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CU-2010—A Novel Small Molecule Protease Inhibitor with Antifibrinolytic and Anticoagulant Properties

Wulf Dietrich, M.D., Ph.D., Silke Nicklisch, M.Sc.,† Andreas Koster, M.D., Ph.D.,‡ Michael Spannagl, M.D., Ph.D.,§ Helmut Giersiefen, Ph.D.,† Andreas van de Locht, Ph.D.†*

Background: **In cardiac surgery, the contact of blood with the artificial surfaces of the cardiopulmonary bypass results in activation of coagulation, fibrinolysis, and platelets, which is recognized as reason for increased bleeding tendency. Antifibrinolytics like tranexamic acid or the broad-spectrum protease inhibitor aprotinin attenuate this response. The marketing of aprotinin has been suspended after a recent clinical trial suggested increased risks associated with aprotinin. Moreover, aprotinin is a protein of animal origin and has antigenic properties. As a result, alternative antifibrinolytic compounds are desirable.**

Methods: **This *in vitro* study compared the antifibrinolytic efficacy of the synthetic small molecule CU-2010 with aprotinin and tranexamic acid. Antifibrinolytic activity in plasma and whole blood of ten healthy volunteers was examined with a turbidometric method and with tissue factor-activated thromboelastometry (ROTEM®; Pentapharm, Munich, Germany). In addition, anticoagulant effects were assessed through measurement of plasma and whole blood clotting times and thrombin generation.**

Results: **With its high affinity for plasmin (K_i, 2 nM), CU-2010 inhibited fibrinolysis comparable to aprotinin (K_i, 4 nM) and was ten times more potent than tranexamic acid. CU-2010 also inhibited plasma kallikrein (K_i < 1 nM) and factors Xa (K_i, 45 nM) and XIa (K_i, 18 nM), which was reflected in prolongation of coagulation times and an attenuation of thrombin generation.**

Conclusion: **These findings suggest that CU-2010 has similar antifibrinolytic potency compared to aprotinin, is more potent than tranexamic acid, and possesses some anticoagulant effects.**

IN cardiac surgery, the use of a 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 period.[1] Despite administration of large amounts of heparin, contact with the artificial surfaces of the heart-lung machine results in generation of thrombin[2] and activation of plasmin.

Prophylactic antifibrinolytic therapies during cardiac surgery are now widely accepted as a strategy to improve hemostasis and ameliorate 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 analogue 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 noncardiac[7] surgery. However, the currently available therapies do no eliminate all problems associated with CPB.

Aprotinin is a broad spectrum serine protease inhibitor, with its major targets being plasmin, trypsin, and plasma kallikrein. In addition, less thrombin generation has been observed with the use of aprotinin.[8] Such multimodal interference is thought to constitute the basis for aprotinin exerting multiple effects on many homeostatic functions, including coagulation, platelet activation, and inflammation.[9] In contrast, the lysine analogues tranexamic acid and epsilon amino caproic acid are solely antifibrinolytics, without anticoagulatory[10] or antiinflammatory properties.[11] Aprotinin is a protein of bovine origin and is associated with anaphylaxis.[12] Aprotinin's safety has recently been questioned because of suspected excess mortality[13,14] and impaired renal function,[15,16] which, despite some controversy,[17–19] finally led to suspension of worldwide marketing.[13]

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,[20] 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 to aprotinin or tranexamic acid and an improved safety profile is desirable.

* Professor of Anesthesia, Institute for Research in Cardiac Anesthesia, Munich, Germany, and Associate Professor, Working Group on Perioperative Hemostasis, Department of Hemostasiology, Ludwig Maximilian University, Munich, Germany. † Senior Scientist, Curacyte Discovery GmbH, Leipzig, Germany. ‡ Associate Professor, Institute for Anesthesiology, German Heart Center, Berlin, Germany. § Associate Professor, Working Group on Perioperative Hemostasis, Department of Hemostasiology, Ludwig Maximilian University, Munich, Germany.

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Address correspondence to Dr. Dietrich: Institute for Research in Cardiac Anesthesia, and Department of Hemostasiology, Ludwig Maximilian University, Munich, Germany, Winthirstr. 4, 80639 Munich, Germany. wulf.dietrich@t-online.de.

CU-2010 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 *et al.*[21] for a review of this class of compounds). CU-2010 shows rapid clearance after intravenous infusion, with an elimination half-life of approximately 20 min in rats and dogs (unpublished animal experiment data, Silke Nicklisch, M.Sc., Curacyte Discovery, Leipzig, Germany, January 2007).

The aim of the current study was to evaluate the antifibrinolytic efficacy and potency of CU-2010 in comparison to aprotinin and tranexamic acid *in vitro*. In addition, the potential anticoagulant properties of CU-2010 were investigated. First, the inhibition constants (K_i) against several human serine proteases were determined. Second, the antifibrinolytic activity in plasma and whole blood was evaluated using a turbidometric assay in plasma and rotation thrombelastometry in whole blood. Third, the impact of CU-2010 on coagulation was assessed from plasma and whole blood clotting times and from thrombin generation assays[22] in platelet-rich plasma (PRP).

Materials and Methods

Venous blood was withdrawn from the antecubital vein of healthy volunteers after we secured written informed consent in accordance with local and federal guidelines with approval of the local review board (Ethikkommission, Klinikum Charité, Berlin, Germany). The blood was mixed with 0.11 M sodium citrate (1:10). For ROTEM®(Pentapharm, Munich, Germany) analysis, whole citrated blood was used within 8 h after collection. PRP was prepared from citrated blood by centrifugation at 330*g* for 10 min at room temperature, platelet-poor plasma (PPP) was collected after centrifugation at 1220*g* for 12 min. The PRP was then adjusted to 3×10^8 platelets per ml using the autologous PPP. PRP was maintained at room temperature for less than 4 h before analysis. The remaining PPP was subjected to high-speed centrifugation at 40,000*g* for 30 min at 4°C to remove any particulate material and was then stored at −70°C until use.

CU-2010 was synthesized by Curacyte Discovery GmbH, Leipzig, Germany. Aprotinin and tranexamic acid were purchased from SIGMA (Schnelldorf, Germany). Human factor Xa, factor XIa, factor XIIa , thrombin, and plasma kallikrein were purchased from Enzyme Research Laboratories, and human plasmin was purchased from Chromogenix (both *via* Hemochrom, Essen, Germany). Various synthetic peptide chromogenic substrates used for determination of inhibition constants were obtained from Pentapharm (Basel, Switzerland), Roche (Mannheim, Germany), and Chromogenix: Chromozyme PL for plasmin, S2302 for plasma kallikrein, Pefachrome FXa for factor Xa, Chromozym XII for FXIIa, Pefachrom tPA for thrombin, and Pefachrome PCa for factor XIa. Tissue-type plasminogen activator was obtained from Boehringer Ingelheim (Ingelheim, Germany). INTEM, EXTEM, and STARTEM reagents and disposables for ROTEM® measurements were obtained from Pentapharm (Munich, Germany).

Determination of Inhibition Constants (K_i) against Human Serine Proteases

Inhibition of purified human serine proteases by CU-2010 and aprotinin was studied using established methods.[23] Enzyme kinetic experiments were carried out in 96-well flat-bottom plates (Brand, Wertheim, Germany) in 50 mM Tris-HCl pH 8.0, 154 mM NaCl in the presence of different substrate and inhibitor concentrations. Steady-state velocities of substrate conversion were obtained from progress curves generated by continuous monitoring of the absorbance at 405 nM with a microplate reader (Multiskan Ascent; Thermo Electron Corporation, Dreieich, Germany). For determination of K_i values less than 1 nM, measurements were performed in acrylic cuvettes (Brand) using a ultraviolet-visible spectrophotometer (Specord® M-400; Carl Zeiss, Jena, Germany). Inhibition constants (K_i) were calculated from nonlinear fits of individual data sets to the Michaelis-Menten equation for competitive inhibitors using an enzyme kinetic analysis software (SIGMA Plot® 9.0 Enzyme Kinetics Module; SSI, San Jose, CA). Dixon plot analysis was applied to confirm the competitive inhibition mode.

Establishment of Antifibrinolytic Potency

The antifibrinolytic activity of CU-2010 in comparison to aprotinin and tranexamic acid was investigated in plasma and whole blood assays. In both assays, tissue factor was added to initiate rapid clot formation *via* the extrinsic pathway, which produces a clot that remains stable for several hours under normal conditions. Supplementation of plasma or whole blood with tissue-type plasminogen activator (t-PA) before stimulation results in activation of endogenous plasminogen and thus fibrinolysis while the initial clot formation is not impaired. The amount of t-PA added was found to determine lysis time. A final concentration of 50 and 100 U/ml in plasma and whole blood, respectively, was chosen to achieve complete lysis within approximately 60 min. In the presence of antifibrinolytics, clot lysis is delayed in a concentration-dependent manner.

Plasma Fibrinolysis Assay. Inhibition of fibrinolysis in plasma was examined using a turbidometric method in 96-well flat-bottom plates. The time course of clot formation and lysis, reflected by an initial increase and subsequent decrease in turbidity, was recorded by continuously measuring the optical density at 405 nm. Similar models have been widely used to study the clot lysis

process in human plasma[24] and its inhibition by antifibrinolytic drugs.[10] Frozen and thawed human plasma (PPP) was preincubated with test compound or vehicle (Owren Veronal buffer) for 5 min at 37°C. Coagulation and subsequent fibrinolysis was started by adding tissue factor (Innovin®; Dade Behring, Eschborn, Germany; 1:9000 final dilution), $CaCl_2$ (12 mM final concentration), and t-PA (50 U/ml final concentration) simultaneously to the wells. Optical density at 405 nm was monitored every 45 s for 180 min at 37°C with a microplate reader (POLARstar OPTIMA; BMG Labtech, Offenburg, Germany). Fibrinolysis was quantified as the relative decrease in optical density at 45 min after the maximum optical density was reached. CU-2010 and aprotinin were each tested at concentrations of 60, 100, 200, 300, 600, 1000, and 3000 nM, and tranexamic acid was tested at concentrations of 600, 1000, 3000, 6000, 10,000, 20,000, and 30,000 nM to cover the complete concentration-response for each compound. These aprotinin concentrations range between 2.8 and 140 kallikrein inhibiting units (KIU)/ml based on the conversion factor of 7.14 KIU/μg. Concentration-response curves were established by plotting percentage fibrinolysis *versus* test compound concentration.

Whole Blood Fibrinolysis Assay. Fibrinolysis in whole blood was studied with rotational thromboelastometry[25] by using a computerized, multichannel ROTEM® instrument.[26] Activation of test samples accelerated the measurement process and enhanced reproducibility compared with conventional thromboelastography. To allow observation of fibrinolysis, ROTEM® analysis with tissue factor activation (EXTEM) was modified through addition of t-PA.[27] Citrated blood was preincubated at 37°C for 5 min with test compound or saline before tissue factor, $CaCl_2$, and t-PA (100 U/ml final concentration) were added to start the reaction. Fibrinolysis was determined by measuring loss of clot strength with time and was recorded as Ly60 (percentage reduction of the maximum amplitude at 60 min after the onset of clotting). In control samples without inhibitor, clots were lysed completely within 60 min, such that Ly60 was above 90%. IC_{50} values for each compound were calculated by plotting Ly60 *versus* test compound concentration.

Assessment of Anticoagulant Potency

Since CU-2010 inhibits multiple proteases of the coagulation system, we investigated possible anticoagulant properties by using established tests *in vitro*. Whereas tissue factor is the physiologic trigger of coagulation, both extrinsic and contact-mediated stimulation contribute to hemostatic activation under conditions like CPB.[2,20] We therefore studied the impact of CU-2010 and aprotinin on coagulation in plasma and whole blood after both intrinsic and extrinsic stimulation. Tranexamic acid has no influence on other proteases than plasminogen; therefore, it is not included in these experiments.

Plasma Coagulation Times. Prothrombin time and activated partial thromboplastin time were determined after human plasma (PPP) was supplemented with test compound solution or saline using a conventional coagulation analyzer (Sysmex CA-560; Dade Behring). Reagents used were Innovin® (extrinsic activator containing tissue factor) for prothrombin time and Actin® FSL (contact activator containing ellagic acid and phospholipids) for activated partial thromboplastin time (both from Dade Behring).

Whole Blood Coagulation Assay. The influence on whole blood clotting was assayed with rotational thrombelastometry[26] using ellagic acid (INTEM reagent) as activator of the intrinsic system or tissue factor (EXTEM reagent) as extrinsic coagulation activator. After a 5-min preincubation with test compounds or saline, citrated human blood was subjected to ROTEM® analysis according to the manufacturer's instructions. ROTEM® clotting time (equal to reaction time, r) and maximum clot strength (equal to maximum amplitude) were obtained as coagulation parameters.

Thrombin Generation Assay. The impact of CU-2010 and aprotinin on thrombin generation was studied in PRP by using the commercially available Technothrombin® TGA kit (Technoclone, Vienna, Austria). This method allows assessment of the dynamics of thrombin generation, *i.e.*, initiation, propagation, and inactivation phases, including the contribution of platelet function to the clotting process.[22] PRP was spiked with test compounds at different concentrations and prewarmed to 37°C in a black 96-well flat-bottom plate (Nunc, Wiesbaden, Germany). Thrombin generation was then initiated by adding a mixture of activator and fluorogenic thrombin substrate. Two different activators were used: a tissue factor-containing reagent provided by the manufacturer for extrinsic stimulation, and Actin FSL® at 1:120 final dilution for intrinsic stimulation of thrombin generation. Starting immediately after addition of reagents, fluorescence was recorded every 60 s for 120 min by using the BMG POLARstar microplate reader (BMG Labtech) set at 390-nm excitation and 460-nm emission and maintaining a temperature of 37°C. Data analyses were performed with the Technothrombin® software. A typical thrombin generation curve is generated by plotting the first derivative (dF/dt) of the original fluorescence *versus* time curve and comparing it to a standard run containing known amounts of thrombin in buffer. From these curves representing the time course of thrombin activity, the following parameters are derived: the lag phase (in min) from time zero until the start of thrombin generation, peak thrombin level (in nM) and the area under the thrombin generation curve (endogenous thrombin potential, in nM · min).

Table 1. Inhibition Constants (K_i) of CU-2010 and Aprotinin against Human Serine Proteases

	CU-2010 K_i, nM	Aprotinin K_i, nM
Plasmin	2.2 ± 0.2	4.2 ± 0.4
Plasma kallikrein	0.019 ± 0.003	38 ± 2
Factor Xa	45 ± 5	55,600 ± 400
Factor XIa	18 ± 1	1840 ± 40
Factor XIIa (alpha)	5200 ± 400	5400 ± 100
Thrombin	1700 ± 200	76,000 ± 2,000

Enzyme kinetic analysis was performed with purified enzymes using chromogenic substrates. CU-2010 competitively inhibits the amidolytic activity of human serine proteases with the strongest affinity for plasma kallikrein and plasmin. Compared to aprotinin, the compound is substantially more potent against plasma kallikrein and factors Xa and XIa, but both inhibitors are much less effective against thrombin. As tranexamic acid has no direct effect on the amidolytic serine protease activity, K_i values can not be determined for this compound.

Data represent mean ± SD of three independent measurements, each carried out in duplicate.

Statistical Analyses

Statistical analyses were performed using SigmaPlot® 9.0 (SSI) and SPSS software (SPSS Inc., Chicago, IL). Data are presented as mean ± SD (SD) or median with 25th and 75th percentile for nonnormal distributed measurements.

Differences among groups were assessed by nonparametric Kruskal-Wallis test and pairwise *post hoc* comparisons using Mann–Whitney U test. Assessment of differences between two related samples was conducted by Wilcoxon signed ranks test. To reduce multiple test issues, Bonferroni correction of *P* values was applied within each many-one group comparison (several concentrations *vs.* one control) as well in any pairwise *post hoc* comparison. All *P* values were two-tailed, and a *P* value < 0.05 was considered to outline statistical significance.

Results

Results of enzyme kinetic experiments are summarized in table 1. CU-2010 and aprotinin show comparable inhibition of plasmin, whereas CU-2010 displayed substantially stronger inhibition of plasma kallikrein (2000-fold), FXa (1200-fold), and FXIa (100-fold).

The effect of CU-2010, aprotinin, and tranexamic acid on the dynamics of clot formation and lysis in whole blood ROTEM® is depicted in figure 1. All three agents have equivalent antifibrinolytic efficacy; however, potency differs significantly: CU-2010 and aprotinin largely suppress clot lysis at concentrations of 600 and 1000 nM, respectively, and tranexamic acid requires concentrations between 3000 and 10,000 nM for effective inhibition. Both CU-2010 and aprotinin produce a concentration-dependent decrease of Ly60 (fig. 2A). The concentrations resulting in 50% suppression of clot lysis (IC_{50}, median [25th, 75th, percentile]) are 150 (115, 210) nM and 345 (304, 497) nM (corresponding to 16 KIU/ml) for CU-2010 and aprotinin, respectively ($P < 0.001$ for comparison of CU-2010 *vs.* aprotinin). Tranexamic acid also reduced clot lysis in a concentration-dependent manner, although with substantially lower potency (IC_{50} = 2750 [1875, 3225] nM; $P < 0.001$ *vs.* CU-2010, $P = 0.002$ *vs.* aprotinin).

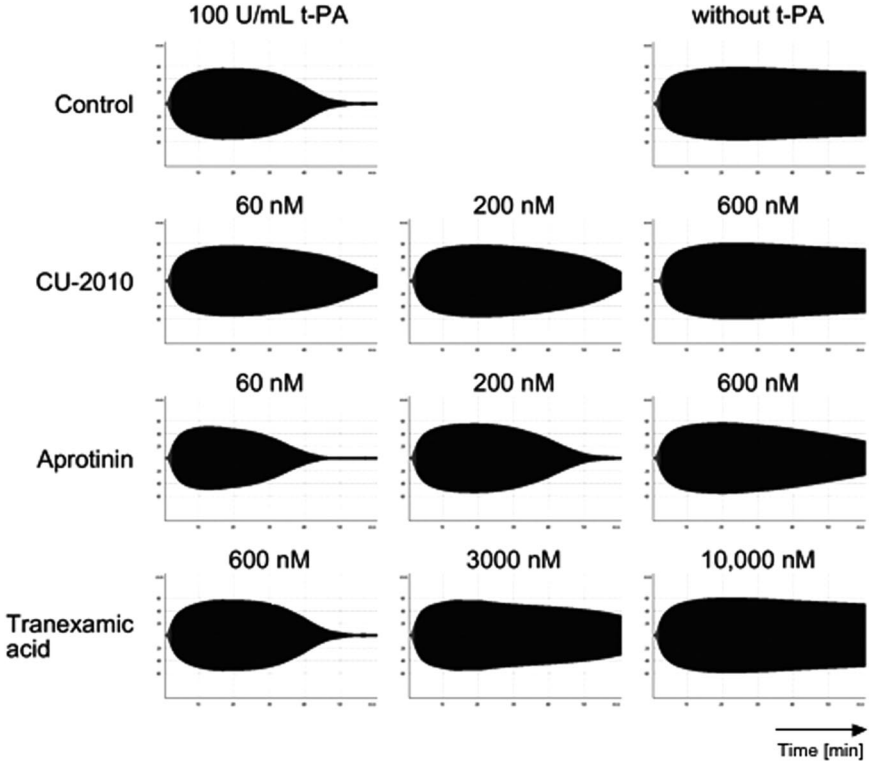

Fig. 1. Inhibitory effects of CU-2010, aprotinin, and tranexamic acid on tissue-type plasminogen activator (t-PA)–induced lysis of whole blood clots. Antifibrinolytic effects of CU-2010, aprotinin, and tranexamic acid were compared by using the ROTEM® analyzer (Pentapharm, Munich, Germany). Coagulation and subsequent lysis were stimulated by simultaneous administration of tissue factor and t-PA (at 100 U/ml final concentration) to recalcified citrated blood. The top panel shows control tracings with and without t-PA. The other tracings are representative of the antifibrinolytic effect of the respective concentrations of CU-2010, aprotinin, and tranexamic acid. Whereas CU-2010 and aprotinin are effective at nanomolar concentrations, much higher concentrations of tranexamic acid are required for equivalent inhibition of lysis.

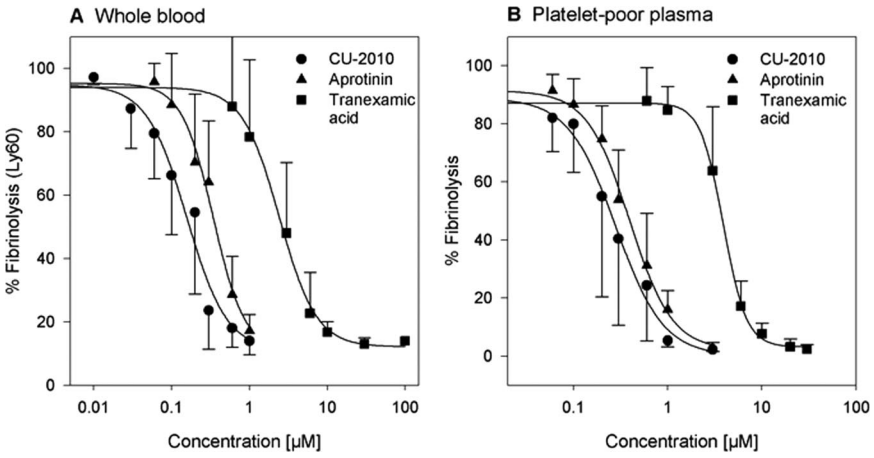

Fig. 2. Concentration-response curves of antifibrinolytic efficacy of CU-2010, aprotinin, and tranexamic acid in human whole blood and plasma. (*A*) To compare the concentration-dependent antifibrinolytic effects of CU-2010, aprotinin, and tranexamic acid in whole blood, Ly60 (*i.e.*, percentage reduction of clot strength at 60 min after the onset of clotting) was obtained from ROTEM®-based fibrinolysis assays (Pentapharm, Munich, Germany) as shown in figure 1 and was plotted *versus* inhibitor concentration. IC_{50} values (median [25th, 75th percentile]) of 150 (115, 210) nM for CU-2010, 345 (304, 397) nM for aprotinin and 2750 (1875, 3225) nM for tranexamic acid were derived from fitted curves. (*B*) Fibrinolysis was similarly examined in platelet-poor plasma activated by simultaneous addition of tissue factor and tissue-type plasminogen activator (t-PA; 50 U/ml final concentration) using a turbidometric method. CU-2010, aprotinin, and tranexamic acid suppressed plasma fibrinolysis with IC_{50} values of 315(135, 506) nM, 327 (280, 537) nM, and 4225 (3050, 4280 nM, respectively. Data points represent means ± SD of (*A*) 8 donors and (*B*) 10 donors, respectively.

Similar results were obtained when human plasma was used instead of whole blood as shown in figure 2B. CU-2010 and aprotinin exhibit comparable potency on t-PA-induced fibrinolysis in plasma, with IC_{50} values of 315 (135, 506) nM and 327 (280, 537) nM (15 KIU/ml), respectively ($P = 0.9$ for comparison of CU-2010 *vs.* aprotinin). Tranexamic acid exhibits an IC_{50} of 4225 (3050, 4280 nM ($P < 0.001$ *vs.* CU-2010 and aprotinin), indicating its significantly lower antifibrinolytic potency.

In addition to its inhibition of plasma kallikrein, CU-2010 also affects factors Xa and XIa. Hence, a significant prolongation of plasma and whole blood coagulation times was observed in the presence of CU-2010 at antifibrinolytic concentrations ranging from 100 to 1000 nM (table 2). These effects were more pronounced upon intrinsic activation (reflected in activated partial thromboplastin time and INTEM results) compared to tissue factor activation (represented by prothrombin time and EXTEM clotting time). Aprotinin had almost no influence on plasma or whole blood coagulation at equivalent concentrations (table 2). A marked prolongation of both activated partial thromboplastin time and intrinsic ROTEM® clotting times occurred at higher aprotinin concentrations, whereas extrinsic coagulation was not affected (data not shown).

Neither CU-2010 nor aprotinin impaired clot strength (reflected in ROTEM® maximum amplitude) in any of the ROTEM® assays over the concentration range tested (data not shown).

CU-2010 also had a similar effect on thrombin generation after both intrinsic and extrinsic activation that was statistically significant compared to aprotinin (fig. 3, table 2). A concentration-dependent delay in the onset of thrombin generation as well as a reduction in the peak thrombin level was observed, whereas there was no impact on endogenous thrombin potential in the presence of 100 to 1000 nM CU-2010.

Discussion

This study demonstrates *in vitro* the efficacy and potency of the novel synthetic, small-molecule direct serine protease inhibitor CU-2010. The findings can be summarized as follows. First, CU-2010 and aprotinin have almost similar nanomolar potency (2.3 *vs.* 4.2 nM) regarding inhibition of plasmin enzymatic activity. Second, consistent with K_i data, CU-2010 and aprotinin display similar nanomolar potencies at inhibiting clot lysis in whole blood (IC_{50}, 150 *vs.*. 345 nM) and plasma (IC_{50}, 315 *vs.* 327 nM), both drugs being approximately 10-fold more potent than tranexamic acid. Third, CU-2010 and aprotinin, but not tranexamic acid, display anticoagulant properties, with CU-2010 being more potent than aprotinin as assessed by ROTEM®, global plasma coagulation tests, and inhibition of thrombin generation. We have thus identified CU-2010 as an inhibitor of fibrinolysis that is at least equivalent to aprotinin and more potent compared to tranexamic acid in all investigations *in vitro* so far.

Beyond pure antifibrinolytic qualities, CU-2010 also demonstrated anticoagulant properties as was expected from its significantly higher affinity for plasma kallikrein and particularly for factor Xa. 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. Therefore, in contrast to aprotinin, CU-2010 behaves as a moderate contact-phase inhibitor already at antifibrinolytic concentrations. However, the extent of its anticoagulant effects is rather small compared to dedicated anticoagulants like heparin or the factor Xa inhibitor rivaroxaban.[28] Importantly, CU-2010 did not compromise clot strength measured by thrombelastometry. This might be of relevance because

Table 2. Influence of CU-2010 and Aprotinin on Coagulation Parameters *In Vitro*

	Plasma Clotting Times		Whole Blood Clotting Times (ROTEM®)	
	PT, s	aPTT, s	Extrinsic CT, s	Intrinsic CT, s
Control	10.1 (8.4, 10.8)	29.8 (26.8, 33.9)	51 (41, 58)	159 (147, 209)
CU-2010				
100 nM	10.0 (9.6, 10.3)	30.3*† (29.8, 30.7)	53 (49, 54)	188*† (174, 205)
300 nM	10.2*† (9.8, 10.4)	33.9*† (33.2, 36.3)	58*† (55, 59)	243*† (218, 257)
1000 nM	11.0*† (10.5, 11.2)	51.6*† (49.8, 54.4)	82*† (75, 93)	326*† (293, 359)
Aprotinin				
100 nM	9.9 (9.5, 10.1)	30.8* (30.3, 31.3)	46 (44, 50)	157* (151, 162)
300 nM	9.9 (9.5, 10.1)	31.6* (30.6, 31.7)	47* (45, 49)	163* (152, 163)
1000 nM	10.0 (9.6, 10.2)	34.7* (33.8, 35.4)	48 (47, 53)	178* (167, 172)

reduced ROTEM® amplitude has been associated with increased blood loss after cardiac surgery.[29,30]

Sperzel *et al.*[10] evaluated aprotinin and tranexamic acid *in vivo* in models of coagulation. In a rat tail-bleeding model, both were equally effective in stopping bleeding tendency, although at different concentrations. Surprisingly, in an arteriovenous shunt model, aprotinin reduced thrombus formation, whereas tranexamic acid increased it. These findings provide further grounds for objections against pure antifibrinolytics, and they stress the relevance of balanced impact on the intricate and finely tuned system of hemostasis.

Due to its inhibition of other targets involved in hemostasis, CU-2010 may provide additional advantages beyond pure antifibrinolytic efficacy. Plasma kallikrein plays a central role in contact activation and in the kinin-generating pathways.[31,32] During CPB, activation of the kallikrein-kinin system results in elevated bradykinin release and represents one major stimulus for complement and neutrophil activation, contributing to the systemic inflammatory response, which is one of the major causes of CPB-related organ injury.[10,33–35] As a result, CU-2010s affinity for plasma kallikrein may provide additional benefits by mitigating prothrombotic processes without compromising hemostasis[36]; at the concentrations tested for efficacious inhibition of fibrinolysis, plasma kallikrein activity was completely suppressed. This is in contrast to aprotinin, for which the high-dose regimen aims to achieve plasma levels of 200 KIU/ml (4300 nM)[37] to markedly inhibit plasma kallikrein. However, this targeted plasma concentration is rarely achieved in the clinical setting.[38]

Furthermore, heparin is not able to inhibit clot-bound thrombin,[39] and continuous thrombin generation occurs

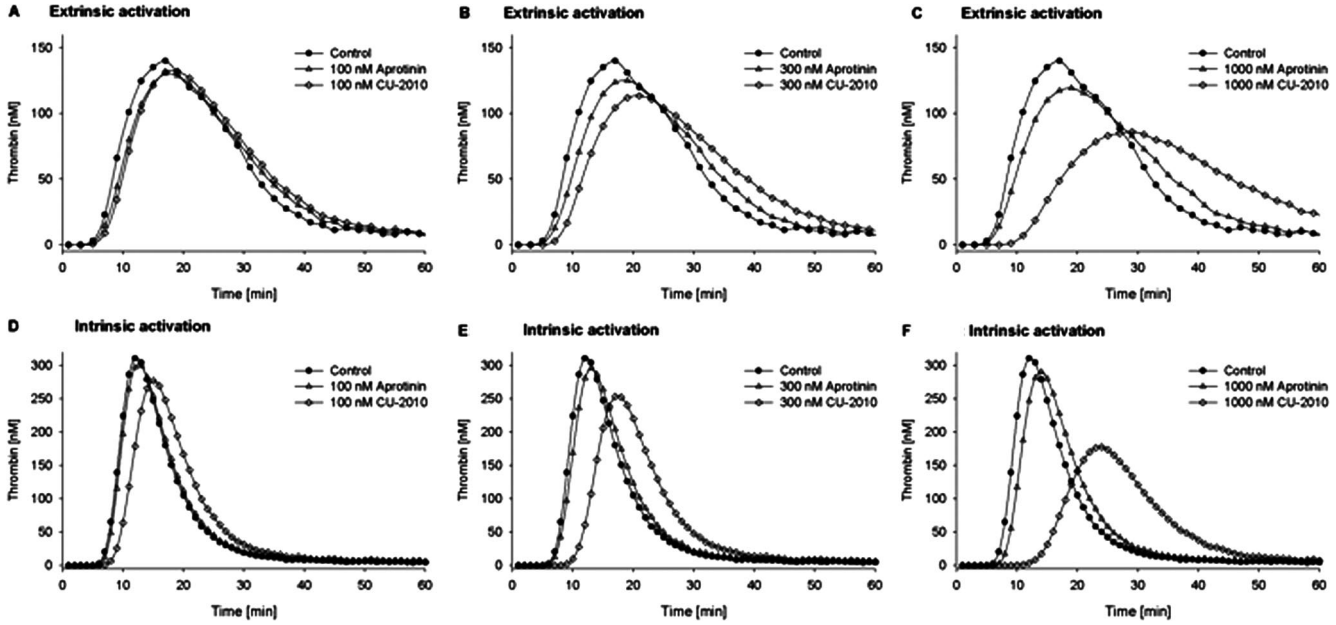

Fig. 3. Effects of CU-2010 and aprotinin on thrombin generation in platelet-rich human plasma Thrombin generation assays were performed with extrinsic (*A*, *B*, *C*) or intrinsic (*D*, *E*, *F*) stimulation. The time course of thrombin activity in the absence (control) or presence of various drug concentrations (as indicated) was measured in platelet-rich plasma from nine donors. Individual curves represent the average of all measurements. A concentration-dependent delay of thrombin generation and a reduction of peak thrombin levels were observed in the presence of CU-2010 in both assays. Compared to CU-2010, aprotinin at identical concentrations was much less effective.

Table 2. Influence of CU-2010 and Aprotinin on Coagulation Parameters *In Vitro*

Extrinsic Thrombin Generation			Intrinsic Thrombin Generation		
Lag Phase, min	Peak Thrombin, nM	ETP, nM/min	Lag Phase, min	Peak Thrombin, nM	ETP, nM/min
9.0 (8.0, 14.0)	156 (125, 237)	3304 (2973, 3837)	9.0 (8.0, 10.0)	338 (274, 375)	3444 (2982, 3957)
10.0* (9.5, 10.5)	142* (113, 169)	3283 (3171, 3845)	11.0*† (10.0, 12.0)	296*† (277, 317)	3475 (3430, 3910)
11.5*† (10.0, 12.0)	130* (98, 143)	3215 (3114, 3818)	13.0*† (12.0, 14.0)	282*† (243, 296)	3456 (3416, 3870)
16.0*† (14.0, 16.5)	80*† (77, 111)	3010* (2994, 3643)	16.5*† (16.0, 21.0)	192*† (192, 244)	3306* (3209, 3684)
9.0 (9.0, 10.0)	134* (117, 150)	3207* (3057, 3650)	9.5 (9.0, 10.0)	325* (295, 334)	3401* (3362, 3818)
9.0* (9.0, 10.0)	125* (117, 146)	3201* (3075, 3651)	10.0 (9.0, 10.0)	323* (285, 332)	3449 (3402, 3852)
9.0* (9.0, 11.0)	122* (111, 143)	3200* (3111, 3689)	10.0* (10.0, 10.5)	311 (271, 324)	3446 (3413, 3870)

Data represent median (25th, 75th percentile) of nine donors. Human whole blood and plasma samples, respectively, were supplemented with CU-2010 or aprotinin at the concentrations indicated. PT and aPTT were determined in platelet-poor plasma, whole blood coagulation was measured by thrombelastometry (ROTEM® analysis; Pentapharm, Munich, Germany). Thrombin generation parameters lag phase, peak thrombin, and ETP were derived from TGA measurements (figure 3) in platelet-rich plasma. Both the extrinsic and intrinsic pathway of coagulation were studied by using tissue factor reagents (PT, extrinsic ROTEM®, and extrinsic TGA) and contact-phase activators (aPTT, intrinsic ROTEM®, and intrinsic TGA) as trigger. At antifibrinolytic concentrations ranging from 100 to 1000 nM, CU-2010 concentration-dependently caused a slight but significant delay in the onset of both clotting and thrombin generation (lag time) as well as a reduction in peak thrombin levels. This effect was more pronounced in whole blood compared to plasma and also when triggered *via* contact activation compared to tissue factor stimulation. Aprotinin at the same final concentrations had only a small effect on the onset of intrinsically stimulated thrombin generation and clotting but none after extrinsic activation. Neither compound affected ETP notably, irrespective of the activator used.

* $P < 0.05$ *vs.* control. † $P < 0.05$ CU-2010 *vs.* aprotinin at the same final concentration.

aPTT = activated partial thromboplastin time; CT = clotting time; ETP = endogenous thrombin potential; PT = prothrombin time; TGA = thrombin generation assay.

during CPB.[20] Therefore, additional or complementary attenuation of procoagulant processes on top of heparin administration may provide benefits for clinical outcome.[20] However, putative benefits as well as potential impact of CU-2010s anticoagulant properties on bleeding will have to be investigated in future studies.

CU-2010 offers a number of potential benefits compared to aprotinin. It is a synthetic compound with no risks of transmitting animal-derived diseases. It has a low molecular weight, so it is unlikely to elicit anaphylactic reactions. Finally, its shorter half-life (terminal half-life is 20 min in rats and dogs) means that stable plasma concentrations may be more easily controlled.

These conclusions are subject to several limitations. First, our studies *in vitro* can only approximate the situation *in vivo* because these models lack important components of hemostasis. Second, our experiments were performed with blood from healthy volunteers not obtained during cardiac surgery. The significance of our results may thus be limited; during CPB, blood is activated extensively, the temperature is different,[40] and coagulation interacts with other drugs like starch.[41] Third, whether the effects of CU-2010 on coagulation are beneficial in a clinical scenario is speculative and remains to be examined. Possibly, the stronger anticoagulative quality of CU-2010 may affect heparin management.

In summary, CU-2010 is a small synthetic antifibrinolytic compound that concentration-dependently inhibits several serine proteases of the hemostatic system. It is not of animal origin, and its profile is comparable to that of aprotinin with a stronger impact on the coagulation enzymes factor Xa and plasma kallikrein. Its low molecular weight makes antigenicity unlikely. CU-2010 is a promising compound that deserves further investigation for clinical application.

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References

1. Ferraris VA, Ferraris SP, Saha SP, Hessel EA 2nd, Haan CK, Royston BD, Bridges CR, Higgins RS, Despotis G, Brown JR, Spiess BD, Shore-Lesserson L, Stafford-Smith M, Mazer CD, Bennett-Guerrero E, Hill SE, Body S: Perioperative blood transfusion and blood conservation in cardiac surgery: The Society of Thoracic Surgeons and The Society of Cardiovascular Anesthesiologists clinical practice guideline. Ann Thorac Surg 2007; 83:S27–86

2. Boisclair MD, Lane DA, Philippou H, Esnouf MP, Sheikh S, Hunt B, Smith KJ: Mechanisms of thrombin generation during surgery and cardiopulmonary bypass. Blood 1993; 82:3350–7

3. Mannucci PM, Levi M: Prevention and treatment of major blood loss. N Engl J Med 2007; 356:2301–11

4. Brown JR, Birkmeyer NJ, O'Connor GT: Meta-analysis comparing the effectiveness and adverse outcomes of antifibrinolytic agents in cardiac surgery. Circulation 2007; 115:2801–13

5. Henry DA, Carless P, Moxey A, O'Connell D, Stokes B, McClelland B, Laupacis A, Fergusson D: Anti-fibrinolytic use for minimising perioperative allogeneic blood transfusion. Cochrane Database Syst Rev 2007:CD001886

6. Sedrakyan A, Treasure T, Elefteriades JA: Effect of aprotinin on clinical outcomes in coronary artery bypass graft surgery: A systematic review and meta-analysis of randomized clinical trials. J Thorac Cardiovasc Surg 2004; 128: 442–8

7. Zufferey P, Merquiol F, Laporte S, Decousus H, Mismetti P, Auboyer C, Samama CM, Molliex S: Do antifibrinolytics reduce allogeneic blood transfusion in orthopedic surgery? ANESTHESIOLOGY 2006; 105:1034–46

8. Landis RC, Asimakopoulos G, Poullis M, Haskard DO, Taylor KM: The antithrombotic and antiinflammatory mechanisms of action of aprotinin. Ann Thorac Surg 2001; 72:2169–75

9. Asimakopoulos G, Thompson R, Nourshargh S, Lidington EA, Mason JC, Ratnatunga CP, Haskard DO, Taylor KM, Landis RC: An anti-inflammatory property of aprotinin detected at the level of leukocyte extravasation. J Thorac Cardiovasc Surg 2000; 120:361–9

10. Sperzel M, Huetter J: Evaluation of aprotinin and tranexamic acid in different *in vitro* and *in vivo* models of fibrinolysis, coagulation and thrombus formation. J Thromb Haemost 2007; 5:2113–8

11. Hill GE, Robbins RA: Aprotinin but not tranexamic acid inhibits cytokine-induced inducible nitric oxide synthase expression. Anesth Analg 1997; 84:1198–202

12. Dietrich W, Ebell A, Busley R, Boulesteix AL: Aprotinin and anaphylaxis: Analysis of 12,403 exposures to aprotinin in cardiac surgery. Ann Thorac Surg 2007; 84:1144–50

13. Fergusson DA, Hebert PC, Mazer CD, Fremes S, MacAdams C, Murkin JM, Teoh K, Duke PC, Arellano R, Blajchman MA, Bussieres JS, Cote D, Karski J, Martineau R, Robblee JA, Rodger M, Wells G, Clinch J, Pretorius R: The BI: A Comparison of aprotinin and lysine analogues in high-risk cardiac surgery. N Engl J Med 2008; 358:2319–31

14. Shaw AD, Stafford-Smith M, White WD, Phillips-Bute B, Swaminathan M, Milano C, Welsby IJ, Aronson S, Mathew JP, Peterson ED, Newman MF: The effect of aprotinin on outcome after coronary-artery bypass grafting. N Engl J Med 2008; 358:784–93

15. Mangano DT, Tudor IC, Dietzel C: The risk associated with aprotinin in cardiac surgery. N Engl J Med 2006; 354:353–65

16. Karkouti K, Beattie WS, Dattilo KM, McCluskey SA, Ghannam M, Hamdy A, Wijeysundera DN, Fedorko L, Yau TM: A propensity score case-control comparison of aprotinin and tranexamic acid in high-transfusion-risk cardiac surgery. Transfusion 2006; 46:327–38

17. Mouton R, Finch D, Davies I, Binks A, Zacharowski K: Effect of aprotinin on renal dysfunction in patients undergoing on-pump and off-pump cardiac surgery: A retrospective observational study. Lancet 2008; 371:475–82

18. Westaby S: Aprotinin: Twenty-five years of claim and counterclaim. J Thorac Cardiovasc Surg 2008; 135:487–91

19. Dietrich W, Busley R, Boulesteix AL: Effects of aprotinin dosage on renal function: An analysis of 8,548 cardiac surgical patients treated with different dosages of aprotinin. ANESTHESIOLOGY 2008; 108:189–98

20. Edmunds LH Jr, Colman RW: Thrombin during cardiopulmonary bypass. Ann Thorac Surg 2006; 82:2315–22

21. Katz BA, Sprengeler PA, Luong C, Verner E, Elrod K, Kirtley M, Janc J, Spencer JR, Breitenbucher JG, Hui H, McGee D, Allen D, Martelli A, Mackman RL: Engineering inhibitors highly selective for the S1 sites of Ser190 trypsin-like serine protease drug targets. Chem Biol 2001; 8:1107–21

22. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Beguin S: Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003; 33:4–15

23. Sturzebecher J, Prasa D, Hauptmann J, Vieweg H, Wikstrom P: Synthesis and structure-activity relationships of potent thrombin inhibitors: Piperazides of 3-amidinophenylalanine. J Med Chem 1997; 40:3091–9

24. Kim PY, Stewart RJ, Lipson SM, Nesheim ME: The relative kinetics of clotting and lysis provide a biochemical rationale for the correlation between elevated fibrinogen and cardiovascular disease. J Thromb Haemost 2007; 5:1250–6

25. Luddington RJ: Thrombelastography/thromboelastometry. Clin Lab Haematol 2005; 27:81–90

26. Ganter MT, Hofer CK: Coagulation monitoring: Current techniques and clinical use of viscoelastic point-of-care coagulation devices. Anesth Analg 2008; 106:1366–75

27. Nielsen VG, Cohen BM, Cohen E: Elastic modulus-based thrombelastographic quantification of plasma clot fibrinolysis with progressive plasminogen activation. Blood Coagul Fibrinolysis 2006; 17:75–81

28. Gerotziafas GT, Elalamy I, Depasse F, Perzborn E, Samama MM: *In vitro* inhibition of thrombin generation, after tissue factor pathway activation, by the oral, direct factor Xa inhibitor rivaroxaban. J Thromb Haemost 2007; 5:886–8

29. Avidan MS, Alcock EL, Da Fonseca J, Ponte J, Desai JB, Despotis GJ, Hunt BJ: Comparison of structured use of routine laboratory tests or near-patient assessment with clinical judgement in the management of bleeding after cardiac surgery. Br J Anaesth 2004; 92:178–86

30. Cammerer U, Dietrich W, Rampf T, Braun SL, Richter JA: The predictive value of modified computerized thromboelastography and platelet function analysis for postoperative blood loss in routine cardiac surgery. Anesth Analg 2003; 96:51–7

31. Wachtfogel YT, Kucich U, Hack CE, Gluszko P, Niewiarowski S, Colman RW, Edmunds LH Jr: Aprotinin inhibits the contact, neutrophil, and platelet activation systems during simulated extracorporeal perfusion. J Thorac Cardiovasc Surg 1993; 106:1–9

32. Bhoola KD, Figueroa CD, Worthy K: Bioregulation of kinins: Kallikreins, kininogens, and kininases. Pharmacol Rev 1992; 44:1–80

33. Laffey JG, Boylan JF, Cheng DC: The systemic inflammatory response to cardiac surgery: Implications for the anesthesiologist. ANESTHESIOLOGY 2002; 97: 215–52

34. Levy JH, Tanaka KA: Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 2003; 75:S715–20

35. Mojcik CF, Levy JH: Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. Ann Thorac Surg 2001; 71:745–54

36. Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, Renne C, Gailani D, Nieswandt B, Renne T: Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J Exp Med 2006; 203:513–8

37. Fritz H, Wunderer G: Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneim Forsch/Drug Res 1983; 33: 479–94

38. Dietrich W, Busley R, Kriner M: High-dose aprotinin in cardiac surgery: is high-dose high enough? An analysis of 8281 cardiac surgical patients treated with aprotinin. Anesth Analg 2006; 103:1074–81

39. Weitz JI: Activation of blood coagulation by plaque rupture: Mechanisms and prevention. Am J Cardiol 1995; 75:B18–B22

40. Augoustides JG: Vascular thrombosis associated with aprotinin and deep hypothermic circulatory arrest: Where are we in 2006? ANESTHESIOLOGY 2007; 106:873

41. Madjdpour C, Thyes C, Buclin T, Frascarolo P, von Roten I, Fisch A, Burmeister M, Bombeli T, Spahn DR: Novel starches: Single-dose pharmacokinetics and effects on blood coagulation. ANESTHESIOLOGY 2007; 106:132–43