6 experimental groups. Control animals received placebo. Dogs of the aprotinin treatment group received bovine aprotinin; the treatment scheme was applied according to Hammersmith (ie, intravenous bolus/pump prime/continuous infusion). The applied dose of aprotinin was adjusted to the actual body weight of the animals. Accordingly, the animals received 28,333 KIU/kg as an initial bolus and 28333 KIU/kg into the pump prime. During CPB 10,000 KIU/kg aprotinin was infused over 90 minutes. The remaining dogs were divided into 4 treatment groups and received CU-2010 at 4 different doses in a slow infusion for 90 minutes starting 5 minutes after administration of heparin. The applied dose of CU-2010 was adjusted to the actual body weight of the animals in all groups as follows: 0.5 mg/kg, 0.83 mg/kg, 1.25 mg/kg, and 1.66 mg/kg.

Measurements of Blood Loss and Coagulation Parameters

The primary end point of the study was the total blood loss after weaning from CPB during the first 2 hours after application of protamine. Blood loss

was measured by gauze bandages after weaning from CPB and 15 minutes after finishing of protamine at different time points (in 120, 160, and 220 minutes after initiation of CPB). Bandages were placed into the operating area (pericardial sack and surrounding tissues). Weight of gauze bandages was measured before and after cleaning the operating area. We calculated blood loss from the difference in the weight of gauze bandages. The same number of bandages (3 pieces at once) were removed every 10 minutes during the first 30 minutes of the observation period and thereafter every 20 minutes to standardize the blood loss measurement protocol and to avoid overfilling of the operation area with blood.

Secondary end points were parameters of coagulation: (activated clotting time (ACT), prothrombin time (PT), and partial thromboplastin time (aPTT). These parameters were monitored before CPB and before application of heparin and study medication, at 45 and 90 minutes on CPB, and after weaning from CPB at 105 and 220 minutes from the time point of the initiation of CPB. ACT (Celite method), PT, and aPTT were assessed by routine clinical assays.

The influence on whole blood clotting was assayed with rotational thrombelastometry using ellagic acid (INTEM reagent; Pentapharm GmbH, Munich, Germany) as activator of the intrinsic system or tissue factor (EXTEM reagent; Pentapharm) as extrinsic coagulation activator. After a 5-minute preincubation with test compounds or saline, citrated human blood was subjected to rotation thromboelastography analysis (ROTEM; Pentapharm) according to the manufacturer's instructions. ROTEM clotting time (equal to reaction time) and maximum clot strength (MCF; equal to maximum amplitude) were obtained as coagulation parameters. In addition, fibrinolysis in whole blood was also studied with ROTEM. Activation of test samples accelerated the measurement process and enhanced reproducibility compared with conventional thromboelastography. To allow observation of fibrinolysis, we modified ROTEM analysis with tissue factor activation (EXTEM) through addition of tissue plasminogen activator.¹⁹ Citrated blood was preincubated at 37°C for 5 minutes with test compound or saline before tissue factor, CaCl₂, and tissue plasminogen activator (100 U/mL final concentration) were added to start the reaction. Fibrinolysis was observed over a period of 60 minutes and determined by measuring loss of clot strength with time (lysis time) and was recorded at the decline of the amplitude to 10% of MCF.

Hemodynamic Measurements

Aortic pressure was monitored with 5F Millar catheter-tip manometer (Millar Instruments Inc, Houston, Tex) inserted via the femoral artery. A 6F combined Millar conductance–pressure catheter was inserted into the left ventricle via the apex to register left ventricular pressures and volumes as well as intracardiac electrograms. A perivascular ultrasonic flow probe was attached to the ascending aorta.

Heart rate, aortic pressure, left ventricular end-systolic pressure, maximum and minimum pressure development (dP/dt max, dP/dt min), end-diastolic pressure, and the time constant of the isovolumetric pressure decay (τ), as well as cardiac output as the equivalent of aortic flow, were monitored continuously. Stroke volume was calculated from the integrated flow signal and was used to calibrate the volume signal from the conductance catheter. Parallel conductance was estimated by rapid injection of 1 mL of hypertonic saline into the left atrium.

The volume signal provided by the conductance catheter was registered continuously (Sigma F5, Leycom; Leiden, The Netherlands) and computed by the Conduct PC software (Leycom). Left ventricular pressure–volume loops were constructed online. Vena caval occlusions were performed to obtain a series of loops for calculation of the slope and intercept of the left and right ventricular end-systolic pressure–volume relationships. In addition, the slope of the left ventricular end-systolic pressure–volume relationship (ESPVR) and preload recruitable stroke work (PRSW) were calculated as load-independent indices of myocardial contractility.

Coronary blood flow was measured by an ultrasonic flow meter placed on the left anterior descending coronary artery. Coronary vascular resistance was calculated as the difference between mean aortic pressure and central

venous pressure divided by coronary blood flow. Coronary endothelium-dependent vasodilatation was assessed after intracoronary administration of a single bolus of acetylcholine (10^{-7} mol/L) and endothelium-independent vasodilatation after nitroglycerin (10^{-4} mol/L). The vasoresponse was expressed as percent change of baseline coronary vascular resistance.

Biochemical Measurements

Plasma myeloperoxidase concentrations an index of neutrophil activation and tissue malonaldehyde concentrations as an index of free radical generation and lipid peroxidation were determined by standard enzyme-linked immunosorbent assay kits from IBL, Hamburg, Germany. Tissue probes for malonaldehyde analysis were taken by biopsy before CPB and at the end of the experiments.

Statistics

All values were expressed as mean $\pm$ SEM. Paired *t* test was used to compare 2 means within groups. Individual means between the groups were compared by 1-way analysis of variance followed by an unpaired *t* test with Bonferroni correction for multiple comparisons and the post hoc Scheffé test. In the figures only the significances between the groups were indicated. Significant changes over the time within each group were indicated in the text.

RESULTS

Blood Loss

Postoperative blood loss during the first 2 hours after injection of protamine was significantly reduced in the aprotinin-treated group in comparison with control ($P < .05$). Application of CU-2010 resulted in a significant, dose-dependent reduction of postoperative blood loss (Figure 1).

Blood Coagulation Parameters

As expected, aPTT and ACT increased significantly in all groups after heparin and remained elevated during CPB. After protamine, ACT and aPTT returned to baseline levels in the control and aprotinin groups, but remained elevated (ACT only slightly, aPTT remarkably so) in the CU-2010 treatment groups (Figure 1).

The PT values increased slightly during CPB and remained at this level until the end of the observation period in all groups (Figure 1).

ROTEM parameters are shown in Table 1 at baseline and at the end of the observation period. Baseline values were similar in all groups. At the end of the observation period, clotting time (both EXTEM and INTEM) was prolonged in the CU-2010 groups in comparison with the vehicle and the aprotinin groups. EXTEM MCF showed a significant decrease after CPB but did not differ among the groups at baseline and at the end of the observation period. If INTEM reagents were used, similar data could be obtained, except that no significant decrease could be observed in the aprotinin group in comparison with baseline, and the MCF was significantly lower in comparison with aprotinin in the CU-2010 1.25 mg/kg group. However, in all groups except the aprotinin group, INTEM MCF still did not reach its maximum in one or more individuals within the measurement time and therefore the values are somewhat

FIGURE 1. Blood loss after weaning from cardiopulmonary bypass (CPB) and protamine (left top). Activated clotting time (ACT, right top), partial thromboplastin time (aPTT, left bottom), and prothrombin time (PT, right bottom). Time points indicate the time from the initiation of CPB (t0); t90 corresponds the end of CPB, t105, directly as well as 20 (T120), 50 (T150), and 120 (T220) minutes after protamine, respectively. All values are mean $\pm$ SEM. * $P < .05$ versus control; # $P < .05$ versus aprotinin.

underestimated. Lysis time (EXTEM) reached the designated end point (reduction of the amplitude to 10% MCF) neither in the CU-2010 nor in the aprotinin groups, but did so in the control group.

Hemodynamic Parameters

Heart rate, cardiac output, left ventricular end-systolic pressure, end-diastolic pressure, time constant of relaxation (τ), maximum and minimum pressure development (dP/dt max, dP/dt min), mean arterial pressure, and coronary blood flow are shown in Table 2. Baseline values did not differ among the groups. Although most hemodynamic parameters did not change significantly, mean arterial pressure and dP/dt min decreased in all groups. dP/dt max and coronary blood flow showed a significant decrease in the control group after CPB ($P < .05$), whereas they remained unchanged in the treatment groups.

After CPB, both left ventricular ESPVR and PRSW decreased significantly in the control and the aprotinin group;

these decreases could be partly up to completely preserved by application of CU-2010 (Table 2). Owing to the physiologic variations of the absolute values of ESPVR and PRSW, a comparison between the groups was somewhat difficult; therefore, percent recovery values were calculated (Figure 2). CU-2010 in higher doses significantly improved percent recovery in comparison with controls (and in case of PRSW in comparison with the aprotinin group as well).

Coronary Vascular Function

Vasoreponse to acetylcholine was similar in all groups before CPB. After CPB, endothelial function (vasodilative response to acetylcholine) was significantly reduced in the control group and was abolished by aprotinin and all doses of CU-2010 (Figure 2). Endothelium-independent vasodilatation after nitroglycerin did not differ between the groups and over time (Figure 2).

TABLE 1. ROTEM parameters

	Control	Aprotinin	CU-2010 0.5 mg/kg	CU-2010 0.83 mg/kg	CU-2010 1.25 mg/kg	CU-2010 1.66 mg/kg
<i>Baseline</i>						
EXTEM CT (s)	34 ± 4	33 ± 1	35 ± 1	31 ± 1	33 ± 4	38 ± 4
EXTEM MCF (mm)	65 ± 2	67 ± 2	66 ± 1	67 ± 2	65 ± 2	62 ± 2
EXTEM LT (s)	2466 ± 252	2498 ± 248	2331 ± 256	3051 ± 281	2505 ± 302	2729 ± 220
INTEM CT (s)	169 ± 8	201 ± 11	187 ± 12	178 ± 9	182 ± 14	172 ± 9
INTEM MCF (mm)	65 ± 3	65 ± 3	65 ± 1	67 ± 2	64 ± 3	66 ± 3
<i>Two hours of reperfusion</i>						
EXTEM CT (s)	43 ± 5	39 ± 3	58 ± 4*†‡	86 ± 8*†‡	82 ± 6*†‡	51 ± 5*†‡
EXTEM MCF (mm)	50 ± 7*	53 ± 5*	48 ± 2*	45 ± 4*	43 ± 3*	54 ± 3*
EXTEM LT (s)	2736 ± 360	>3600	>3600	>3600	>3600	3290 ± 222
INTEM CT (s)	312 ± 28*	268 ± 31*	845 ± 112*†‡	1587 ± 180*†‡	1520 ± 120*†‡	823 ± 67*†‡
INTEM MCF (mm)	49 ± 4*	57 ± 7	41 ± 3*	45 ± 5*	30 ± 8*†	47 ± 4*

The influence on whole blood clotting was assayed with rotational thrombelastometry using ellagic acid (INTEM reagent) as activator of the intrinsic system or tissue factor (EXTEM reagent) as extrinsic coagulation activator. Clotting time (CT), maximum clot strength (MCF), and lysis time (LT) were measured at baseline (before heparin and test substance) and after 2 hours of reperfusion. All values are given as mean ± SEM. **P* < .05 versus baseline; †*P* < .05 versus control; ‡*P* < .05 versus aprotinin.

Biochemical Measurements

Plasma myeloperoxidase levels increased significantly in all groups in comparison with baseline, but there were no differences among the groups (Table 3). Cardiac tissue malonaldehyde levels increased after CPB in the control group, but this increase was partly reversed by the use of the serine protease inhibitors. The most pronounced decrease was observed at the highest doses of CU-2010.

DISCUSSION

In the present study, we demonstrated that the novel synthetic small molecule serine protease inhibitor CU-2010 dose-dependently reduced postoperative blood loss after CPB to a degree comparable with that produced by aprotinin. This decrease of blood loss in the CU-2010 groups occurred despite prolonged ACT and aPTT values. Both aprotinin and CU-2010 attenuated endothelial dysfunction

TABLE 2. Hemodynamic variables

	Control	Aprotinin	CU-2010 0.5 mg/kg	CU-2010 0.83 mg/kg	CU-2010 1.25 mg/kg	CU-2010 1.66 mg/kg
<i>Baseline</i>						
HR (beats/min)	114 ± 8	140 ± 9	151 ± 7	144 ± 6	135 ± 5	118 ± 5
CO (mL/min)	2599 ± 353	2344 ± 290	2261 ± 288	2534 ± 259	1449 ± 141	1596 ± 180
LVEBP (mm Hg)	104 ± 9	106 ± 6	107 ± 2	91 ± 6	104 ± 5	97 ± 4
LVEDP (mm Hg)	9.92 ± 1.53	8 ± 0.91	6.8 ± 1	7 ± 1	9.83 ± 1	12.5 ± 1.76
τ (ms)	32 ± 1	34 ± 1	32 ± 1	27 ± 3	42 ± 3	46 ± 6
dP/dt max (mm Hg/s)	1811 ± 78	1784 ± 236	2076 ± 230	1503 ± 47	1575 ± 80	1570 ± 137
dP/dt min (mm Hg/s)	-1566 ± 160	-1866 ± 108	-2012 ± 63	-1609 ± 99	-1727 ± 100	-1542 ± 130
MAP (mm Hg)	104 ± 3.67	95 ± 11	88 ± 4	79 ± 8	95 ± 6	87 ± 6
CBF (mL/min)	40 ± 5	46 ± 4	46 ± 2	40 ± 3	44 ± 5	39 ± 4
ESPVR (mm Hg/mL)	3.04 ± 0.61	2.99 ± 0.33	2.77 ± 0.33	3.34 ± 0.51	4.20 ± 0.33	3.05 ± 0.80
PRSW (kerg)	68 ± 7	66 ± 5	62 ± 6	54 ± 4	51 ± 8	56 ± 6
<i>Two hours of reperfusion</i>						
HR (beats/min)	128 ± 4	136 ± 8	162 ± 8†	136 ± 6	145 ± 4†	133 ± 8
CO (mL/min)	2275 ± 344	2320 ± 264	2236 ± 286	1952 ± 224	1917 ± 209	1648 ± 198
LVEBP (mm Hg)	87 ± 4	89 ± 3	85 ± 6*	78 ± 5	93 ± 5	84 ± 3*
LVEDP (mm Hg)	11 ± 1	8 ± 0.	7 ± 1	8 ± 2	10 ± 1	11 ± 2
τ (ms)	34 ± 2	35 ± 1	28 ± 1	32 ± 5	42 ± 5	42 ± 5
dP/dt max (mm Hg/s)	1244 ± 93*	1394 ± 165	1620 ± 132†	1202 ± 149	1377 ± 128	1388 ± 147
dP/dt min (mm Hg/s)	-1111 ± 107*	-1444 ± 78*	-1473 ± 117*	-1215 ± 108*	-1377 ± 64*	-1220 ± 49*
MAP (mm Hg)	68 ± 2*	62 ± 4	60 ± 3*	58 ± 5	70 ± 5.9	59 ± 3
CBF (mL/min)	24.00 ± 3*	45 ± 5†	51 ± 7†	42 ± 6†	47 ± 5†	37 ± 3†
ESPVR (mm Hg/mL)	1.45 ± 0.5*	1.82 ± 0.32*	2.32 ± 0.34	2.81 ± 0.23	3.57 ± 0.58	2.96 ± 0.63†
PRSW (kerg)	47 ± 6*	37 ± 5*	48 ± 3*	40 ± 3*	55 ± 6†	57 ± 7†

Heart rate (HR), cardiac output (CO), left ventricular end-systolic pressure (LVEBP), end-diastolic pressure (LVEDP), time constant of relaxation (τ), maximum and minimum pressure development (dP/dt max, dP/dt min), mean arterial pressure (MAP), coronary blood flow (CBF), slope of the endsystolic pressure–volume relationship (ESPVR), and preload recruitable stroke work (PRSW). All values are given as mean ± SEM. **P* < .05 versus baseline; †*P* < .05 versus control; ‡*P* < .05 versus aprotinin.

FIGURE 2. Percent recovery of the load-independent slope of the end-systolic pressure–volume relationship (*ESPVR*, left top) and preload recruitable stroke work (*PRSW*, right top) 2 hours after weaning from cardiopulmonary bypass. Endothelium-dependent relaxation after application of acetylcholine (*ACh*, left bottom). Endothelium-independent relaxation after application of nitroglycerin (*NTG*, right bottom). *CVR*, Coronary vascular resistance. All values are mean $\pm$ SEM. **P* < .05 versus control; °*P* < .05 versus baseline; #*P* < .05 versus aprotinin; \$*P* < .05 versus all other groups.

after cardioplegic arrest and reperfusion. Furthermore, high-dose CU-2010 but not aprotinin improved postischemic recovery of left ventricular function.

Apart from the concerns raised about possible side effects of aprotinin, there is evidence that aprotinin reduces postoperative blood loss and transfusion requirements up to 40% to 50% in different population of patients (eg, first time, redo, patients with aspirin) undergoing cardiac surgery with²⁰⁻²²

or without CPB.²³ This study demonstrates for the first time that a member of a new class of synthetic small molecule serine protease inhibitors, CU-2010, significantly decreases postoperative blood loss after CPB in a canine model. The effects of CU-2010 were comparable with those of aprotinin.

The novel serine protease inhibitors may have some potential advantages that may also overcome some concerns raised currently about the use of aprotinin and leading to its

TABLE 3. Biochemical parameters

	Control	Aprotinin	CU-2010 0.5 mg/kg	CU-2010 0.83 mg/kg	CU-2010 1.25 mg/kg	CU-2010 1.66 mg/kg
<i>Baseline</i>						
MPO (ng/L)	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2	1.6 $\pm$ 0.2	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	1.3 $\pm$ 0.2
MDA (μ mol/L/g dry weight)	0.9 $\pm$ 0.1	1.2 $\pm$ 0.1	1.1 $\pm$ 0.1	1.0 $\pm$ 0.2	1.4 $\pm$ 0.1	1.1 $\pm$ 0.2
<i>Two hours of reperfusion</i>						
MPO (ng/L)	2.5 $\pm$ 0.2*	2.7 $\pm$ 0.3*	3.1 $\pm$ 0.3*	2.4 $\pm$ 0.2*	2.5 $\pm$ 0.4*	2.6 $\pm$ 0.2
MDA (μ mol/L/g dry weight)	5.3 $\pm$ 0.2*‡	3.4 $\pm$ 0.2*‡	2.9 $\pm$ 0.2°‡	3.1 $\pm$ 0.3*‡	2.4 $\pm$ 0.3*‡	1.9 $\pm$ 0.2*‡‡

Myeloperoxidase (MPO) and Malonaldehyde (MDA). All values are given as mean $\pm$ SEM, **P* < .05 versus baseline; †*P* < .05 versus control; ‡*P* < .05 versus aprotinin.

withdrawal from the market. First of all, in contrast to the biologic product aprotinin (bovine peptide), CU-2010 is a synthetic small molecule and its low molecular weight makes antigenicity less likely.²⁴ Beyond pure antifibrinolytic qualities, CU-2010 also demonstrated anticoagulant properties in previous studies,^{18,25} as was expected from its significantly higher affinity for plasma kallikrein and particularly for factor Xa and factor XIa. At concentrations between 100 and 1000 nm, which were found to be effective in inhibition of fibrinolysis, CU-2010 progressively delayed coagulation after both tissue factor and contact-phase stimulation, with the intrinsic assay being more sensitive to the compound's effect.²⁵ Therefore, in contrast to aprotinin, CU-2010 behaves like a moderate contact-phase inhibitor already at antifibrinolytic concentrations, although, the extent of its anticoagulant effects is smaller than that of dedicated anticoagulants like heparin or the factor Xa inhibitor rivaroxaban.²⁶ Importantly, in vitro, CU-2010 did not compromise clot strength measured by thrombelastometry.²⁵ This might be of relevance because reduced ROTEM amplitude has been associated with increased blood loss after cardiac surgery.^{19,27} In the present study, we could confirmed previous in vitro data showing real anticoagulatory properties (ie, prolonged whole blood coagulation times) that were in line with the ACT and aPTT measurements. The ROTEM measurements also clearly demonstrated that this anticoagulatory action does not influence clot firmness. Furthermore, clear antifibrinolytic property could be demonstrated, expressed as prolonged lysis times. As the ROTEM measurements were stopped at 1 hour and both aprotinin and CU-2010 still showed no signs of partial or complete clot lysis, exact lysis times could not be determined (>3600 seconds). The values are clearly higher as in the control group, but we do not have any evidence whether CU-2010 is superior to aprotinin. Here further studies are warranted. The unique combination of anticoagulatory and antifibrinolytic effects may provide a real advantage in comparison with aprotinin and most probably other antifibrinolytics, with a potentially higher safety profile against thromboembolic complications. Nevertheless, further studies are necessary in models of thrombosis or emboli before a final conclusion can be reached whether CU-2010 is able to reduce thromboembolic risk.

A second important aim of the study was to investigate whether the serine protease inhibitors can be translated to improved postischemic recovery. In the past decade, different studies showed that aprotinin may reduce ischemia/reperfusion injury. McCarthy and associates²⁸ showed for the first time that aprotinin improves regional systolic function after reversible coronary ligation in a canine model. Subsequently in mice,²⁹ rat,³⁰ rabbit,³¹ and pig models,^{32,33} protective effects could be demonstrated. The effectiveness of aprotinin on myocardial function was controversial. McEvoy and colleagues²⁹ found improved myocardial function in a mice model if aprotinin was applied in a lower dose,

and this effect was diminished at a high dose. In the study of Khan and colleagues,³² aprotinin reduced myocardial infarct size and improved coronary microvascular function but not myocardial function. Corroborating these preclinical findings to observations in the clinical setting, Wendel and associates³⁴ found that full-dose aprotinin reduced serum creatine kinase MB and troponin T levels in patients at 1 and 3 days after coronary artery bypass graft surgery compared with controls. As suggested by retrospective analysis of the Trasylol Proprietary Database, the ability of full-dose aprotinin to preserve myocardial function after ischemia/reperfusion may result in the decreased use of inotropic agents, vasopressors, and antiarrhythmic agents after coronary artery bypass grafting.³⁵ Aprotinin's actions are multiple: myocardial ischemia/reperfusion induces leukocyte accumulation, inflammation, gene expression, protein expression, and finally tissue injury, and aprotinin limits reperfusion injury through each of these stages, even after 24 hours of reperfusion. In both murine and pig models^{29,33} aprotinin reduces leukocyte-endothelial interaction, leukocyte migration, expression of adhesion molecules, apoptosis rates, and the expression of inflammatory genes (in particular tumor necrosis factor- α) and protein synthesis.

In the present study, we could demonstrate that aprotinin improved coronary endothelial function after myocardial ischemia/reperfusion, but not myocardial contractility, which is in line with the observations of Khan and associates.³² The novel serine protease inhibitor CU-2010 improved coronary endothelial function at all applied doses, and this improvement was comparable with aprotinin. In higher doses, CU-2010 could also improve the recovery of contractility in comparison with controls and thereby provided improved myocardial protection in comparison with aprotinin.

Although we did not measure inflammatory cytokines, we assume that the beneficial effects of CU-2010 are carried out on the same pathways as shown previously for aprotinin (see discussion above) as the anti-inflammatory potential of both substances is based on their plasma-kallikrein inhibitory effect. Cardiac tissue malonaldehyde levels were decreased in the serine proteinase inhibitor-treated groups, which was more pronounced at higher doses of CU-2010. This indicates lower levels of free radical generation and lipid peroxidation and subsequently anti-inflammatory properties. This, however, is unlikely carried out at the level of neutrophil activation inasmuch as myeloperoxidase levels were nearly identical among the groups.

In summary, CU-2010 is a small synthetic antifibrinolytic compound that dose-dependently reduces blood loss. Its profile is comparable with that of aprotinin with a stronger impact on the coagulation enzyme factor Xa and plasma kallikrein. Thereby it provides favorable anticoagulant properties and improved myocardial and endothelial protection. We believe that this compound deserves further investigations for clinical application.

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Discussion

Dr John A. Eleftheriades (New Haven, Conn). I congratulate the authors on an excellent experimental investigation. This is an important topic because patients with complex cardiac surgical disease currently have excess bleeding since the withdrawal of aprotinin. The members of our group are strong proponents of aprotinin. In 2006 in this *Journal* we published a study of patients with aneurysms operated on with and without aprotinin. Everything was better with aprotinin, not only blood product transfusion but also respiratory function and other parameters.

I have a few simple questions for you. The first one has to do with your measurement of blood loss. Why did you use sponges rather than chest tube drainage? Also, were the operators blinded as to study drug or no study drug?

Dr Szabo. First of all, the operator was blinded to control and the de novo compound but not aprotinin, because aprotinin is a different dosing scheme. The control group received continuous infusion as placebo.

Regarding the measurement of blood loss, in our experience, gauze bandages were more exact than using a chest tube drain. We measured blood loss volume in the first 2 hours at the open

chest situation. With drying the operative area with the gauze bandages, we could remove all blood from the operative area.

Dr Elefteriades. Since you were doing this experiment, why didn't you allow the animals to survive and look for renal function or evidence of thrombosis?

Dr Szabo. These experiments sought to establish a dose-response relationship and to explore whether the compound has some cardioprotective effects. As such it was designed as an acute experiment without keeping the animals alive. However, safety issues are very important, before moving to the clinical arena. These experiments are required from the authorities in Germany and they are running now.

Dr Elefteriades. Some of your cardiac functional parameters exceeded the preoperative baseline. Do you mean to imply that your drug has a positive inotropic effect?

Dr Szabo. Actually, it was within the physiologic ranges of a few percent, and I think it is just the physiologic variation. However, we also did pharmacokinetic experiments as dose finding previous to these experiments and in that case we measured hemodynamics, even at the larger range of doses. We did not see any direct inotropic effect of this drug.

Dr Elefteriades. Congratulations and I hope your drug is borne out in further studies.

Dr Szabo. We hope so as well. Thank you very much.