(54) Titre : SERPINES MODIFIEES POUR LE TRAITEMENT DE TROUBLES DE SAIGNEMENT
(54) Title: MODIFIED SERPINS FOR THE TREATMENT OF BLEEDING DISORDERS

(57) Abrégé/Abstract:

This invention relates pro-coagulant serpin molecules engineered by modification of the P4, P2, P1 and/or P1' residues within the reactive center loop (RCL) to display increased specificity for anticoagulant proteases. These modified serpin molecules may be useful in therapy, for example as pro-coagulants for the treatment of bleeding.

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(54) Title: MODIFIED SERPINS FOR THE TREATMENT OF BLEEDING DISORDERS

(57) **Abstract**: This invention relates pro-coagulant serpin molecules engineered by modification of the P4, P2, P1 and/or P1' residues within the reactive center loop (RCL) to display increased specificity for anticoagulant proteases. These modified serpin molecules may be useful in therapy, for example as pro-coagulants for the treatment of bleeding.

Modified Serpins for the Treatment of Bleeding Disorders

Field

This invention relates to modified serpin molecules with altered
5   specificity, in particular serpin molecules modified to have
increased specificity for anticoagulant proteases, such as activated
Protein C (APC).

Background

10 Hemophilias are bleeding disorders, which are caused by a deficiency
in circulating plasma fVIII (hemophilia A, HA) or fIX (hemophilia B,
HB) (reviewed in Bolton-Maggs & Pasi, 2003). This reduces the
activity of the intrinsic tenase (Xase) and thereby the amount of
thrombin generated when tissue injury occurs. This leads to
15 uncontrolled bleeding after injury as well as spontaneous bleeding
into joints and soft tissue.

Hemophilia affects around 1 in 5,000 people. The 170,000 patients
identified in the World Federation of Hemophilia Global Survey is an
20 underestimate of the global health burden (World Federation of
Hemophilia, 2011). The treatment costs are very high and treatment
is frequent and lifelong.

Standard treatments for hemophilia entail replacement of the
25 clotting factor affected, using either recombinant or plasma-derived
factors (reviewed in Mannucci, 2003; 2008). However, a significant
proportion of patients treated in this manner will develop
inhibitory antibodies against the supplemented coagulation factor,
rendering the treatment ineffective (reviewed in Brettler, 1996).
30 Inhibitors occur in 30% of treated patients with hemophilia
(reviewed in Teitel & Sholzberg 2013) but global estimates are low
due to high mortality in untreated inhibitor patients and a low
prevalence of inhibitors in many countries in which factor VIII
replacement therapy is not available. Another drawback of
35 conventional therapies is their expense, as well as the short half-
life of the injected clotting factor, necessitating frequent
treatments (reviewed in Lee et al, 2006).

In the case where patients develop inhibitory antibodies, bypassing agents are used for treatment of bleeding events (reviewed in (Negrier et al, 2006)). Bypassing agents reduce bleeding without directly supplying the clotting factor affected; they 'bypass' the activity of the tenase complex. Examples of current bypassing agents include recombinant fVIIa and FEIBA (Factor Eight_Bypassing Activity), a prothrombin complex concentrate. These replacement treatments are very expensive (Bohn et al, 2004; Di Minno et al, 2010; Gringeri et al, 2003; Escobar, 2010) and need to be given even more frequently than the conventional therapies and in high doses due to the short half-lives of both products (reviewed in Haya et al, 2007). In addition, patient response has been shown to be variable and unpredictable (reviewed in Berntorp, 2009).

In addition, the short half-life of factor concentrates renders standard replacement therapy of hemophilia suboptimal. This is particularly evident in hemophilia A as factor VIII has a half-life of less than 12 hours. Consequently, despite the availability of treatment for both hemophilia A and B the bleeding rates are higher in hemophilia A and chronic hemophilic arthropathy is more common. This may be related to the short half-life of factor VIII and consequently the difficulty in maintaining a hemostatic level of factor VIII (Escobar and Sallah 2013). In a national review of treatment the annual frequency of bleeding in patients with severe hemophilia A without inhibitors was 14 compared to 9 in patients with hemophilia B (Nagel, et al 2011). The need for musculoskeletal surgery was 3-times greater in patients with hemophilia A. Tagariello et al found that patients with hemophilia A required joint replacement three times more often than patients with hemophilia B (Tagariello, et al 2009). Lowe et al found that hospitalization was required three times more frequently for patients with hemophilia A compared to hemophilia B (Lowe and Ludlam 2008).

Current treatments for bleeding disorders, such as hemophilia therefore have a range of drawbacks.

Summary
The present inventors have recognised that the specificity of serpin
molecules can be engineered by modification of residues within the
5   reactive center loop (RCL), and have identified modified serpin
molecules with increased specificity for anticoagulant proteases.
These modified serpin molecules may be useful in therapy, for
example for the treatment of bleeding.

10   An aspect of the invention provides a modified serpin having
mutations at one or more of residues P4, P2, P1 and P1' in the
reactive center loop (RCL) thereof.

Another aspect of the invention provides a modified serpin having
15   mutations at one or both of residues P1' and P2 and optionally
residues P4 and/or P1 in the reactive center loop (RCL) thereof.

The mutations may increase the inhibition of activated Protein C
relative to the inhibition of thrombin.
20

The mutations may also increase the inhibition of activated Protein
C relative to the inhibition of other procoagulant proteases, such
as fVIIa, fIXa, fXa and fXIa.

25   Other aspects of the invention relate to the use of modified serpins
as described herein for the treatment of bleeding, for example
bleeding in patients with heritable bleeding disorders and acquired
bleeding, including trauma, surgery and in patients receiving
anticoagulant therapy.
30

Brief Description of Figures
Figure 1 shows the coagulation cascade and the regulatory role of
serpins in this cascade.

35   Figure 2 shows the results of a prothrombin time (PT) assay to
determine the effect of Protein C Inhibitor (PCI) with a 21 residue
N-terminal truncation (N-terminal residue is A22 of the wild-type

sequence, when counting residue 1 of the propeptide as residue 1 of
the mature protein) and having K residues at the P2 and P1'
positions within the RCL (A22 P2KP1'K PCI) on coagulation through
the tissue factor pathway (extrinsic). Pooled normal plasma from
5   three separate plasmas was incubated with either no PCI (black bar,
-) or 5 μM A22 wild-type (WT) PCI (grey bar, WT) or 5 μM A22 P2KP1'K
PCI (white bar, P2KP1'K). Coagulation was initiated by the addition
of PT reagent, to initiate coagulation via the extrinsic pathway and
the time until clot formation measured. The assay was performed in
10   triplicate, error bars show the standard deviation. 2-fold diluted
plasma was used to increase the sensitivity of the assay for
inhibitors of coagulation. A22 P2KP1'K PCI has no effect on
coagulation in this prothrombin time (PT) assay. This result
indicates that there is no significant inhibition of TF: fVIIa,
15   thrombin and other procoagulant proteases by A22 P2KP1'K PCI.

Figure 3 shows the results of an activated partial thromboplastin
time (aPTT) assay to determine the effect of A22 P2KP1'K PCI on
coagulation through the contact activation pathway (intrinsic). Fig
20   3A shows pooled normal plasma from three separate plasmas incubated
with either no PCI (black bar, -) or 5 μM of A22 wild-type (WT) PCI
(grey bar, WT) or A22 P2KP1'K PCI (white bar, P2KP1'K). aPTT reagent
was added and the samples incubated for 5 min at 37˚C. Coagulation
was then initiated by the addition of $CaCl_2$, to initiate coagulation
25   via the intrinsic pathway and the time until clot formation
measured. Bars show averages of at least three measurements, error
bars show the standard deviation. The assay was stopped at 300 s.
Samples shown at 300 s did not clot within the time of the
experiment and are marked with asterisks. Fig 3B shows the data from
30   A without A22 WT PCI samples to show a small effect on the clotting
time.

Figure 4 shows the results of a prothrombin time assay (PT)
determining the effect of a full-length (FL) α_1-antitrypsin
35   Pittsburgh (Pitts) mutant (M358R = P1R) that further comprises a
C232S mutation and P357K (P2) and S359K (P1'K) mutations within the
RCL (FL α_1AT Pitts C232S P2KP1'K) on coagulation through the tissue

factor pathway (extrinsic). Pooled normal plasma from three separate plasmas was incubated with either no α_1AT (-, black bar) or 5 µM FL α_1AT Pitts C232S (Pitts, grey bar) or 5 µM FL α_1AT Pitts C232S P2KP1'K (P2KP1'K, white bar). Coagulation was initiated by the

5   addition of PT reagent and the time until clot formation measured. Bars show averages of at least three measurements, error bars show the standard deviation. 2-fold diluted plasma was used to increase the sensitivity of the assay for inhibitors of coagulation. FL α_1AT Pitts C232S P2KP1'K, unlike FL α_1AT Pitts C232S does not prolong the

10   PT. This indicates there is no significant inhibitory effect towards any of the procoagulant proteases, including TF-fVIIa, thrombin, or fXa.

Figure 5 shows the results of an activated partial thromboplastin

15   time assay (aPTT) to determine the effect of FL α_1AT Pitts C232S P2KP1'K on coagulation through the contact activation pathway (intrinsic). Fig 5A shows pooled normal plasma from three separate plasmas incubated with either no α_1AT (black bar) or increasing concentrations of FL α_1AT Pitts C232S (grey bars) or FL α_1AT Pitts

20   C232S P2KP1'K (white bars). aPTT reagent was added and the samples incubated for 5 min at 37°C. Coagulation was then initiated by the addition of $CaCl_2$ and the time until clot formation measured. Bars show averages of at least three measurements, error bars show the standard deviation. The assay was stopped at 300 s. Samples shown at

25   300s did not clot within the time of the experiment and are marked with asterisks. Fig 5B shows the data from A without FL α_1AT Pitts C232S samples to show a small effect on the clotting time by FL α_1AT Pitts C232S P2KP1'K. However, this effect does not increase dose-dependently.

30
Figure 6 shows that FL α_1AT Pitts C232S inhibits thrombin generation in normal human plasma (NP). Figs A-C show representative thrombin generation curves for reactions containing increasing concentrations of FL α_1AT Pitts C232S in the presence of (A) no thrombomodulin (TM)

35   (B) 1.25 nM thrombomodulin (TM) (C) 10 nM thrombomodulin (TM). Curves show an average of duplicates. All assays were performed in pooled normal human plasma (NP) from George King Biomedical.

Coagulation was initiated by the addition of $CaCl_2$ and
TF/phospholipid (RB low TF and phospholipid reagent #5006210,
Technoclone GmbH) to activate coagulation through the extrinsic
pathway. Thrombin generation was measured through the cleavage of a
5 fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Fluorescence units were
converted to thrombin concentration by calibrating fluorescence
units against known concentrations of thrombin, using the
Technothrombin calibration kit (Technoclone). Fig 6D shows mean
ETPs (endogenous thrombin potentials), representing the total amount
10 of thrombin generated during the reactions. Bars show the mean of
two independent experiments performed in duplicate. Error bars
represent the standard deviation. The thrombomodulin (TM) used was
recombinantly produced from HEK-EBNA cells and consists of the
extracellular domain of TM.
15

Figure 7 shows that FL α_1AT Pitts C232S P2KP1'K rescues the
anticoagulant effect of TM in normal human plasma (NP). Fig 7A-C
show representative thrombin generation curves for reactions
containing increasing concentrations of FL α_1AT Pitts C232S P2KP1'K
20 in the presence of (A) no TM (B) 1.25 nM TM (C) 10 nM TM. Curves
show an average of duplicates. All assays were performed in pooled
normal human plasma (NP) from George King Biomedical. Coagulation
was initiated by the addition of $CaCl_2$ and TF/phospholipid (RB low
TF and phospholipid reagent #5006210 Technoclone GmbH) to activate
25 coagulation through the extrinsic pathway. Thrombin generation was
measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-
Arg-AMC). Fluorescence units were converted to thrombin
concentration by calibrating fluorescence units against known
concentrations of thrombin, using the Technothrombin calibration kit
30 (Technoclone GmbH). Fig 7D shows mean ETPs (endogenous thrombin
potentials), representing the total amount of thrombin generated
during the reactions. Bars show the mean of three independent
experiments performed in duplicate. Error bars represent the
standard deviation.
35

Figure 8 shows that FL α_1AT Pitts C232S abolishes thrombin
generation in human hemophilia A plasma (HA, fVIII-deficient), and

human hemophilia B plasma (HB, fIX-deficient). Graphs show the mean
ETPs from thrombin generation experiments spiked with increasing
concentrations of FL α_1AT Pitts C232S in either (A) fVIII-deficient
plasma (less than 1% fVIII activity) or (B) fIX-deficient plasma
5   (less than 1% fIX activity) with the indicated amounts of added
thrombomodulin (TM). All plasmas were from George King Biomedical.
Reactions were initiated by adding $CaCl_2$ and TF/phospholipid (RB low
TF and phospholipid reagent #5006210 Technoclone GmbH) with 1:4,000
final dilution of Dade Innovin (Siemens) to activate coagulation
10 through the extrinsic pathway. Thrombin generation was measured
through the cleavage of a fluorogenic substrate (Z-Gly-Gly-Arg-AMC).
Fluorescence units were converted to thrombin concentration by
calibrating fluorescence units against known concentrations of
thrombin, using the Technothrombin calibration kit (Technoclone).
15 Mean ETPs are shown from at least two independent experiments
performed in duplicate. Error bars show standard deviations.

Figure 9 shows that FL α_1AT Pitts C232S P2KP1'K rescues the effect
of TM on human HA plasma (fVIII-deficient plasma). Figs 9A-C show
20 representative thrombin generation curves for reactions containing
increasing concentrations of FL α_1AT Pitts C232S P2KP1'K in the
presence of (A) no TM (B) 1.25 nM TM (C) 5 nM TM. Curves show an
average of duplicates. All assays were performed in fVIII-deficient
plasma (less than 1% fVIII activity) from George King Biomedical.
25 Coagulation was initiated by the addition of $CaCl_2$ and
TF/phospholipid (RB low TF and phospholipid reagent #5006210,
Technoclone GmbH) and 1:4,000 Dade Innovin (Siemens) to activate
coagulation through the extrinsic pathway. Thrombin generation was
measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-
30 Arg-AMC). Fluorescence units were converted to thrombin
concentration by calibrating fluorescence units against known
concentrations of thrombin, using the Technothrombin calibration kit
(Technoclone). Fig 9D shows mean ETPs (endogenous thrombin
potentials), representing the total amount of thrombin generated
35 during the reactions. Bars show the mean of two independent
experiments performed in duplicate. Error bars represent the
standard deviation.

Figure 10 shows that FL α_1AT Pitts C232S P2KP1'K rescues the effect
of TM on human HB plasma (fIX-deficient plasma). (A-C)
Representative thrombin generation curves are shown for reactions

5   containing increasing concentrations of FL α_1AT Pitts C232S P2KP1'K
in the presence of (A) no TM (B) 1.25 nM TM (C) 5 nM TM. Curves show
an average of duplicates. All assays were performed in fIX-deficient
plasma (less than 1% fIX activity) from George King Biomedical.
Coagulation was initiated by the addition of $CaCl_2$ and

10   TF/phospholipid (RB low TF and phospholipid reagent #5006210,
Technoclone GmbH) and 1:4,000 Dade Innovin (Siemens) to activate
coagulation through the extrinsic pathway. Thrombin generation was
measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-
Arg-AMC). Fluorescence units were converted to thrombin

15   concentration by calibrating fluorescence units against known
concentrations of thrombin, using the Technothrombin calibration kit
(Technoclone). Fig 10D shows mean ETPs (endogenous thrombin
potentials), representing the total amount of thrombin generated
during the reactions. Bars show the mean of at least two independent

20   experiments performed in duplicate. Error bars represent the
standard deviation.

Figure 11 shows the effect of FL α_1AT Pitts C232S and FL α_1AT Pitts
C232S P2KP1'K in HB mouse plasma. Fig 11A-D show representative

25   thrombin generation curves for reactions containing increasing
concentrations of (A-B) FL α_1AT Pitts C232S or (C-D) FL α_1AT Pitts
C232S P2KP1'K in the presence of (A and C) no TM (B and D) 750 nM
soluble human TM. All assays were performed in HB mouse plasma
collected by tail bleed into citrate, centrifuged to remove red

30   cells and frozen at -80°C. Coagulation was initiated by the addition
of $CaCl_2$, TF/phospholipid (RB low TF and phospholipid reagent,
Technoclone) and 1:12,000 Dade Innovin (Siemens) to activate
coagulation through the extrinsic pathway. Thrombin generation was
measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-

35   Arg-AMC). Fluorescence units were converted to thrombin
concentration by calibrating fluorescence units against known
concentrations of thrombin, using the Technothrombin calibration kit

(Technoclone). Fig 11E shows mean ETPs (endogenous thrombin
potentials), representing the total amount of thrombin generated
during the reactions. Bars show the mean of four independent
experiments. Error bars represent the standard deviation. All assays
5   were performed at 33°C.

Figure 12 shows that FL α_1AT Pitts C232S P2KP1'K reduces bleeding in
HB mice in a tail clip assay. Tail clip results show the total blood
loss over a 10 min collection period after tail transection at 3 mm
10   diameter for WT mice or HB mice. Mice were injected either with PBS,
FL α_1AT Pitts C232S or FL α_1AT Pitts C232S P2KP1'K at the indicated
doses in a total injection volume of 200 µl. Protein solutions were
made up to 200 µl using PBS. The volume blood loss was determined by
collection of blood from the tail into 14 ml saline. The collected
15   blood was spun down and the red cells lysed, followed by measurement
of the absorbance at 575 nm. A standard curve was constructed by
determining the absorbance at 575 nm after red cell lysis using
known volumes of collected blood. Blood loss in the experimental
samples was then calculated from this standard. Each point shown
20   indicates data from a single mouse and the horizontal lines show the
mean of all animals per group. P values were calculated using an
unpaired t-test. Circles indicate WT mice injected with PBS, squares
indicate HB mice injected with PBS, triangles indicate HB mice
injected with 7.5 mg/kg FL α_1AT Pitts C232S, inverted triangles
25   indicate HB mice injected with 7.5 mg/kg FL α_1AT Pitts C232S P2KP1'K
and diamonds indicate HB mice injected with 15 mg/kg FL α_1AT Pitts
C232S P2KP1'K.

Figure 13 shows that FL α_1AT Pitts C232S P2KP1'K increases stable
30   clot formation in HB mice in a cremaster arteriole laser injury
model. HB mice were infused with a fluorescently tagged α-fibrin
antibody and a fluorescently tagged antibody for labeling platelets
through a jugular vein cannula. Controls indicate the baseline level
of clot formation after injury in mice infused with antibodies only.
35   m indicates the number of mice per condition, n indicates the number
of injuries performed for that condition. Light grey indicates no
clot, dark grey indicates a platelet only clot, and black represents
clots containing platelets and fibrin.

Figure 14 shows that FL α_1AT Pitts C232S P2KP1'K increases stable
clot formation in HB mice in a cremaster arteriole laser injury
model. Figure 14 shows representative raw data of the results shown

5   in Figure 13. Fluorescence was quantified over time from all
injuries for each condition. Graphs display the median value from
all injuries in the indicated condition. Number of mice and total
number of injuries were as follows: Control: 5 mice, 8 injuries; FL
α_1AT Pitts C232S at 7.5 mg/kg: 1 mouse, 7 injuries; FL α_1AT Pitts

10   C232S P2KP1'K at 7.5 mg/kg: 4 mice, 18 injuries; FL α_1AT Pitts C232S
P2KP1'K at 15 mg/kg: 3 mice, 20 injuries.

Figure 15 shows the inhibition of thrombin and fXa by mutants of
α_1AT specific for APC over thrombin that were rescreened for fXa

15   inhibition. The resulting four mutants, which should have reduced
fXa inhibition were then tested for inhibition of (A) thrombin and
(B) fXa, to determine if the screening had been successful in
producing APC-specific mutants which did not inhibit thrombin or
fXa. 1 µM serpin was incubated with 12.5 nM protease for different

20   amounts of time. At the indicated timepoints, the reaction was
stopped by addition of an excess of chromogenic substrate (S2238 for
thrombin, S2222 for fXa). The residual enzyme activity was divided
by the initial enzyme activity and the natural log of this value
plotted against time. The slope of these plots is the observed rate

25   constant, k_{obs}. To obtain an estimate of the second-order rate
constant k_2, k_{obs} was divided by the serpin concentration. Selected
values are shown in the figure, to illustrate the highest inhibition
of thrombin and fXa, as well as the value for the only mutant that
did not substantially inhibit fXa (P2RP1'Q). The mutants shown all

30   have the Pitts (M358R, P1R) mutation.

Detailed Description
This invention relates to the modification of serpins to increase
their specificity for APC relative to other coagulation proteases.

35   Modified serpin variants specific for APC may be useful, for example
as procoagulants.

Since they are specific to APC, the modified serpins described herein are expected to have few or no off-target effects and are specific to pathways that are activated on injury. This allows the modified serpins to be applied therapeutically and prophylactically,

5   as well as in emergency situations, i.e. trauma. The dosing of modified serpins is predictable since they are suicide inhibitors, meaning that one molecule of serpin cannot inhibit more than one molecule of protease. In addition, because efficient natural clearance mechanisms are specific for serpin: protease complexes

10   over serpins alone, the plasma half-lives of modified serpins are likely to exceed those of current bypassing agents. For example, the half-life of a modified serpin as described herein may be about 5 days.

15   Protein C (Gene ID 5624) is a vitamin K-dependent plasma glycoprotein that is cleaved to its activated form (activated Protein C, APC) by the thrombin-thrombomodulin complex. Human Protein C has the reference amino acid sequence of NP_000303.1 GI: 4506115 and may be encoded by the reference nucleotide sequence of

20   NM_000312.3 GI: 270483720. APC is an anticoagulant protease that proteolytically cleaves fVa and fVIIIa (Figure 1), thereby attenuating the production of thrombin.

A modified serpin as described herein may have one or more mutations

25   in its reactive center loop (RCL). For example, the modified serpin may have one, two, three, four, or more than four mutations in its RCL. The residues at one, two, three or all four of positions P4, P2, P1 and P1' may be mutated. For example, the residues at one or both of positions P1' and P2 and optionally P1 and/or P4 may be

30   mutated.

RCL residues are numbered herein according to the Schechter-Berger nomenclature for substrates and inhibitors of serine proteases (Schechter & Berger, 1967). This standard nomenclature allows the

35   residue at specific positions in the RCL, such as positions P1', P1, P2 and/or P4, to be easily identified in any serpin sequence.

Preferably, the one or more mutations are the only mutations in the RCL of the modified serpin. For example, the RCL may consist of the sequence of the RCL of the wild-type serpin with mutations at one or both of positions P1' and P2 and optionally P1 and/or P4.

The RCL of the modified serpin may have mutations at the P1' and P2 positions; mutations at the P1', P2 and P1 positions; mutations at the P1', P2 and P4 positions or mutations at the P1', P2, P1 and P4 positions; a mutation at the P1' position; mutations at the P1' and P1 positions; mutations at the P1' and P4 positions or mutations at the P1', P1 and P4 positions; a mutation at the P2 position; mutations at the P2 and P1 positions; mutations at the P2 and P4 positions, mutations at the P2, P1 and P4 positions, a mutation at the P1 position, a mutation at the P4 position; or mutations at the P1 and P4 positions. The residues at other positions in the RCL may be unmutated wild-type residues.

Preferably, the residues at position P1'; positions P1' and P2; positions P1', P1 and P2; positions P2 and P1; positions P1 and P1'; positions P1', P2 and P4 or positions P1', P1, P2 and P4 of the RCL are mutated. In some preferred embodiments, the residues at positions P1', P1 and P2 are mutated.

The reactive center loop (RCL) of a serpin is typically about 20 residues in length and contains the scissile P1-P1' bond that is cleaved by the target protease. The RCL extends from strand 5 of beta sheet A to strand 1 of beta sheet C of the serpin. Residues P17 Glu, P15 Gly and P14 Thr are conserved in serpins. For example, the RCL of a serpin may comprise the consensus sequence P17 E, P16 E/K/R, P15 G, P14 T/S, P12-P9 (A/G/S)$_4$ (Hopkins et al. 1993; Irving et al. 2000). The RCL starts at residue P17 and usually ends at residue P3'. RCLs may be extended in some serpins, such as PCI, by additional residues on the P' side. For example, the RCL of α_1-antitrypsin consists of residues P17-P3' and the RCL of PCI consists of residues P17-P6'. Examples of serpins with residues P1', P1, P2 and P4 highlighted are shown in SEQ ID NOS: 1 to 11 below. The

residues that constitute the mature serpin sequences are also indicated.

The residues in the other positions of the RCL in the serpin may be
5   unmodified i.e. they may be the native residues of the wild-type serpin sequence. The modified serpin may therefore comprise an RCL having a wild-type sequence with mutations at positions P1, P1', P2 and/or P4 as described above.

10   The one or more mutations in the reactive center loop (RCL) of the modified serpin may comprise or consist of a mutation at the P1' position. Preferably, the mutation is a substitution. The native P1' residue in the RCL of the wild-type serpin may be replaced with a non-native residue in the modified serpin. For example, the native S
15   residue at the P1' position in the wild-type sequence of α_1AT or PCI may be replaced with a residue other than S in the modified serpin.

The native P1' residue in the RCL of the wild-type serpin may be replaced with a large polar residue, such as Q, N, Y; a large
20   hydrophobic residue, such as I, M and V; a positively charged residue such as R, H or K; or another residue such as C, A, S and E.

In some preferred embodiments, the P1' residue may be modified to a large polar residue, such as Q, N, Y; a large hydrophobic residue,
25   such as V; or a positively charged residue such as R, H or K; more preferably, a positively charged or large polar residue, such as H, K, R, or Q; most preferably K.

In other embodiments, the P1' residue may be unmodified in the
30   modified serpin. For example, the residue at the P1' position in the RCL of the modified serpin may be the residue that is present at the P1' residue in the wild-type serpin sequence.

The one or more mutations in the reactive center loop (RCL) of the
35   modified serpin may comprise or consist of a mutation at the P2 residue. Preferably, the mutation is a substitution. For example,

the native P2 residue in the RCL of the wild-type serpin may be replaced with a non-native residue in the modified serpin.

5   The native P2 residue in the RCL of the wild-type serpin may be replaced with a large polar residue, such as D, Q, N, Y, a large hydrophobic residue such as W, L, I, V and F, a positively charged residue, such as R, H or K or another residue, such as C, A, T, S or P.

10   In some embodiments the P2 residue in the modified serpin may be other than P.

        In some preferred embodiments, the P2 residue may be modified to a large polar residue, such as Q, N, Y, a large hydrophobic residue
15   such as W or a positively charged residue such as R, H or K, most preferably a positive residue, such as H, K or R, preferably K.

        In some embodiments, the P2 residue may be unmodified in the modified serpin. For example, the residue at the P2 position in the
20   RCL of the modified serpin may be the residue that is present at the P2 residue in the wild-type serpin sequence.

        The one or more mutations in the RCL of the modified serpin may comprise or consist of substitutions at the P1' and/or P2 residues
25   i.e. the residues located at the P1' and/or P2 positions in the RCL of the wild-type serpin sequence may be replaced by other residues in the modified serpin.

        In preferred embodiments, the modified serpin has mutations at both
30   the P2 and the P1' positions of the RCL.

        Suitable residues at the P2 and P1' positions of the modified serpin are described above.

35   In some modified serpins described herein, at least one of the P1' and P2 residue may be a positively charged residue, such as R, H or K or a large polar residue, such as D, Y, Q or N; a large

hydrophobic residue, such as W, L, F, V, M or I; or another residue, such as L, C, A, E, T, S or P. Preferably, at least one of the P1' and P2 residue is a positively charged residue, such as R, H or K; a large polar residue, such as Y, Q or N; or a large hydrophobic residue, such as W, L, F, V or I.

Examples of P2 and the P1' residues respectively in a modified serpin as described herein include KK, FK, RK, VK, LK, QK, CK, PK, FR, HR, IR, SR, TR, VR, YR, AR, PR, RS, KS, QV, RV, RI, RH, KH, TH, RC, RA, LY, QY, TY, DM, TM, WN, RN, HN, TN, KN, NN, PE, RQ, KQ and TQ.

In some preferred embodiments, both the P1' and the P2 residue may be modified to positively charged residues, such as K, H or R, most preferably K.

In some embodiments, the P2 and the P1' residues respectively in a modified serpin as described herein may be other than PN, FS, QS, AS, TS, HS, TA, PT, CC, PS, PT, PM, PH, PA or PC. For example, the P2 and P1' residues may be other than PN, FS, QS, AS, TS, HS, TA, PT, CC or PC in a PCI scaffold or other than PS, PT, PM, PH or PA in an α₁AT scaffold,

In some embodiments, the P1 residue may be unmodified in the modified serpin. For example, the residue at the P1 position in the RCL of the modified serpin may be the residue that is present at the P1 residue in the wild-type serpin sequence. For example, the P1 residue in a modified PCI may be an R residue.

In other embodiments, the P1 residue may be mutated in the modified serpin. For example, the one or more mutations in the reactive center loop (RCL) of the modified serpin further comprise a mutation at the P1 residue. Preferably, the mutation is a substitution. The native P1 residue in the RCL of the wild-type serpin may be replaced with a non-native residue in the modified serpin.

In some embodiments, the P1 residue may be mutated or modified to a positively charged residue such as H, K or R, preferably R.

Preferably, a native residue that is non-positively charged at the
5   P1 position of a wild-type serpin may be replaced by a positively charged residue in the modified serpin. For example, M at the P1 position of wild-type α_1AT may be replaced by a positively charged residue, such as R, in a modified α_1AT. The Pittsburgh (Pitts) variant of α_1AT has a mutation at residue 358 which replaces the M
10   residue at the P1 position with an R residue.

In some embodiments, the P4 residue may be unmodified in the modified serpin. For example, the residue at the P4 position in the RCL of the modified serpin may be the residue that is present at the
15   P4 residue in the wild-type serpin sequence. For example, the P4 residue in a PCI scaffold may be F and the P4 residue in an α_1AT scaffold may be A.

In other embodiments, the P4 residue may be mutated in the modified
20   serpin. For example, the one or more mutations in the reactive center loop (RCL) of the modified serpin further comprise a mutation at the P4 residue.

Preferably, the mutation is a substitution. The residue at the P4
25   residue in the RCL of the wild-type serpin may be replaced with a different residue in the modified serpin. For example, the P4 residue in a modified PCI may be mutated or modified to a residue other F and the P4 residue in a modified α_1AT may be mutated or modified to a residue other than A.
30
Suitable residues in the P4 position of the RCL of the modified serpin include S, R, V, C, W, K, G, L, H, F, T, Q and A.

In examples of modified procoagulant serpins as described herein,
35   (1)   the P4 residue is Q, the P2 residue is R, the P1 residue is R and the P1' residue is N;

(2)   the P4 residue is K, the P2 residue is R, the P1 residue is R
and the P1' residue is H;

(3)   the P4 residue is S, the P2 residue is L, the P1 residue is R
and the P1' residue is K;

(4)   the P4 residue is H, the P2 residue is R, the P1 residue is R
and the P1' residue is V;

(5)   the P4 residue is F, the P2 residue is K, the P1 residue is R
and the P1' residue is K;

(6)   the P4 residue is F, the P2 residue is R, the P1 residue is R
and the P1' residue is K;

(7)   the P4 residue is F, the P2 residue is V, the P1 residue is R
and the P1' residue is K;

(8)   the P4 residue is C, the P2 residue is L, the P1 residue is R
and the P1' residue is K;

(9)   the P4 residue is F, the P2 residue is F, the P1 residue is R
and the P1' residue is R;

(10) the P4 residue is S, the P2 residue is H, the P1 residue is R
and the P1' residue is R;

(11) the P4 residue is G, the P2 residue is I, the P1 residue is R
and the P1' residue is R;

(12) the P4 residue is R, the P2 residue is Q, the P1 residue is R
and the P1' residue is V;

(13) the P4 residue is T, the P2 residue is R, the P1 residue is R
and the P1' residue is V

(14) the P4 residue is R, the P2 residue is R, the P1 residue is R
and the P1' residue is I;

(15) the P4 residue is V, the P2 residue is R, the P1 residue is R
and the P1' residue is I;

(16) the P4 residue is L, the P2 residue is R, the P1 residue is R
and the P1' residue is I;

(17) the P4 residue is T, the P2 residue is L, the P1 residue is R
and the P1' residue is Y;

(18) the P4 residue is A, the P2 residue is Q, the P1 residue is R
and the P1' residue is Y;

(19) the P4 residue is K, the P2 residue is D, the P1 residue is R
and the P1' residue is M;

(20) the P4 residue is W, the P2 residue is W, the P1 residue is R
and the P1' residue is N;

(21) the P4 residue is A, the P2 residue is K, the P1 residue is R,
and the P1' residue is S;

(22) the P4 residue is A, the P2 residue is R, the P1 residue is R,
and the P1' residue is S;

(23) the P4 residue is A, the P2 residue is P, the P1 residue is R,
and the P1' residue is E;

(24) the P4 residue is A, the P2 residue is P, the P1 residue is R,
and the P1' residue is R;

(25) the P4 residue is A, the P2 residue is P, the P1 residue is R,
and the P1' residue is K;

(26) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is M;

(27) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is H;

(28) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is Q;

(29) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is N;

(30) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is Y;

(32) the P4 residue is A, the P2 residue is T, the P1 residue is R,
and the P1' residue is R;

(33) the P4 residue is A, the P2 residue is R, the P1 residue is R,
and the P1' residue is A;

(34) the P4 residue is A, the P2 residue is R, the P1 residue is R,
and the P1' residue is H;

(35) the P4 residue is A, the P2 residue is R, the P1 residue is R,
and the P1' residue is C;

(36) the P4 residue is A, the P2 residue is R, the P1 residue is R,
and the P1' residue is N;

(37) the P4 residue is A, the P2 residue is S, the P1 residue is R,
and the P1' residue is R;

(38) the P4 residue is A, the P2 residue is K, the P1 residue is R,
and the P1' residue is N;

(39) the P4 residue is A, the P2 residue is K, the P1 residue is R, and the P1' residue is H;

(40) the P4 residue is A, the P2 residue is K, the P1 residue is R, and the P1' residue is K;

5   (41) the P4 residue is A, the P2 residue is V, the P1 residue is R, and the P1' residue is R;

(42) the P4 residue is A, the P2 residue is Y, the P1 residue is R, and the P1' residue is R;

(43) the P4 residue is A, the P2 residue is A, the P1 residue is R,
10   and the P1' residue is R;

(44) the P4 residue is A, the P2 residue is C, the P1 residue is R, and the P1' residue is K;

(45) the P4 residue is A, the P2 residue is W, the P1 residue is R, and the P1' residue is N;

15   (46) the P4 residue is A, the P2 residue is H, the P1 residue is R, and the P1' residue is N;

(47) the P4 residue is A, the P2 residue is Q, the P1 residue is R, and the P1' residue is K; or,

(48) the P4 residue is A, the P2 residue is N, the P1 residue is R,
20   and the P1' residue is N.

(49) the P4 residue is F, the P2 residue is F, the P1 residue is R, and the P1' residue is K.

(50) the P4 residue is A, the P2 residue is K, the P1 residue is R, and the P1' residue is Q,

25   (51) the P4 residue is A, the P2 residue is R, the P1 residue is R, and the P1' residue is Q.

In some preferred modified serpins, the P4 residue is A, the P2 residue is K, the P1 residue is R, and the P1' residue is K.

30

In some embodiments, the residues at the P4, P2 and the P1' positions in a modified procoagulant serpin as described herein may be other than HPN, DKN, HPE, FFS, LQS, HAS, YTS, AHS, ATA, LPT, ACC, APT, APA, APM, APH, APS and VPC, respectively. For example, a
35   modified PCI may have residues other than HPN, DKN, HPE, FFS, LQS, HAS, YTS, AHS, ATA, LPT, ACC and VPC at the P4, P2 and the P1' positions and a modified α_1AT may have residues other than APT, APA,

APM or APH at the P4, P2 and the P1' positions. In some embodiments,
the combination of residues at the P4, P2 and the P1' positions in a
modified procoagulant serpin as described herein may be non-
naturally occurring i.e. the combination of residues at the P4, P2
and the P1' positions is not found in the parent wild-type (i.e.
unmodified) serpin or in other wild-type serpins.

A modified serpin as described herein may comprise the sequence of a
wild-type (i.e. unmodified) serpin, preferably a mature wild-type
serpin, with one or more mutations in the RCL thereof as described
above, and optionally one or more additional mutations outside the
RCL.

The sequences of wild-type serpins are well-known in the art, and
may include SEQ ID NOS: 1 to 11 as set out herein. The sequences of
wild-type serpins may include the sequence of mature wild-type
proteins.

The mature protein C inhibitor (PCI) sequence including its
propeptide corresponds to residues 20 to 406 of SEQ ID NO: 1. The
mature α_1-antichymotrypsin corresponds to residues 26 to 423 of SEQ
ID NO: 2. The mature C1-esterase inhibitor sequence corresponds to
residues 23-500 of SEQ ID NO: 3. The mature α_2-antiplasmin sequence
corresponds to residues 28-491 of SEQ ID NO: 4. The mature
antithrombin (ATIII) sequence corresponds to residues 33-464 of SEQ
ID NO: 5. The mature heparin cofactor II sequence corresponds to
residues 20-499 of SEQ ID NO: 6. The mature α_1-antitrypsin (α_1AT)
sequence corresponds to residues 25-418 of SEQ ID NO: 7. The mature
kallistatin sequence corresponds to residues 21-427 of SEQ ID NO: 8.
The mature plasminogen activator inhibitor sequence corresponds to
residues 24-402 of SEQ ID NO: 9. The mature protein Z dependent
inhibitor sequence corresponds to residues 22-444 of SEQ ID NO: 10.
The mature protease nexin 1 isoform a sequence corresponds to
residues 20-398 of SEQ ID NO: 11.

Other than mutations of residues in the RCL as described above, a
modified serpin may have 50 or fewer amino acid residues altered

relative to a wild-type serpin amino acid sequence (for example the
mature serpin sequence of one of SEQ ID NOS 1 to 11, preferably SEQ
ID NO: 1 or 7), preferably 45 or fewer, 40 or fewer, 30 or fewer, 20
or fewer, 15 or fewer, 10 or fewer, 5 or fewer or 3 or fewer. For

5   example, a modified serpin may comprise the sequence of a wild-type
serpin with 50 or fewer, 45 or fewer, 40 or fewer, 30 or fewer, 20
or fewer, 15 or fewer, 10 or fewer, 5 or fewer or 3 or fewer amino
acid residues mutated or altered, in addition to the one, two, three
or four amino acid residues in the RCL of the serpin that are

10   mutated or altered as described above (i.e. the residues at
positions P1' and/or P2 and optionally P1 and/or P4).

An amino acid residue in the wild-type amino acid sequence may be
altered or mutated by insertion, deletion or substitution,

15   preferably substitution for a different amino acid residue. Such
alterations may be caused by one or more of addition, insertion,
deletion or substitution of one or more nucleotides in the encoding
nucleic acid.

20   For example, a modified serpin may comprise the amino acid sequence
of residues 25-418 of SEQ ID NO: 12 having 50 or fewer mutations,
wherein said mutations are at positions other than P4, P2, P1 and
P1' i.e. the P4 residue at in the RCL of the modified serpin is A,
the P2 residue is K, P1 residue is R and the P1' residue is K.

25
The P4 residue in the modified serpin of SEQ ID NO: 12 is located at
position 379 (355 of the mature protein), the P2 residue is located
at position 381 (357 of the mature protein), the P1 residue is
located at position 382 (358 of the mature protein), and the P1'

30   residue is located at position 383 (359 of the mature protein).

The modified serpin may share at least 50% sequence identity with
the wild-type amino acid sequence of a wild-type serpin, for example
the mature serpin sequence of any one of SEQ ID NOS: 1 to 11,

35   preferably SEQ ID NO: 1 or 7, at least 55%, at least 60%, at least
65%, at least 70%, at least about 80%, at least 90%, at least 95%,
at least 98% or at least 99% sequence identity.

For example, a modified serpin may comprise an amino acid sequence
having at least 50% sequence identity to residues 25-418 of SEQ ID
NO: 12, wherein the P4 residue in the RCL of the modified serpin is

5   A, the P2 residue is K, P1 residue is R and the P1' residue is K.

Sequence identity is commonly defined with reference to the
algorithm GAP (Wisconsin GCG package, Accelerys Inc, San Diego USA).
GAP uses the Needleman and Wunsch algorithm to align two complete

10   sequences that maximizes the number of matches and minimizes the
number of gaps. Generally, default parameters are used, with a gap
creation penalty = 12 and gap extension penalty = 4. Use of GAP
may be preferred but other algorithms may be used, e.g. BLAST (which
uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 405-

15   410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS
USA* 85: 2444-2448), or the Smith-Waterman algorithm (Smith and
Waterman (1981) *J. Mol Biol.* 147: 195-197), or the TBLASTN program,
of Altschul et al. (1990) supra, generally employing default
parameters. In particular, the psi-Blast algorithm may be used

20   (Nucl. Acids Res. (1997) 25 3389-3402). Sequence identity and
similarity may also be determined using Genomequest™ software (Gene-
IT, Worcester MA USA).

Sequence comparisons are preferably made over the full-length of the
25   relevant sequence described herein.

Preferably, a modified procoagulant serpin as described herein
comprises the RCL consensus P17 E, P16 S/E/K/R, P15 G, P14 T/S, P12-
P9 (A/G/S)₄.

30

A modified serpin may further comprise one or more residues that are
conserved in wild-type serpin sequences. For example, a modified
serpin may comprise some or all of the following residues (numbered
according to their position in α1AT): 33F, 49N, 53S, 54P, 56S, 61L,
35   67G, 72T, 80L, 130F, 147F, 157I, 158N, 161V, 165T, 167G, 169I, 180T,
184L, 186N, 190F, 191K, 192G, 194W, 198F, 203T, 208F, 218V, 220M,
221M, 277Y, 254L, 255P, 289P, 290K, 299L, 303L, 307G, 312F, 316A,

327L, 334H, 342E, 344G, 347A, 369P, 370F, 383L, 384F, 386G, and 391P
(Irving et al 2008). The corresponding conserved residues in other
serpin sequences may be readily determined using routine sequence
analysis.

5

Mutations or variations outside the RCL of the modified serpin may
include replacement of one or more Cys residues in the modified
serpin, such as the C232 (numbering according to the mature
sequence) residue of α_1AT, to abolish disulfide bridge formation or
10 other modifications; deletion or substitution of residues at the N
terminus of the wild-type sequence, for example to facilitate
expression; or mutation or modification of residues in the heparin
binding sites of the modified serpins (i.e. helix D or helix H) in
order to alter the heparin binding activity of the modified serpin.

15

In some embodiments, the modified serpin may have an N terminal
truncation relative to the wild-type serpin. For example, the
modified serpin may have a 10 to 30 residue truncation at the N
terminus, preferably about 20 residues.

20

One or more residues in the modified serpin may be non-natural amino
acids, modified amino acids or D-amino acids. The use of such amino
acids is well-known to those of skill in the art

25 A modified serpin as described herein may display the secondary
structure of the wild-type serpin, for example a modified serpin
may display a structure comprising 3 beta sheets, 8-9 alpha helices
and a flexible RCL of about 20 residues.

30 In some preferred embodiments, the modified serpin may consist of
the amino acid sequence of a wild-type serpin with one or more
mutations in the reactive center loop (RCL) thereof as described
herein.

35 Preferably, the modified serpin is non-immunogenic in a human. For
example, the wild-type serpin may be a human serpin, preferably a
human plasma serpin.

24

The wild-type serpin may be α₁-antichymotrypsin (SERPINA3), α₂-antiplasmin (SERPINF2) antithrombin (ATIII) (SERPINC1), heparin cofactor II (HCII) (SERPIND1), protein C inhibitor (PCI) (SERPINA5), α₁-antitrypsin (α₁AT) (SERPINA1), Kallistatin (SERPINA4), Plasminogen activator inhibitor-1 (SERPINE1), C1-esterase inhibitor (SERPING1), protease nexin 1 (SERPINE2) or Protein Z-dependent inhibitor (SERPINA10) (Whisstock et al JBC. 285 32 24307-24312, Rau et al Journal of Thrombosis and Hemostasis, 5 (Suppl. 1): 102-115, Huntington, Journal of Thrombosis and Hemostasis, 9 (Suppl. 1): 26-34).

Preferably, the wild-type serpin is ATIII, HCII, PCI or α₁AT, most preferably PCI or α₁AT (Huntington *et al* Cell. Mol. Life Sci. 66 (2009) 113-121; Li *et al* JBC. 283 51 36039-36045; and Li *et al* PNAS 2008 105 4661-4666).

α₁-antichymotrypsin (SERPINA3; Gene ID 12) may have the reference amino acid sequence of NP_001076.2 GI:50659080 (SEQ ID NO: 2) and may be encoded by the reference nucleotide sequence of NM_001085.4 GI:73858562.

C1-esterase inhibitor (SERPING1; Gene ID 710) may have the reference amino acid sequence of NP_000053.2 GI: 73858568(SEQ ID NO: 3) and may be encoded by the reference nucleotide sequence of NM_000062.2 GI:73858567.

α₂-antiplasmin (SERPINF2 Gene ID 5345) may have the reference amino acid sequence of NP_000925.2 GI:115583663(SEQ ID NO: 4)and may be encoded by the reference nucleotide sequence of NM_001165920.1 GI:260064047

Antithrombin (ATIII) (SERPINC1 Gene ID 462) may have the reference amino acid sequence of NP_000479.1 GI: 4502261 (SEQ ID NO: 5) and may be encoded by the reference nucleotide sequence of NM_000488.3 GI:254588059

Heparin cofactor II (HCII) (SERPIND1 Gene ID3053) may have the reference amino acid sequence of NP_000176.2 GI: 73858566 (SEQ ID NO: 6) and may be encoded by the reference nucleotide sequence of NM_000185.3 GI:73858565

Protein C inhibitor (PCI) (SERPINA5 Gene ID 5104) may have the reference amino acid sequence of NP_000615.3 GI: 194018472 and may be encoded by the reference nucleotide sequence of NM_000624.5 GI:401782581. In some preferred embodiments, the protein C inhibitor (PCI) may be an allelic variant resulting from a substitution at SNP rs6115, VAR_013081 (the S45N variant, numbering according to mature protein including the propeptide) and having the sequence shown in SEQ ID NO: 1. Residues 1-19 of SEQ ID NO: 1 correspond to the signal sequence. In plasma, PCI may exist in a full-length form which includes the propeptide of residues 20-25 of SEQ ID NO: 1 (i.e. residues 20-406 of SEQ ID NO: 1) or N terminally cleaved form which lacks the propeptide (i.e. residues 26-406 of SEQ ID NO: 1).

α_1-antitrypsin (α_1AT) (SERPINA1 Gene ID 5265) may have the reference amino acid sequence of NP_000286.3 GI:50363217(SEQ ID NO: 7) and may be encoded by the reference nucleotide sequence of NM_000295.4 GI:189163524.

Kallistatin (SERPINA4 Gene ID 5267) may have the reference amino acid sequence of NP_006206.2 GI: 21361302(SEQ ID NO: 8) and may be encoded by the reference nucleotide sequence of NM_006215.2 GI: 21361301.

Plasminogen activator inhibitor-1 (SERPINE1 Gene ID 5054) may have the reference amino acid sequence of NP_000593.1 GI: 10835159(SEQ ID NO: 9) and may be encoded by the reference nucleotide sequence of NM_000602.4 GI: 383286745.

Protein Z-dependent inhibitor (PZI) (SerpinA10; Gene ID 51156) may have the reference amino acid sequence of NP_057270.1 GI: 7705879 (SEQ ID NO: 10) and may be encoded by the reference nucleotide sequence of NM_016186.2 GI: 154759289.

Protease nexin 1 (PN1) (SerpinE2; Gene ID 5270) may have the
reference amino acid sequence of NP_001130000.1 GI: 24307907,
NP_001130002.1 GI: 211904152 or NP_006207.1 GI: 211904156 (SEQ ID
5   NO: 11) and may be encoded by the reference nucleotide sequence of
NM_001136528.1 GI: 211904151, NM_001136530.1 GI: 211904155 or
NM_006216.3 GI: 211904150.

The P1', P1, P2 and P4 residues that may be mutated as described
10   above are highlighted in bold in SEQ ID NOS: 1 to 11.

The one or more mutations in the RCL alter the specificity of the
modified serpin relative to the unmodified wild-type serpin. The
modified serpin displays increased selectively for anticoagulant
15   proteases over procoagulant proteases compared to the wild-type
serpin.

Preferably, the one or more mutations within the RCL increase the
inhibition of APC by the modified serpin relative to the inhibition
20   of other coagulation proteases, in particular one or more
procoagulant proteases out of thrombin, fXa, fVIIa, fIXa and fXIa.

For example, the one or more mutations in the RCL of the modified
serpin may increase the inhibition of APC by the modified serpin
25   relative to the inhibition of thrombin. The selective inhibition of
APC relative to thrombin may be increased in the presence or absence
of heparin.

In addition, the one or more mutations in the RCL of the modified
30   serpin may increase the inhibition of APC by the modified serpin
relative to the inhibition of 1, 2, 3 or all 4 of the procoagulant
proteases fXa, fVIIa, fIXa and fXIa.

A serpin modified as described herein displays greater inhibition of
35   APC relative to thrombin and other procoagulant proteases than the
unmodified wild-type serpin.

The modified serpin may show greater inhibition of APC than inhibition of thrombin. For example, inhibition of APC by the modified serpin may be at least 5 fold more, at least 10 fold more at least 100 or at least 1000 fold more than inhibition of thrombin

5   by the modified serpin. In some embodiments, the modified serpin may inhibit APC with a second-order rate constant (k_2) that is at least 5 fold more, at least 10 fold more at least 100 or at least 1000 fold more than the second-order rate constant for the inhibition of thrombin. Preferably the stoichiometry of inhibition of the modified

10   serpin for APC is 1.

Preferably, a modified serpin as described herein may bind and inhibit APC but display no binding or inhibition or substantially no binding or inhibition of thrombin.

15

The one or more mutations in the RCL may also increase the inhibition of APC relative to the inhibition of 1, 2, 3, or all 4 of fVIIa, fIXa, fXa and fXIa. Inhibition of APC relative to fVIIa, fIXa, fXa and/or fXIa may be increased in the presence or absence of

20   heparin.

For example, the modified serpin may display greater inhibition of APC relative to 1, 2, 3, or all 4 of fVIIa, fIXa, fXa and fXIa than the wild-type serpin.

25

The modified serpin may inhibit APC more than it inhibits fVIIa. For example, inhibition of APC by the modified serpin may be at least 2 fold more, at least 10 fold more, at least 100 more, or at least 1000 fold more than the inhibition of fVIIa by the modified serpin.

30   The modified serpin inhibits APC with a second-order rate constant (k_2) that is at least 2 fold more, at least 10 fold more, at least 100 more, or at least 1000 fold more than the second-order rate constant for the inhibition of fVIIa.

35   The modified serpin may inhibit APC more than it inhibits fIXa. For example, inhibition of APC by the modified serpin may be at least 2 fold more, at least 10 fold more, at least 100 more or at least 1000

fold more than the inhibition of fIXa by the modified serpin. The modified serpin inhibits APC with a second-order rate constant (k_2) that is at least 2 fold more, at least 10 fold more, at least 100 more, or at least 1000 fold more than the second-order rate constant for the inhibition of fIXa.

The modified serpin may inhibit APC more than it inhibits fXa. For example, inhibition of APC by the modified serpin may be at least 1.5 fold more, at least 2 fold more, at least 10 fold more at least 100 or at least 1000 fold more than the inhibition of fXa by the modified serpin. The modified serpin inhibits APC with a second-order rate constant (k_2) that is at least 1.5 fold more, at least 2 fold more, at least 10 fold more, at least 100 more, or at least 1000 fold more than the second-order rate constant for the inhibition of fXa.

The modified serpin may inhibit APC more than it inhibits fXIa. For example, inhibition of APC by the modified serpin may be at least 2 fold more, at least 10 fold more at least 100 or at least 1000 fold more than the inhibition of fXIa by the modified serpin. The modified serpin inhibits APC with a second-order rate constant (k_2) that is at least 2 fold more, at least 10 fold more, at least 100 more, or at least 1000 fold more than the second-order rate constant for the inhibition of fXIa.

A modified serpin as described herein may be part of a fusion protein which contains one or more heterologous amino acid sequences additional to the modified serpin sequence. For example, the fusion protein comprising the modified serpin may further comprise one or more additional domains which improve the stability, pharmacokinetic, targeting, affinity, purification and production properties of the modified serpin.

Suitable additional domains include immunoglobulin Fc domains. Immunoglobulin Fc domains are well-known in the art and include the human IgG1 Fc domain. A human immunoglobulin Fc domain may be located at the N-terminal or C- terminal end of the modified serpin.

Modified serpins as described herein may be provided using synthetic
or recombinant techniques which are standard in the art.

5   In some embodiments, the modified serpin may be produced as a fusion
protein further comprising an affinity tag, which may, for example,
be useful for purification. An affinity tag is a heterologous
peptide sequence which forms one member of a specific binding pair.
Polypeptides containing the tag may be purified by the binding of
10   the other member of the specific binding pair to the polypeptide,
for example in an affinity column. For example, the tag sequence may
form an epitope which is bound by an antibody molecule.

Suitable affinity tags include for example, glutathione-S-
15   transferase, (GST), maltose binding domain (MBD), MRGS(H)$_6$, DYKDDDDK
(FLAG™), T7-, S- (KETAAAKFERQHMDS), poly-Arg (R$_{5-6}$), poly-His (H$_{2-10}$),
poly-Cys (C$_4$) poly-Phe(F$_{11}$) poly-Asp(D$_{5-16}$), SUMO tag (Invitrogen
Champion pET SUMO expression system), Strept-tag II (WSHPQFEK), c-
myc (EQKLISEEDL), Influenza-HA tag (Murray, P. J. et al (1995) *Anal*
20   *Biochem* 229, 170-9), Glu-Glu-Phe tag (Stammers, D. K. et al (1991)
FEBS Lett 283, 298-302), Tag.100 (Qiagen; 12 aa tag derived from
mammalian MAP kinase 2), Cruz tag 09™ (MKAEFRRQESDR, Santa Cruz
Biotechnology Inc.) and Cruz tag 22™ (MRDALDRLDRLA, Santa Cruz
Biotechnology Inc.). Known tag sequences are reviewed in Terpe
25   (2003) Appl. Microbiol. Biotechnol. 60 523-533. In preferred
embodiments, a poly-His tag such as (H)$_6$, His-SUMO tag (Invitrogen
Champion pET SUMO expression system), or MRGS(H)$_6$ may be used.

The affinity tag sequence may be separated from the modified serpin
30   after purification, for example, using site-specific proteases.

In some embodiments, the modified serpin may be coupled to an
appropriate signal leader peptide to direct secretion of the fusion
polypeptide from cell into the culture medium. A range of suitable
35   signal leader peptides are known in the art. The signal leader
peptide may be a serpin signal sequence or may be heterologous to
the modified serpin i.e. it may be a non-serpin signal sequence. For

example, an α-factor secretion signal or BiP signal sequence may be employed. Preferably, the signal peptide is removed by post-translational processing after expression of the polypeptide.

5   Modified serpins as described herein may be isolated, in the sense of being free from contaminants, such as unmodified serpins and other polypeptides and/or serum components.

Modified serpins as described herein may inhibit one or more
10   activities of activated protein C (APC). For example, modified serpins as described herein may inhibit the proteolytic cleavage of one or more APC substrates, such as fVa or fVIIIa. For example, binding of the modified serpin to APC may result in an at least 5-fold, at least 10-fold, or at least 15-fold decrease in the
15   proteolytic cleavage of fVa, fVIIIa and/or another APC substrate. In some embodiments, binding of APC by the modified serpin may result in no detectable cleavage of fVa or fVIIIa substrate by APC.

Techniques for measuring APC activity, for example by measuring the
20   proteolytic cleavage of APC substrates *in vitro* are standard in the art and are described herein. Suitable assays for use in determining APC activity include standard kinetic assays, for example to measure rate constants, and coagulation assays, including thrombin generation assays (TGA).

25

Techniques for measuring the activity of procoagulant proteases, for example by measuring the proteolytic cleavage of chromogenic substrates *in vitro* are standard in the art and are described herein. Suitable assays for use in determining protease activity
30   include standard kinetic assays, for example to measure rate constants, and coagulation assays, including thrombin generation assays (TGA), prothrombin time assays (PT) and activated partial thromboplastin time assays (aPTT).

35   In some embodiments, modified serpins as described herein may be further modified by chemical modification, for example by PEGylation, or by incorporation in a liposome, to improve their

pharmaceutical properties, for example by increasing *in vivo* half-life.

A modified serpin as described herein may be attached to one or more polyethylene glycol (PEG) or other moieties to increase the in vivo half-life of the modified serpin (Cantin et al. 2002, Am. J. Respir. Cell Mol. Biol. 27; 659-665). For example, a modified serpin may be mono-pegylated or poly-pegylated (for example, with 2-6 PEG moieties). Suitable pegylation methods are well known in the art.

The effect of a modified serpin on coagulation and bleeding may be determined. Suitable techniques are standard in the art. For example, the effect of a modified serpin on thrombin generation may be determined using a thrombin generation assay (TGA) or an activated partial thromboplastin time assay or a prothrombin time assay described herein. Suitable *in vivo* models include cremaster arteriole laser injury models, $FeCl_3$ carotid artery models and tail clip assays as described herein. Other suitable coagulation models are well known in the art.

Other aspects of the invention provide a nucleic acid encoding a modified serpin as described above and a vector comprising such a nucleic acid.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Preferably, the vector contains appropriate regulatory sequences to drive the expression of the nucleic acid in mammalian cells. A vector may also comprise sequences, such as origins of replication, promoter regions and selectable markers, which allow for its selection, expression and replication in bacterial hosts such as *E. coli*.

Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 3rd edition, Russell et al., 2001,

Cold Spring Harbor Laboratory Press. Many known techniques and
protocols for manipulation of nucleic acid, for example in
preparation of nucleic acid constructs, mutagenesis, sequencing,
introduction of DNA into cells and gene expression, are described in
detail in Current Protocols in Molecular Biology, Ausubel et al.
eds. John Wiley & Sons, 1992.

A nucleic acid or vector as described herein may be introduced into
a host cell.

Another aspect of the invention provides a recombinant cell
comprising a nucleic acid or vector that expresses a polypeptide
comprising or consisting of a modified serpin as described above.

A range of host cells suitable for the production of recombinant
modified serpins are known in the art. Suitable host cells may
include prokaryotic cells, in particular bacteria such as
Escherichia coli and *Lactococcus lactis* and eukaryotic cells,
including mammalian cells such as CHO and CHO-derived cell lines
(Lec cells), HeLa, COS, HEK293 and HEK-EBNA cells, amphibian cells
such as Xenopus oocytes, insect cells such as *Trichoplusia ni*, Sf9
and Sf21 and yeast cells, such as *Pichia pastoris*.

Techniques for the introduction of nucleic acid into cells are well
established in the art and any suitable technique may be employed,
in accordance with the particular circumstances. For eukaryotic
cells, suitable techniques may include calcium phosphate
transfection, DEAE-Dextran, electroporation, liposome-mediated
transfection and transduction using retrovirus or other virus, e.g.
adenovirus, AAV, lentivirus or vaccinia. For bacterial cells,
suitable techniques may include calcium chloride transformation,
electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may
be used in identifying clones containing nucleic acid of interest,
as is well-known in the art.

The introduced nucleic acid may be on an extra-chromosomal vector within the cell or the nucleic acid may be integrated into the genome of the host cell. Integration may be promoted by inclusion of sequences within the nucleic acid or vector which promote

5   recombination with the genome, in accordance with standard techniques.

In some embodiment, nucleic acid encoding a modified serpin as described herein may be contained in a vector suitable for

10   administration to an individual e.g. for gene therapy applications. Suitable vectors include retroviral vectors, lentiviral vectors, adenoviral vectors and AAT vectors.

The introduction may be followed by expression of the nucleic acid

15   to produce the encoded modified serpin. In some embodiments, host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) may be cultured _in vitro_ under conditions for expression of the nucleic acid, so that the encoded serpin polypeptide is produced. When an

20   inducible promoter is used, expression may require the activation of the inducible promoter.

The expressed polypeptide comprising or consisting of the modified serpin may be isolated and/or purified, after production. This may

25   be achieved using any convenient method known in the art. Techniques for the purification of recombinant polypeptides are well known in the art and include, for example HPLC, FPLC or affinity chromatography. In some embodiments, purification may be performed using an affinity tag on the polypeptide as described above.

30

Another aspect of the invention provides a method of producing a modified serpin comprising expressing a nucleic acid encoding a modified serpin as described above in a host cell and optionally isolating and/or purifying the modified serpin thus produced.

35

Polypeptides comprising or consisting of a modified serpin produced as described may be investigated further, for example the

34

pharmacological properties and/or activity may be determined.
Methods and means of protein analysis are well-known in the art.

5   A modified serpin as described herein, nucleic acid encoding a
     modified serpin or a recombinant cell expressing a modified serpin,
     may be useful in therapy. For example, a modified serpin as
     described herein, nucleic acid encoding a modified serpin or a
     recombinant cell expressing a modified serpin may be administered to
     an individual for the treatment of bleeding.
10

     Whilst a modified serpin may be administered alone, modified serpins
     will usually be administered in the form of a pharmaceutical
     composition, which may comprise at least one component in addition
     to the modified serpin e.g. a nucleic acid encoding the modified
15   serpin or recombinant cell expressing the modified serpin. Thus
     pharmaceutical compositions may comprise, in addition to the
     modified serpin, nucleic acid or cell, a pharmaceutically acceptable
     excipient, carrier, buffer, stabilizer or other materials well known
     to those skilled in the art. The term "pharmaceutically acceptable"
20   as used herein pertains to compounds, materials, compositions,
     and/or dosage forms which are, within the scope of sound medical
     judgement, suitable for use in contact with the tissues of a subject
     (e.g., human) without excessive toxicity, irritation, allergic
     response, or other problem or complication, commensurate with a
25   reasonable benefit/risk ratio. Each carrier, excipient, etc. must
     also be "acceptable" in the sense of being compatible with the other
     ingredients of the formulation. The precise nature of the carrier or
     other material will depend on the route of administration, which may
     be by bolus, infusion, injection or any other suitable route, as
30   discussed below.

     In some embodiments, modified serpins, nucleic acids or cells may be
     provided in a lyophilised form for reconstitution prior to
     administration. For example, lyophilised serpins may be re-
35   constituted in sterile water and mixed with saline prior to
     administration to an individual.

For parenteral, for example sub-cutaneous or intra-venous
administration, e.g. by injection, the pharmaceutical composition
comprising the modified serpin, nucleic acid or cell may be in the
form of a parenterally acceptable aqueous solution which is pyrogen-
5   free and has suitable pH, isotonicity and stability. Those of
relevant skill in the art are well able to prepare suitable
solutions using, for example, isotonic vehicles, such as Sodium
Chloride Injection, Ringer's Injection, Lactated Ringer's Injection.
Preservatives, stabilizers, buffers, antioxidants and/or other
10   additives may be employed as required including buffers such as
phosphate, citrate and other organic acids; antioxidants, such as
ascorbic acid and methionine; preservatives (such as
octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride;
benzalkonium chloride; benzethonium chloride; phenol, butyl or
15   benzyl alcohol; alkyl parabens, such as methyl or propyl paraben;
catechol; resorcinol; cyclohexanol; 3'-pentanol; and m-cresol); low
molecular weight polypeptides; proteins, such as serum albumin,
gelatin or immunoglobulins; hydrophilic polymers, such as
polyvinylpyrrolidone; amino acids, such as glycine, glutamine,
20   asparagines, histidine, arginine, or lysine; monosaccharides,
disaccharides and other carbohydrates including glucose, mannose or
dextrins; chelating agents, such as EDTA; sugars, such as sucrose,
mannitol, trehalose or sorbitol; salt-forming counter-ions, such as
sodium; metal complexes (e.g. Zn-protein complexes); and/or non-
25   ionic surfactants, such as TWEEN™, PLURONICS™ or polyethylene
glycol (PEG). Suitable carriers, excipients, etc. can be found in
standard pharmaceutical texts, for example, Remington's
Pharmaceutical Sciences, 18th edition, Mack Publishing Company,
Easton, Pa., 1990.
30

Pharmaceutical compositions and formulations may conveniently be
presented in unit dosage form and may be prepared by any methods
well known in the art of pharmacy. Such methods include the step of
bringing into association the modified serpin with the carrier which
35   constitutes one or more accessory ingredients. In general, the
compositions are prepared by uniformly and intimately bringing into
association the active compound with liquid carriers or finely

divided solid carriers or both, and then if necessary shaping the product.

Preferably, modified serpins, nucleic acids or cells as described
5   herein are formulated in a pharmaceutical composition for intra-venous or sub-cutaneous administration.

A pharmaceutical composition comprising a modified serpin, nucleic acid or cell may be administered alone or in combination with other
10 treatments, either simultaneously or sequentially dependent upon the condition to be treated.

A modified serpin, nucleic acid or cell as described herein may be used in a method of treatment of the human or animal body, including
15 therapeutic and prophylactic or preventative treatment (e.g. treatment before the onset of a condition in an individual to reduce the risk of the condition occurring in the individual; delay its onset; or reduce its severity after onset). The method of treatment may comprise administering a modified serpin to an individual in
20 need thereof.

An individual suitable for treatment as described above may be a mammal, such as a rodent (e.g. a guinea pig, a hamster, a rat, a mouse), murine (e.g. a mouse), canine (e.g. a dog), feline (e.g. a
25 cat), equine (e.g. a horse), a primate, simian (e.g. a monkey or ape), a monkey (e.g. marmoset, baboon), an ape (e.g. gorilla, chimpanzee, orang-utan, gibbon), or a human.

In some preferred embodiments, the individual is a human. In other
30 preferred embodiments, non-human mammals, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g. murine, primate, porcine, canine, or rabbit animals) may be employed.   The inhibition of human and murine APC by modified serpins without the inhibition of human or murine
35 thrombin is shown below.

Administration is normally in a "therapeutically effective amount"
or "prophylactically effective amount", this being sufficient to
show benefit to a patient. Such benefit may be at least amelioration
of at least one symptom. The actual amount administered, and rate

5   and time-course of administration, will depend on the nature and
severity of what is being treated, the particular mammal being
treated, the clinical condition of the individual patient, the cause
of the disorder, the site of delivery of the composition, the method
of administration, the scheduling of administration and other

10   factors known to medical practitioners.

A composition may be administered alone or in combination with other
treatments, either simultaneously or sequentially dependent upon the
circumstances of the individual to be treated.

15

Prescription of treatment, e.g. decisions on dosage etc, is within
the responsibility of general practitioners and other medical
doctors and may depend on the severity of the symptoms and/or
progression of a disease being treated. Appropriate doses of

20   therapeutic polypeptides are well known in the art (Ledermann J.A.
et al. (1991) Int. J. Cancer 47: 659-664; Bagshawe K.D. et al.
(1991) Antibody, Immunoconjugates and Radiopharmaceuticals 4: 915-
922). Specific dosages may be indicated herein or in the Physician's
Desk Reference (2003) as appropriate for the type of medicament

25   being administered may be used. A therapeutically effective amount
or suitable dose of a modified serpin may be determined by comparing
its *in vitro* activity and *in vivo* activity in an animal model.
Methods for extrapolation of effective dosages in mice and other
test animals to humans are known. The precise dose will depend upon

30   a number of factors, including whether the modified serpin is for
prevention or for treatment, the size and location of the area to be
treated, the precise nature of the modified serpin and the nature of
any detectable label or other molecule attached to the modified
serpin.

35

A typical modified serpin dose will be in the range of 0.1 mg/kg to
100mg/kg, for example 1 to 80mg/kg. For example, a dose in the range

100 µg to 1 g may be used for systemic applications, and 1 µg to 1 mg for topical applications. An initial higher loading dose, followed by one or more lower doses, may be administered. This is a dose for a single treatment of an adult patient, which may be

5   proportionally adjusted for children and infants, and also adjusted for other modified serpin formats in proportion to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician. The treatment schedule for an individual may be dependent on the pharmocokinetic

10 and pharmacodynamic properties of the modified serpin composition, the route of administration and the nature of the condition being treated.

Treatment may be periodic, and the period between administrations

15 may be about one week or more, e.g. about two weeks or more, about three weeks or more, about four weeks or more, about once a month or more, about five weeks or more, or about six weeks or more. For example, treatment may be every two to four weeks or every four to eight weeks. Treatment may be given before, and/or after surgery,

20 and/or may be administered or applied directly at the anatomical site of trauma, surgical treatment or invasive procedure. Suitable formulations and routes of administration are described above.

In some embodiments, modified serpins as described herein may be

25 administered as sub-cutaneous injections. Sub-cutaneous injections may be administered using an auto-injector, for example for long term prophylaxis/treatment.

In some preferred embodiments, the therapeutic effect of the

30 modified serpin may persist for several half-lives, depending on the dose.

Modified serpins described herein inhibit APC without inhibiting or substantially inhibiting procoagulant factors, such as thrombin, and

35 may be useful in the treatment of bleeding and bleeding disorders; in particular disorders caused by reduced thrombin generation or increased APC anticoagulant pathway activity.

Hemostasis is the normal coagulation response to injury i.e. the prevention of bleeding or hemorrhage, for example from a damaged blood vessel. Hemostasis arrests bleeding and hemorrhage from blood

5   vessels in the body. Modified serpins may promote hemostasis i.e. they may promote or increase the arrest of bleeding and hemorrhage from blood vessels in the body, for example in individuals with bleeding disorders or trauma.

10   Aspects of the invention provide; a modified serpin as described herein for use in a method of treatment of the human or animal body; a modified serpin as described herein for use in a method of treatment of bleeding or the promotion of hemostasis; the use of a modified serpin as described herein in the manufacture of a

15   medicament for the treatment of bleeding or the promotion of hemostasis; and a method of treatment of bleeding or the promotion of hemostasis comprising administering a modified serpin as described herein to an individual in need thereof.

20   Bleeding may include bleeding or hemorrhage from blood vessels in the body.

An individual suitable for treatment with a modified serpin as described herein may have a bleeding disorder.

25

Bleeding disorders may be caused or associated with reduced thrombin generation or increased activity of the APC anticoagulant pathway.

Bleeding disorders may include any heritable or acquired bleeding

30   disorder in which there is reduced thrombin generation, reduced fibrin clot formation or reduced clot stability. For example, bleeding disorders may include congenital deficiencies of factors VII, VIII, X, IX, XI and XIII; combined V and VIII deficiency; prothrombin deficiency; fibrin deficiency; and rare deficiencies of

35   other clotting factors; hemophilia A, B and C; increased bleeding associated with hyperfibrinolysis; increased bleeding due to reduced

platelet count or reduced platelet function; and von Willebrand Disease.

Acquired bleeding disorders may include dilutional coagulopathy associated with major blood loss, bleeding resulting from trauma and surgery and the effects of anticoagulant therapy.

In some embodiments, the individual may be resistant to exogenous coagulation factors, such as exogenous fVIII or fIX. For example, exogenous fVIII or fIX may elicit an immune response in the individual, for example the production of inhibitory alloantibodies.

An individual suitable for treatment with a modified serpin as described herein may have an acquired bleeding disorder, such as bleeding related to trauma, surgery or anti-coagulant therapy. For example, an individual suitable for treatment with a modified serpin as described herein may have suffered a trauma; or may have undergone or be undergoing surgery or anti-coagulant therapy. Suitable individuals may be bleeding or at risk of bleeding from one or more blood vessels in the body.

In some embodiments, a modified serpin as described herein may be useful in the prevention or treatment of i) bleeding in patients with clotting factor alloantibodies; ii) bleeding in patients at high risk of inhibitor development, for example to avoid development of alloantibodies; iii) bleeding in patients with factor VIII deficiency in the absence of inhibitors; iv) bleeding in patients with congenital bleeding disorders, for example a congenital bleeding disorder for which there is no current recombinant optimal replacement therapy, such as severe factor VII deficiency, factor XI deficiency, combined VIII & V deficiency, factor X deficiency and factor V deficiency; v) bleeding in patients with hemophilia, for example patients for whom replacement therapy is inappropriate or unavailable; or vi) acquired bleeding, including bleeding related to trauma, surgery, and anticoagulant therapy.

Other aspects of the invention provide the use of a modified serpin as described herein as a procoagulant and the use of a modified serpin to inhibit APC in the treatment of bleeding.

5   Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

Other aspects and embodiments of the invention provide the aspects
10   and embodiments described above with the term "comprising" replaced by the term "consisting of" and the aspects and embodiments described above with the term "comprising" replaced by the term "consisting essentially of".

15   It is to be understood that the application discloses all combinations of any of the above aspects and embodiments described above with each other, unless the context demands otherwise. Similarly, the application discloses all combinations of the preferred and/or optional features either singly or together with
20   any of the other aspects, unless the context demands otherwise.

Modifications of the above embodiments, further embodiments and modifications thereof will be apparent to the skilled person on reading this disclosure, and as such these are within the scope of
25   the present invention.

30

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if
35   each is set out individually herein.

Certain aspects and embodiments of the invention will now be
illustrated by way of example and with reference to the figures
described above and the tables described below.

5   Table 1 shows second-order rate constants for the inhibition of
thrombin and APC by A22 PCI (PCI with an N-terminal truncation,
starting at Ala22, numbering uses the mature protein sequence,
including the propeptide), FL α_1AT Pitts (full length α_1AT with the
P1R Pittsburgh (Pitts) mutation) and their variants. Standard errors
10   are shown. * The rate constant of inhibition for thrombin by the
P2KP1' variant of PCI is an estimate as after initial inhibition,
reactions do not appear to approach complete inhibition, potentially
due to serpin being shuttled down the substrate pathway or
dissociation of the covalent serpin:protease inhibitory complex

15
Table 2 shows second-order rate constants for the inhibition of
thrombin and APC by PCI and variants in the presence of heparin.
Standard errors are shown. * the rate constant of inhibition for
thrombin by the P2KP1'K variant of PCI is an estimate from the
20   initial slope of the plot of residual thrombin activity versus time.
Complete inhibition is not achieved, potentially because of serpin
being shuttled down the substrate pathway or dissociation of the
covalent serpin:protease inhibitory complex.

25   Table 3 shows second-order rate constants for the inhibition of fXa
by α_1AT Pitts and PCI and their variants. Standard errors are shown.

Table 4 shows second-order rate constants for the inhibition of fXIa
by PCI and the PCI P2KP1'K variant. APC inhibition is shown for
30   comparison (from Table 1). Standard errors are shown.

Table 5 shows second-order rate constants for the inhibition of
thrombin and APC by PCI variants generated by targeted random
mutagenesis. Standard errors are shown. Constants for WT and P2KP1'
35   PCI are shown for comparison. * The rate constant of inhibition for
thrombin by the P2KP1' variant of PCI is an estimate as after
initial inhibition, reactions do not appear to approach complete

inhibition, potentially due to serpin being shuttled down the
substrate pathway or dissociation of the covalent serpin:protease
inhibitory complex.

5   Table 6 shows second-order rate constants for the inhibition of fXIa
        by FL α_1AT Pitts C232S and its P2KP1'K variant. APC inhibition is
        shown for comparison (from Table 1). Standard errors are shown.

        Table 7 shows a fraction of the RCL sequences of the PCI variants
10   determined by targeted random mutagenesis to be specific for APC
        inhibition over thrombin inhibition. Sequences shown are from an
        initial experiment in which 88 mutants were assessed. The P4, P2 and
        P1' residues that were varied in this experiment are shown in bold.
        WT PCI and P2KP1'K PCI sequences are shown for comparison.

15
        Table 8 shows a fraction of the RCL sequences of the PCI variants
        determined by targeted random mutagenesis to be specific for APC
        inhibition over thrombin inhibition. Sequences shown are from a
        larger experiment in which 460 mutants were assessed. The P4, P2 and
20   P1' residues that were varied in this experiment are shown in bold.
        WT PCI and P2KP1'K PCI sequences are shown for comparison.

        Table 9 shows a fraction of the RCL sequences of the α_1AT variants
        determined by targeted random mutagenesis to be specific for APC
25   inhibition over thrombin inhibition. Sequences are shown compared to
        both WT α_1AT and α_1AT Pitts. The P2 and P1' residues are shown in
        bold, the P1 residues are underlined. Prefixes show the library of
        origin for the particular mutant with mutants denoted P2.nr coming
        from the P2 variant library P1'.nr. coming from the P1' variant
30   library and mutants labelled 1-5.nr. coming from plates 1-5 of the
        P2P1' variant library.

        Table 10 shows second-order rate constants for the inhibition of
        thrombin and APC by a subset of variants of α_1AT determined by
35   targeted random mutagenesis to be more specific for APC than for
        thrombin. Standard errors are given. The second-order rate constants

for thrombin and APC inhibition by FL α₁AT Pitts C232S P2KP1'K and
FL α₁AT Pitts C232S are given for comparison.

Table 11 shows the results of PT and aPTT assays to investigate
5   inhibition of procoagulant proteases by hits from random mutagenesis
on the FL α₁AT Pitts C232S background. PT assays were performed
using 1/4 diluted plasma to increase the sensitivity of the assay
and performed in triplicate, except for the reactions shown for FL
α₁AT Pitts C232S P2RP1'C, which were performed in duplicate. The
10 error shown is the standard deviation. aPTT assays were single
experiments, no error is shown. For both PT and aPTT a buffer only
control, where instead of protein, buffer (TBS) was added to the
plasma was used as a control. Increases in PT or aPTT with respect
to the control are an indication of inhibition of procoagulant
15 proteases. For both PT and aPTT assays, the serpin mutants were used
at a 5 µM concentration. For comparison, the P2K and P1'K mutants
are shown as an average of triplicates. As shown from inhibition
rate constants in Tables 1 and 3, these mutants show high
specificity for APC over thrombin, but inhibit fXa significantly.
20 They are therefore good comparator for an inhibition of procoagulant
proteases other than thrombin. ND indicates not determined.

Table 12 shows second-order rate constants for the inhibition of fXa
by α₁AT variants from targeted random mutagenesis. The mutants
25 evaluated here showed specificity for APC over thrombin, substantial
APC inhibition and showed no prolongation of the PT. Most also
showed only minor aPTT prolongation. Because of these features, they
were selected for further analysis. For comparison, fXa inhibition
by FL α₁AT Pitts C232S and FL α₁AT Pitts C232S P2KP1'K is also shown
30 (from Table 3).

Table 13 shows a summary of the characterization of two more mutants
of α₁AT found by combining information from rational and random
mutagenesis. FL α₁AT Pitts C232S and FL α₁AT Pitts C232S P2KP1'K
35 C232S are shown for comparison (data from Tables 1 and 3 and Figure
5). aPTTs for P2RP1'Q and P2KP1'Q are the average of four separate
measurements, the error shown is the standard deviation. The value

obtained for plasma with buffer is shown in brackets, again with the standard deviation shown as the error. The aPTTs shown for Pitts and P2KP1'K are the result of at least three separate measurements, the error shown is the standard deviation. The value shown in brackets is the value obtained for plasma with buffer, shown with the standard deviation as the error. All aPTTs were obtained using a final concentration of 5 µM serpin. FL α_1AT Pitts C232S at this concentration rendered the plasma unclottable. The value of 300 s shown is the cut-off for the assay. Second-order rate constants for the inhibition of thrombin, APC and fXa by the variants are shown with the standard error.

Experiments

The coagulation cascade and the regulatory role of serpins in this cascade are shown in Figure 1. Two pathways lead to activation of the coagulation cascade, the extrinsic cascade (or tissue factor pathway) and the intrinsic pathway (contact activation pathway). The main physiological pathway of activation is believed to be the extrinsic pathway. In this pathway, tissue factor (TF) is exposed on the surface of the damage blood vessel. TF can then bind fVIIa and fVII. TF:fVIIa activates fVII, as well as TF:fVII spontaneously activating to TF:fVIIa. TF:fVIIa activates fX to fXa and this activates prothrombin to thrombin (fIIa); the central protease of the coagulation cascade. Thrombin activates platelets by cleavage of protease activated receptors (PARs) and cleaves fibrinogen to fibrin. Fibrin is crosslinked by fXIIIa, which is itself activated by thrombin, to form the stable fibrin clot. Thrombin in addition activates a positive feedback mechanism to potentiate its own formation. It activates fVIII to fVIIIa and fV to fVa. fVIIIa binds to fIXa to form the intrinsic tenase (Xase) complex. The intrinsic Xase activates more fX. This fXa can bind to fVa to form prothrombinase. Prothrombinase activates prothrombin to thrombin and is responsible for most of the thrombin generated after initiation of coagulation. In addition to thrombin's positive feedback mechanism, thrombin can also shut down its own activation via a negative feedback mechanism. When it binds its cofactor thrombomodulin (TM), the thrombin:TM complex can activate protein C

(PC) to activated protein C (APC). APC cleaves and inactivates both
fVIIIa and fVa, effectively shutting down thrombin generation.
Serpins are important inhibitors of the coagulation cascade. The
inhibitory actions of the serpins protein C inhibitor (PCI),

5   antithrombin (ATIII), heparin cofactor II (HCII) and α_1-antitrypsin
(α_1AT) are shown in Figure 1.

Below we describe the conversion of serpins into specific inhibitors
of APC for use as procoagulant agents (treatment, prophylaxis,

10 bypassing or synergistic) in the treatment of bleeding disorders
such as hemophilia. The modifications described here for both PCI
and α_1AT show as a proof of principle that small changes in these
proteins can be used to create specific APC inhibitors.

15 PCI was first described as the physiological inhibitor of APC and
therefore served as a starting point for these investigations
(Suzuki et al, 1983; 1984). However, PCI is promiscuous and also
inhibits thrombin, thrombin:TM, fXIa, fXa and TF:fVIIa (Elisen et
al, 1998; Mosnier et al, 2001; Suzuki et al, 1984; Fortenberry et

20 al, 2011; Meijers et al, 1988). As a consequence, PCI can function
as both a pro- and an anticoagulant. Its activity is regulated by
binding to glycosaminoglycans, such as heparin and heparan sulfates
(Pratt & Church, 1992; Pratt et al, 1992; Li & Huntington, 2008).
For an overview of the role of PCI in the coagulation cascade, see

25 Figure 1.

α_1AT is the natural inhibitor of neutrophil elastase (Kalsheker,
1989). Unlike other serpins in the coagulation cascade that have
either Arg or Leu at P1, α_1AT has a Met instead. This makes it a

30 very poor inhibitor of coagulation proteases. Nevertheless, because
of the high concentration of α_1AT in plasma, it is believed to
inhibit APC to a physiologically significant degree (Heeb & Griffin,
1988). Mutagenesis of the P1 residue has shown that the use of an
Arg or Leu at P1 drastically improves inhibition of coagulation

35 proteases by α_1AT (Heeb et al, 1990). This is exemplified by the
Pittsburgh (M358R (P1R); Pitts) variant of α_1AT that causes a severe
bleeding disorder (Owen et al, 1983).

To develop serpins specific for inhibition of APC over procoagulant proteases, PCI and α₁AT Pittsburgh (P1R, α₁AT Pitts) were used as template serpin scaffolds. All proteins used in this study were expressed from *Escherichia coli* cultures (Rosetta2(DE3)pLysS, Novagen) using the pETSUMO expression vector (Invitrogen) and purified using a combination of Ni-chromatography, anion-exchange and in the case of PCI, heparin affinity chromatography. The SUMO-tag was subsequently removed by SUMO protease cleavage and the tag removed by tandem Ni-anion exchange chromatography for α₁AT Pittsburgh (Pitts, P1R) and tandem anion exchange-heparin chromatography for PCI. The PCI construct is N-terminally truncated, starting at Ala22 (A22, numbering according to the mature protein sequence, starting with the propeptide). The α₁AT Pitts (P1R) construct is full-length (FL) and has an additional C232S mutation to abolish intermolecular disulfide bond formation and other modifications during expression and purification (Yamasaki *et al*, 2011). Due to the expression vector used, the α₁AT Pitts construct has a Ser (S) as its first residue instead of a Glu (E). The C232S and E1S mutations are not expected to alter the activity of α₁AT.

Lys residues were introduced at different positions into the RCL of PCI and α₁AT Pittsburgh and the resulting mutants tested for inhibition of thrombin and APC. In the initial stages of this study, inhibitors were not screened or tested for their inhibition of other coagulation proteases on the premise that once thrombin inhibition was abolished in favor of APC inhibition, the inhibitor could potentially be additionally modified if it had significant residual inhibitory activity towards other coagulation proteases. The RCL residues are numbered according to the Schechter-Berger nomenclature for serpins (Schechter & Berger, 1967).

Rate constants of thrombin and APC inhibition were measured under pseudo-first-order conditions using an excess of serpin over protease (Table 1). Serpin and protease were incubated together for varying lengths of time and the residual activity was determined by adding an excess of chromogenic substrate for the protease (S2238

for thrombin and S2366 for APC). Residual protease activity was then measured by following the absorbance at 405 nm. Plots of residual protease activity over time gave the observed rate constant k_{obs}. The second-order rate constant, k_2, is the slope of the plot of k_{obs}

5   versus serpin concentration (fitted using a linear regression model). Standard errors of the slope are shown.

The lysine mutations introduced at P2 and P1' were highly effective at increasing the specificity of PCI and α_1AT for APC over thrombin

10   for all variants shown in Table 1. Generally, thrombin inhibition was greatly reduced in all cases. APC inhibition was also reduced for all mutants but not nearly to the same degree. Both serpins initially inhibited thrombin better than APC. This was reversed for all mutants tested.

15

PCI, unlike α_1AT, binds heparin and this binding considerably increases its inhibition of thrombin and APC (Pratt & Church, 1992). We therefore tested the inhibition of thrombin and APC by the P1'K and P2KP1'K PCI mutants in the presence of heparin to see if the

20   swap in specificity seen in Table 1 would persist. Rate constants were measured under pseudo-first-order conditions using an excess of PCI over protease. PCI was preincubated with an equimolar concentration of unfractionated heparin for 30 min prior to the experiment. PCI:heparin and protease were incubated together for

25   varying lengths of time and the residual activity after certain timepoints determined by adding an excess of chromogenic substrate for the protease mixed with polybrene to bind the heparin. Plots of residual protease activity over time gave the observed rate constant k_{obs}. The second-order rate constant, k_2, is the slope of the plot of

30   k_{obs} versus serpin concentration (fitted using a linear regression model). Standard errors of the slope are shown. The value calculated for the inhibition of thrombin by P2KP1'K PCI was an estimate from the initial slope of the plot of residual thrombin activity versus time, as the graph suggests that complete inhibition is not

35   achieved. This might be due to the substrate pathway or complex dissociation. The second-order rate constants are shown in Table 2. As for the inhibition in the absence of heparin, the P1'K and

P2KP1'K mutants of PCI, unlike the WT protein were specific for APC over thrombin (Table 2).

These experiments showed that introducing only one or two
5   modifications in the serpin RCL was sufficient to abolish or greatly reduce the inhibition of thrombin both in the presence and absence of cofactors. The inhibition of APC was reduced but still considerable, especially for the variants of α_1AT and the PCI variants in the presence of heparin. However, the specificity of PCI
10   and α_1AT Pitts is not limited to thrombin and APC. Both these serpins also inhibit fXa, another procoagulant protease. In order not to inhibit coagulation, our variants also need to be specific for APC over fXa. We therefore also determined the rate constants of inhibition of PCI and α_1AT and their variants for fXa (Table 3).
15   Rate constants were measured under pseudo-first-order conditions using an excess of serpin over protease. Serpin and protease were incubated together for varying lengths of time and the residual activity determined by adding an excess of chromogenic substrate (S2222) for the protease. Plots of residual protease activity over
20   time gave the observed rate constant k_{obs}. The second-order rate constant, k_2, is the slope of the plot of k_{obs} versus serpin concentration (fitted using a linear regression model). Standard errors of the slope are shown (Table 3).

25   As seen before for thrombin, WT PCI inhibited fXa better than APC. α_1AT Pitts inhibited APC better than fXa, but the inhibition of fXa was still considerable. The inhibition of fXa was still significant for P1'K PCI, P2K α_1AT Pitts and P1'K α_1AT Pitts (Table 3). The P2KP1'K variants of α_1AT Pitts and PCI were both highly specific for
30   APC over fXa, with absent or negligible inhibition of fXa and were therefore considered lead candidates. The P1'K variant of PCI is also of interest as its inhibition of fXa is very slow in the absence of heparin. The presence of heparin accelerates the rate of APC inhibition significantly, which could potentially skew the
35   specificity ratio in favor of APC.

The PCI lead compounds will be discussed first, followed by the α_1AT

lead compound.

To investigate the properties of A22 P2KP1'K PCI in a more complex
plasma system and to rule out any negative effects on the

5   procoagulant proteases, a prothrombin time assay (PT) was performed.
This assay measures the time until clot formation in plasma after
coagulation is initiated via the extrinsic pathway. A22 WT PCI
showed a small increase in the clotting time, whereas P2KP1'K showed
a smaller increase, consistent with reduced inhibitory activity

10   towards procoagulant proteases (Figure 2).

In addition, we wanted to rule out any effect of the PCI mutant on
the contact activation pathway of coagulation. To do so, rate
constants of inhibition were measured for the inhibition of fXIa and

15   an aPTT assay was done. This assay is similar to the PT assay except
that it measures coagulation initiated via the intrinsic pathway.

Second-order rate constants of inhibition for inhibition of fXIa by
PCI and the P2KP1'K variant were measured under pseudo-first-order

20   conditions using an excess of serpin over protease. Serpin and
protease were incubated together for varying lengths of time and the
residual activity determined by adding an excess of chromogenic
substrate for the protease (S2366). Plots of residual protease
activity over time gave the observed rate constant k_{obs}. The second-

25   order rate constant, k_2, is the slope of the plot of k_{obs} versus
serpin concentration (fitted using a linear regression model).
Standard errors of the slope are given. fXIa inhibition by A22
P2KP1'K PCI did not go to completion over the course of the
experiment, potentially due to serpin cleavage by the protease.

30   Compared to WT, the P2KP1'K mutant showed a much reduced inhibition
towards fXIa and greater specificity for APC than for fXIa (Table
4).

The aPTT assay showed that WT PCI was a potent inhibitor of the

35   contact activation pathway, potentially due to inhibition of fXIa or
fXIIa (Figure 3). The P2KP1'K mutant showed a small increase in the
aPTT. However, since the contact activation pathway primarily

activates coagulation through fIXa activation, inhibition of contact
activation to a small extent is likely to be insignificant in
hemophiliacs as they are deficient in either the main target of
contact activation (fIX) or its cofactor (fVIII).

5

The results shown here so far therefore show that both A22 P1'K PCI
and A22 P2KP1'K PCI are promising, APC-specific lead compounds for
development into bypassing agents for the treatment of hemophilia.

10   To generate additional PCI mutants with specificity for APC over
thrombin, a targeted random mutagenesis strategy was employed on the
PCI scaffold. The residues targeted were P4, P2 and P1'. The random
approach is based on a selection for APC inhibition and against
thrombin inhibition by testing inhibitory activity of bacterial
15   lysates, after PCI expression in a 96-well format.

The assay was calibrated using the most specific PCI mutant
generated from the rational mutagenesis combined with the testing
for specificity outlined above; A22 P2KP1'K PCI. WT PCI was used as
20   an additional control. The negative selection against inhibition of
thrombin was achieved by incubating bacterial lysates for a time
period such that A22 WT PCI showed complete inhibition and the
incubation period extended from that timepoint on to also select
against minor inhibition. Positive selection for APC inhibition was
25   calibrated such that both WT and P2KP1'K PCI fell into the
intermediate range of APC inhibitory activity so we would be able to
determine both increases as well as decreases in inhibition.

In an initial assay, 88 variants were screened for thrombin and APC
30   inhibitory activity. Cultures were grown, induced and protein
expressed in 96-well plates. Cells were lysed by the addition of a
lysis buffer and the lysates assayed for inhibitory activity against
thrombin and APC by incubating the lysate with the protease for 1 h
for thrombin and 30 min for APC. Residual protease activity was then
35   read by addition of a chromogenic substrate to the protease. A22 WT
PCI and A22 P2KP1'K PCI were used as controls. Any lysate with
higher or equivalent residual thrombin activity and lower or

equivalent residual APC activity compared to P2KP1'K PCI was considered to be a promising APC-specific candidate. These sequences are shown in Table 7. P4, P2 and P1' positions are shown in bold.

5   The mutant with the greatest APC inhibitory activity from this set of experiments (D8; P4QP2RP1'N) was characterized in a preliminary fashion and was shown to be more specific for the inhibition of APC than thrombin, whilst utilizing different mutations than P2KP1'K (Table 5). This indicated that it would be possible to make
10   additional mutations in the serpin RCL, which would have equivalent effects to the mutations already described.

       To generate a larger dataset, a further 460 mutants were screened in both the positive and negative selection assays. Cultures were
15   grown, induced and protein expressed in five 96-well plates. Cells were lysed by the addition of a lysis buffer and the lysates assayed for inhibitory activity against thrombin and APC by incubating the lysate with the protease for 1 h for thrombin and 30 min for APC. Residual protease activity was then read by addition of a
20   chromogenic substrate to the protease (S2238 for thrombin, S2366 for APC). A22 WT PCI and A22 P2KP1'K PCI were used as controls. Any lysate with higher or equivalent residual thrombin activity and lower or equivalent residual APC activity compared to P2KP1'K PCI was considered to be a promising APC-specific candidate. From this
25   dataset, colonies were picked and sequenced for serpins that showed better inhibition of APC and worse or equivalent inhibition of thrombin than P2KP1'K and these were retested in triplicate in the same assay to verify that these were indeed true hits and not false positives. From this retest a set of 15 out of the 17 mutants found
30   in the initial screen was determined to be better or equivalent at inhibiting APC than P2KP1'K and worse or equivalent at inhibiting thrombin (Table 8). Sequences of variants that were positive after retesting are shown in Table 8. Interestingly, the random mutagenesis confirmed the beneficial effects of positively charged
35   residues in the P2 and P1' positions. However alternative RCL compositions were also found.

Preliminary second-order rate constants for the inhibition of thrombin and APC by random mutagenesis PCI variants were measured under pseudo first-order conditions using an excess of serpin over protease. Serpin and protease were incubated together for varying lengths of time and the residual activity determined by adding an excess of chromogenic substrate for the protease. Plots of residual protease activity over time gave the observed rate constant k_{obs}. The second-order rate constant, k_2, is the slope of the plot of k_{obs} versus serpin concentration (fitted using a linear regression model). WT and P2KP1'K are shown for comparison. Rate constants are shown in Table 5. The error shown is the standard error of the slope.

Preliminary characterization of some of the selected mutants from the random mutagenesis assay showed that the selected mutants were at least functionally equivalent to P2KP1'K PCI, and some had a slightly improved rate of APC inhibition (Table 5). These experiments strongly suggest that P2KP1'K is not the only composition which could be utilized to generate APC-specific serpins.

The main lead compound based on α_1AT Pitts from the rate constants shown in Tables 1 and 3 was FL α_1AT Pitts C232S P2KP1'K. This mutant was shown not to inhibit thrombin and only slowly inhibited fXa, but retained APC inhibition (Tables 1 and 3).

To investigate the properties of this mutant in a more complex plasma system and to rule out any negative effects on the procoagulant proteases, a prothrombin time assay (PT) was performed. This assay measures the time until clot formation after coagulation is initiated via the extrinsic pathway. As expected, the anticoagulant α_1AT Pitts showed an increase in clotting time, due to its inhibition of thrombin and fXa (Figure 4). In contrast, the P2KP1'K mutant of α_1AT Pitts showed no increase in clotting time and therefore did not interfere with normal coagulation in this assay.

The data so far indicated that the P2KP1'K mutant of α_1AT Pitts did

not interfere with procoagulant proteases in either the extrinsic
(tissue factor) pathway or the common pathway of coagulation. In
addition we wanted to determine whether FL α_1AT Pitts C232S P2KP1'K
affected the intrinsic (contact activation) pathway. Second-order
5   rate constants of inhibition of fXIa by FL α_1AT Pitts C232S and the
P2KP1'K variant were measured under pseudo first-order conditions
using an excess of serpin over protease. Serpin and protease were
incubated together for varying lengths of time and the residual
activity determined by adding an excess of chromogenic substrate for
10   the protease (S2366). Plots of residual protease activity over time
gave the observed rate constant k_{obs}. The second-order rate constant,
k_2, is the slope of the plot of k_{obs} versus serpin concentration
(fitted using a linear regression model). Standard errors of the
slope are given.
15

fXI is activated during the contact activation pathway and it feeds
into the common pathway of coagulation by activating fIX. In
addition, fXI is activated once coagulation is initiated by
thrombin. fXIa was inhibited to a significant degree by FL α_1AT
20   Pitts C232S, however this inhibition was greatly reduced for FL α_1AT
Pitts C232S P2KP1'K (Table 6).

Because a small degree of inhibition of fXIa by the P2KP1'K mutant
of α_1AT Pitts was detected and to determine any potential negative
25   effect on fXIIa, we additionally performed an aPTT assay. This assay
is similar to the PT assay except that it measures coagulation
initiated via the intrinsic pathway of coagulation. The aPTT could
therefore be used to detect any negative effect on fXIa and the
contact activation pathway of coagulation. In this assay, plasma
30   incubated with FL α_1AT Pitts C232S did not clot within the time of
the assay except for one reaction with 0.67 µM serpin (Figure 5A).
FL α_1AT Pitts C232S P2KP1'K showed a small increase in clotting
time, but there was no dose-dependent increase (Figure 5B). This
indicates that the fXIa inhibitory activity of FL α_1AT Pitts C232S
35   P2KP1'K is likely too slow to significantly affect the contact
activation pathway. In addition, the contact activation pathway
activates the coagulation cascade via activation of fIXa. Since

hemophiliacs lack either fIX or its essential cofactor fVIII, the role of a small degree of inhibition of the contact activation pathway in hemophiliacs is likely to be minimal.

5 To investigate whether the P2KP1'K mutant of α_1AT Pitts was able to inhibit APC in a plasma system, we used a modified thrombin generation assay (TGA). Thrombin generation was measured in human pooled normal plasma (NP) in the presence and absence of recombinant soluble thrombomodulin (TM). This TM was expressed and purified from

10 a HEK-EBNA expression system and comprises the soluble extracellular domain. TM is not normally present in the TGA because physiologically it is a transmembrane protein, present on the endothelial membrane and largely absent from plasma. Therefore, there is no activation of the PC pathway in a normal TGA or other

15 coagulation assays utilizing plasma, such as the PT and aPTT assays. Adding TM to the assay allows PC activation and thereby might give a more realistic picture of thrombin generation *in vivo*. Assays shown in Figures 6 and 7 were performed in pooled normal human plasma (NP) from George King Biomedical. Coagulation was initiated by the

20 addition of $CaCl_2$ and TF/phospholipid (RB low TF and phospholipid reagent, Technoclone) to activate coagulation through the extrinsic pathway. Thrombin generation was measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Fluorescence units were converted to thrombin concentration by calibrating fluorescence

25 units against known concentrations of thrombin, using the Technothrombin calibration kit (Technoclone).

Addition of TM to pooled normal plasma reduced thrombin generation in a concentration-dependent manner. From this experiment we chose

30 two concentrations of TM to knock down thrombin generation to either intermediate levels (1.25 nM TM final assay concentration) or to low levels (10 nM TM final assay concentration). These concentrations were used in subsequent assays to evaluate the ability of FL α_1AT Pitts C232S P2KP1'K to inhibit APC in plasma.

35

Addition of FL α_1AT Pitts C232S to normal human plasma (NP) reduced thrombin generation at all concentrations used, likely due to the

inhibition of thrombin as well as fXa (Figure 6). In contrast, FL
α_1AT Pitts C232S P2KP1'K had no effect on NP in the absence of TM
(Figure 7A). However in the presence of TM, FL α_1AT Pitts C232S
P2KP1'K dose-dependently rescued thrombin generation (Figure 7B-D).
5   This effect is the result of specific inhibition of APC by FL α_1AT
Pitts C232S P2KP1'K.

In order to perform the same experiments in fVIII- or fIX-deficient
plasma, it was necessary to increase the amount of tissue factor
10   (TF) used to initiate the assay because at the baseline conditions
(RB trigger only), there was no detectable thrombin generation in
factor deficient plasma. To demonstrate the effect of an increase in
TF on thrombin generation, the reactions were spiked with different
dilutions of TF reagent (Dade Innovin, Siemens) in addition to the
15   RB reagent used to trigger the assay at baseline conditions. The
concentration of TF in the Innovin reagent is not disclosed by the
manufacturer, however previous measurements have shown it to be
around 7.36 nM (Duckers et al, 2010). Increasing the TF trigger
shortened lag-time and increased both peak thrombin and endogenous
20   thrombin potential (ETP) in human NP, fVIII-deficient (HA) and fIX-
deficient (HB) plasma. From these experiments we chose an Innovin
dilution of 1:4,000 in the final reaction to initiate thrombin
generation. RB reagent, which contains both phospholipids and TF was
added because of the need to add phospholipids to the assay.

25

Because the use of factor-deficient plasma required modification of
the assay parameters, we repeated the TM titration experiment for
human normal pooled plasma in human HA plasma with the addition of
1:4,000 Innovin. The assay was performed in human fVIII-deficient
30   plasma (less than 1% fVIII activity) from George King Biomedical.
Coagulation was initiated by the addition of CaCl$_2$ and/or
TF/phospholipid (RB low TF and phospholipid reagent, Technoclone)
with 1:4,000 final dilution of Dade Innovin (Siemens) to activate
coagulation through the extrinsic pathway. Thrombin generation was
35   measured through the cleavage of a fluorogenic substrate (Z-Gly-Gly-
Arg-AMC). Fluorescence units were converted to thrombin
concentration by calibrating fluorescence units against known

concentrations of thrombin, using the Technothrombin calibration kit
(Technoclone). Thrombomodulin (TM) was found to reduce thrombin
generation in the thrombin generation assay (TGA) in fVIII-deficient
plasma (HA).

5

From this experiment, 1.25 nM and 5 nM TM were selected for
subsequent experiments. The high TM concentration used was lower
than for NP, mainly because the total thrombin generation in HA
plasma in the assay conditions used was lower.

10

The effects of both FL α_1AT Pitts C232S and FL α_1AT Pitts C232S
P2KP1'K on HA and HB plasma were comparable to the results from
pooled NP. FL α_1AT Pitts inhibited thrombin generation in the
presence and absence of TM in both HA (fVIII-deficient) and HB

15   plasma (fIX-deficient) (Figure 8). FL α_1AT Pitts C232S P2KP1'K could
rescue the effect of TM on thrombin generation in both fVIII- and
fIX-deficient plasma and had no effect in the absence of TM (Figures
9 and 10). This indicates that FL α_1AT Pitts C232S P2KP1'K can
inhibit APC and have a procoagulant effect in factor-deficient

20   plasma. This means it could potentially promote clot formation and
reduce bleeding in hemophilia patients. The magnitude of this
procoagulant effect will be determined by the relative contribution
of the protein C system to the reduction in thrombin generation *in
vivo*. The *in vitro* experiments shown here cannot be used to predict

25   the likely efficacy of this mutant *in vivo*, however they do show
that in complex plasma systems FL α_1AT Pitts C232S P2KP1'K can
inhibit APC and does not interfere with the procoagulant pathways,
and that these effects are independent of the presence or absence of
fIX and fVIII.

30

In order to verify our *in vitro* data we wanted to use *in vivo* mouse
models of hemophilia. However, to verify that the effect of human
α_1AT on mouse plasma would be comparable to the effect seen in human
plasma we first performed a TGA in mouse plasma. This was done using

35   a modified TGA protocol (Bunce *et al*, 2011; Ivanciu *et al*, 2011).
These modifications were necessary because of the increased
concentrations of inhibitory proteins in mouse plasma that preclude

TGA assays under standard conditions (Tchaikovski *et al*, 2007; Bunce *et al*, 2011; Ivanciu *et al*, 2011). Comparable to the human system, there was no thrombin generation in HB mouse plasma under the baseline conditions of the assay. Therefore, we performed a

5   titration spiking in different concentrations of Innovin. A concentration of 1:12,000 Innovin was chosen for subsequent assays.

Because no murine TM was available, we used soluble human TM as used in the human plasma TGAs to promote APC formation in the mouse TGA

10 assay. The concentration required to see any effect of human TM in HB mouse plasma was ~ 100-fold higher than seen in human plasma. This could be explained by the observation that human TM knock-in mice show reduced ability to activate murine PC (Raife *et al*, 2011). This indicated that human TM is less potent at promoting murine PC

15 activation than murine TM. A concentration of 750nM human TM was used in subsequent experiments.

Different concentrations of both FL α_1AT Pitts C232S and FL α_1AT Pitts C232S P2KP1'K were then added to the determined conditions in

20 mouse HB plasma both in the absence and presence of TM to compare the effect of these mutants in mouse plasma to the previous TGA results in human plasma.

FL α_1AT Pitts C232S reduced thrombin generation in mouse HB plasma

25 in the absence of TM as seen in human plasma (Figure 11A). However in the presence of TM, at the lowest α_1AT concentration, there was a partial rescue of thrombin generation (Figure 11B). This may potentially be due to a difference in the relative rates of inhibition for murine thrombin and murine APC by FL α_1AT Pitts

30 compared to the rates seen in humans, such that the generated APC is inhibited prior to thrombin inhibition. When the concentration of FL α_1AT Pitts C232S is increased to such levels that all APC has been inhibited, thrombin is also inhibited. This could explain the results seen in Figure 11B, but was not further investigated. FL

35 α_1AT Pitts C232S P2KP1'K rescued thrombin generation knocked-down by TM addition in HB mouse plasma as it did in human plasma (Figure 11D). However, when FL α_1AT Pitts C232S P2KP1'K was added to HB

mouse plasma in the absence of TM, an increase in thrombin
generation was also observed (Figure 11C). It is possible that this
effect relates to the method of blood collection employed for these
experiments. To collect plasma, the tail was transected and blood

5   collected into citrate. This was then spun down and the plasma
removed and frozen. The injury inflicted for blood collection leads
to activation of the coagulation system and may cause generation of
APC in the plasma prior to the experiment. Additionally, mice do not
have PCI in their plasma (Zechmeister-Machhart et al, 1996), which

10   may increase the circulating half-life of APC, such that it is not
inactivated prior to the TGA assay. An alternative explanation,
which cannot be ruled out at present would be an off-target
procoagulant effect in mouse plasma. However, since this effect is
not seen in human plasma, this would involve the inhibition of a

15   mouse-specific anticoagulant protease.

To investigate a potential _in vivo_ effect of FL α_1AT Pitts C232S
P2KP1'K and to determine if it could potentially be useful as either
a prophylactic agent or a treatment for hemophilia we used two _in_

20   _vivo_ mouse assays; tail clip and a cremaster arteriole laser injury
model. The mice used were male, fIX knockout mice on the BALB/c
background (Lin et al, 1997; Ivanciu et al, 2011).

In the tail clip assay, protein or buffer was injected through the

25   tail vein and after a 5 min incubation period, the tail was
transected at a diameter of 3 mm and placed in 14 ml 37°C saline
solution in a 37°C water bath. Blood was collected for 10 min and
the resulting blood loss was quantified by measuring total
hemoglobin after red cell lysis by measuring absorbance at 575 nm

30   (Ivanciu et al, 2011). The volume blood loss was calculated by
making a standard curve where known volumes of blood collected by
tail transection were processed in a similar manner to the tail clip
samples. After red cell lysis, the absorbance at 575 nm was
determined and plotted against the volume blood loss to generate the

35   standard curve. Tail clip assays showed a potent procoagulant effect
of FL α_1AT Pitts C232S P2KP1'K (Figure 12). At the dose of 15 mg/kg,
blood loss of the HB mice was restored to the level of WT mice

injected with PBS (Figure 12). Lower dose FL α_1AT Pitts C232S
P2KP1'K also showed a trend to reduction of bleeding with respect to
HB mice although this was not statistically significant. FL α_1AT
Pitts C232S showed no significant effect on blood loss at 7.5 mg/kg.

5

Another *in vivo* model used for evaluating procoagulant agents is the
intravital cremaster arteriole laser-induced injury model (Falati *et*
al, 2002). In this system, a cannula is inserted into the mouse
jugular vein to allow infusion of the therapeutic protein as well as
10   fluorescently-tagged antibodies to fibrin and platelets. The
cremaster muscle is then spread out for imaging. Clot formation
after laser-induced injury to the arterioles is imaged and
quantified using fluorescence microscopy.

15   For an overall qualitative assessment, injuries were sorted into
three categories: no clot (no fluorescence detected), platelet clot
(only platelets visible, these clots were generally unstable and
dissolved over the course of the imaging) and platelets + fibrin
(both platelet and fibrin fluorescence visible and clot remained
20   stable over the course of the imaging). This showed that there was a
dose-dependent increase in stable platelet and fibrin clot formation
with increasing concentration of FL α_1AT Pitts C232S P2KP1'K (Figure
13). All images were quantified for platelet and fibrin
fluorescence. The median value for each timepoint was calculated and
25   the results plotted in Figure 14. These data included the
quantification from all images, regardless of their category
assigned for Figure 13. The plots of the median show that control or
FL α_1AT Pitts C232S infused mice exhibit no clot formation, whereas
both high and low dose FL α_1AT Pitts C232S P2KP1'K showed platelet
30   aggregation and fibrin deposition at the site of the injury. No
difference could be detected between the two doses in terms of
platelet aggregation. For fibrin, there was a dose-dependent
increase in fibrin deposition for FL α_1AT Pitts C232S P2KP1'K and no
fibrin for either control or FL α_1AT Pitts C232S infused mice
35   (Figure 14).

Taken together, these results show that FL α_1AT Pitts C232S P2KP1'K

has a procoagulant effect in both *in vitro* assays and *in vivo* models
of hemophilia. The *in vivo* experiments were all done in mouse models
of hemophilia B, however TGA results in human plasma (Figures 9 and
10) and the proposed mechanism of action of FL α_1AT Pitts C232S

5   P2KP1'K indicate that its procoagulant effect should be independent
of fIX or fVIII deficiency and could therefore be used in both
hemophilia A and B. The procoagulant effect seen *in vivo* was
sufficient to reduce bleeding to the same levels as seen for WT mice
(Figure 12) indicating that serpins that inhibit APC can be used for

10   treatment of bleeding disorders and not only as a prophylactic or an
adjuvant to existing treatments.

A targeted random mutagenesis strategy was also employed on the α_1AT
scaffold in order to explore potential additional APC specific

15   mutants on the α_1AT scaffold.

Three different α_1AT variant libraries were generated on the FL α_1AT
Pitts C232S background: one randomised at P2, one randomised at P1'
and a third library randomised at both P2 and P1'. The resulting

20   plasmid libraries were transformed into the Rosetta2(DE3)pLysS
expression strain and protein expressed in 96-well plates. Bacteria
were lysed and the lysates assayed for thrombin and APC inhibition.

For the single variant libraries, 88 colonies were assayed per

25   library. For the double (P2P1') variant library, 460 colonies were
assayed. FL α_1AT Pitts and FL α_1AT Pitts P2KP1'K (the lead APC
specific variants) were expressed on all assay plates as a
reference. Variants with higher or equal APC inhibitory activity
(lower or equal residual APC activity) and lower or equal thrombin

30   inhibitory activity (higher or equal residual thrombin activity)
compared to P2KP1'K α_1AT were deemed candidates for variants
specific for APC over thrombin. A subset of candidate variants was
picked and re-assayed in the same setup to verify the results from
the first screen. Mutants showing similar properties in both assays

35   were then sequenced. The resulting sequences are shown in Table 9.
To verify the ability of this assay to pick out variants, which were
specific for APC over thrombin, nine variants identified in Table 9

were expressed on a larger scale in *E. coli* and purified. The second-order rate constant of inhibition with respect to thrombin and APC was then determined for each mutant. The results are shown in Table 10. These results confirmed that all variants tested had a higher specificity for APC than for thrombin unlike the FL α₁AT Pitts C232S variant.

Certain types of residues were favored in the α₁AT Pitts scaffold at both P2 and P1' positions (Table 9). Specificity was primarily conferred by the presence of bulky polar (Q, N, Y) bulky hydrophobic (W) or positively charged residues (R, H, K) at P2 and P1'. Other residues seen at these positions included C, A, T, S and V. These medium to small residues were accompanied in the double variant library by a complementing large positively charged residue (R, K) or large polar residue (Y, N, Q) at the other position, which likely has a larger influence on the specificity swap. However, there may be a cooperative effect of these mutations as well, especially where the P1' is R. P1'R showed variable results in the single variant library screen and may have some residual thrombin inhibitory activity. The P2 P is known to be important for thrombin cleavage of substrates (Gallwitz et al, 2012). Simple removal of this residue coupled to a specificity swapping mutation at P1' may be sufficient to generate an APC specific inhibitor with little residual thrombin inhibitory activity. Especially T at P2 might have some effect on its own as, out of its partnering residues at P1' (Q, N, Y, R), only R was identified in the single residue P1' variant library as being sufficient to cause a specificity swap by itself.

Interestingly, non-specific mutants that clustered around the α₁AT Pitts control were shown to be largely α₁AT Pitts. All retained the P2 P, showing its importance in maintaining thrombin inhibitory activity. The P1' was more variable, consistent with the distribution of mutants in the P1' variant library. Comparing the spread of variants in the P2 and P1' libraries, thrombin seemed to be more tolerant to P1' than to P2 mutations. However, the appearance of favorable residues in the double variant libraries

that were not present in the single variant libraries indicate that
the effects of these residues on specificity may be cooperative and
double mutations may be more effective than single mutations at
increasing specificity.

5

The random mutagenesis results presented above showed that it was
possible to generate mutants that showed specificity for APC over
thrombin other than the lysine mutants already identified. So far,
the random mutagenesis strategy was used, assuming that once
10   specificity over thrombin was obtained, these mutants would also
show some degree of specificity for APC over other procoagulant
proteases. To test this, the PT and aPTTs of the random mutants in
Table 10 were tested. These results are displayed in Table 11. None
of the mutants had a significant effect on the PT, unlike previously
15   shown for FL α1AT Pitts C232S (Figure 4). This provides indication,
that the mutants have largely lost their inhibitory activity towards
procoagulant proteases. However, aPTTs are more sensitive to the
presence of inhibitors. Therefore, the measure of the aPTT might
give a more accurate representation of smaller residual inhibition.
20   Previous experiments (Figure 5) showed that FL α1AT Pitts C232S
rendered plasma unclottable in the aPTT assay. In contrast, only one
of the mutants evaluated here showed this effect (P2TP1'N). Some
mutants, such as P2KP1'H and P2KP1'N showed only a relatively small
prolongation of the aPTT and were therefore potentially interesting.

25

From these results, we selected four mutants, P2R, P2QP1'K, P2KP1'H
and P2KP1'N (all on the Pitts, P1R background). These showed either
a high inhibition of APC over thrombin (Table 10) and for some also
a low prolongation of the aPTT (P2KP1'H and P2KP1'N). Because they
30   did not prolong the PT, it is unlikely that they inhibit TF:fVIIa.
The most likely candidates for prolongation of the aPTT would be
inhibition of fXIa or fXa. Of these, fXa inhibition would most
inhibit the initial stages of coagulation and was therefore
considered to be a more significant barrier for a successful
35   inhibitor. Inhibition constants for the inhibition of fXa by these
four mutants were therefore determined as described above (Table
12). P2R showed significant inhibition of fXa, which may be the

64

reason for its prolongation of the aPTT. The other three mutants showed lower fXa inhibition, but none of the mutants were as specific as the previously identified P2KP1'K mutant. Nevertheless, P2KP1'N, P2QP1'K and P2KP1'H represent additional promising

5   candidates for further development as they show specificity for APC over thrombin and fXa. In addition, their inhibition of APC is roughly 2-fold increased with respect to the P2KP1'K mutant described earlier. This faster inhibition might in part compensate for the slightly reduced specificity.

10

However, these results indicated that selecting for specificity for APC over thrombin is not completely sufficient to design an inhibitor which also shows specificity over fXa and other procoagulant proteases. Therefore, the random mutagenesis strategy

15 was extended further. Mutants, which previously had been selected for specificity for APC over thrombin, were rescreened against fXa and mutants selected, which had reduced fXa inhibition, while maintaining low thrombin inhibition and APC inhibition.

20 Four additional mutants were identified from this additional screen. These all had the P1R mutation and in addition had either P2RP1'A, P2RP1'Q, P2WP1'I or P2WP1'H. To verify the specificity of these mutants, an initial experiment was performed, using only one concentration of serpin and testing its inhibition of thrombin and

25 fXa. APC inhibition was not considered at this stage, because mutants were selected based on their low inhibition of thrombin and fXa. Serpin and protease were incubated for different times and at the indicated timepoints, the reaction was stopped by addition of an excess of chromogenic substrate. Residual protease activity was

30 divided by the initial protease activity and the natural log of this value plotted against time (Figure 15). The slope of this line divided by the serpin concentration gives an estimate of the second-order rate constant of inhibition. These assays showed that while all mutants hardly inhibited thrombin, with the fastest inhibitor,

35 P2WP1'H having a second-order rate constant of ~50.3 $M^{-1}.s^{-1}$ (compare to the inhibition constant of FL α_1AT Pitts C232S, 2.928 x 10^5 $M^{-1}.s^{-1}$). However, P2RP1'A, P2WP1'I and P2WP1'H showed significant

inhibition of fXa. The second-order rate constant was only ~10-fold
reduced for these mutants compared to FL α_1AT Pitts C232S (4,070.1 M^{-1}.s^{-1} for P2RP1'A compared to 4.13 x 10^4 M^{-1}.s^{-1} for FL α_1AT Pitts
C232S). P2RP1'A, P2WP1'I and P2WP1'H showed similar fXa inhibition
5   to each other.

Only one mutant showed significant selectivity against both thrombin
and fXa. This mutant had a P2RP1'Q in addition to the Pitts (P1R)
mutation. Because of its selectivity, it was interesting for more
10   thorough investigation. Previous results from both the random and
rational mutagenesis studies indicated that R and K residues perform
reasonably similarly. We therefore also generated a P2KP1'Q mutant
on the P1R background as it was expected from the results shown here
to have similar properties. The results from measurements of
15   inhibition constants and aPTTs (experiments performed as described
before) for both mutants are shown in Table 13. The P2KP1'K mutant
is shown for comparison. Both P2KP1'Q and P2RP1'Q showed very low
inhibition of thrombin and fXa. In addition, there was also hardly
any effect on the aPTT. APC inhibition was significant, being only
20   slightly reduced in comparison to P2KP1'K. Therefore, these two
mutants would be expected to perform similarly to P2KP1'K and may
represent other potentially promising alternative molecules for
further development.

25   We evaluated inhibition of murine thrombin and APC by FL α_1AT Pitts
C232S P2KP1'K. Thrombin and APC were obtained from recombinant
sources. The proteases used are truncated with respect to the plasma
version, including only the EGF2-protease domains for APC (Gla-
domainless APC) and the protease domain for thrombin. Therefore, we
30   also tested the human versions of these proteases to ensure that any
differences were due to a species difference, rather than a
construct difference. Human and murine thrombin showed none or very
little reactivity with FL α_1AT Pitts C232S P2KP1'K by SDS-PAGE,
indicating that in this respect, results from model systems would be
35   relevant to the human system. Second-order rate constants of
inhibition were (8.14 ± 0.58) x 10^3 M^{-1}.s^{-1}, for human Gla-domainless
APC, (3.80 ± 0.37) x 10^3 M^{-1}.s^{-1} for murine Gla-domainless APC,

compared to $(14.88 \pm 1.87) \times 10^3$ $M^{-1}.s^{-1}$ for human plasma APC. These results provided indication that while the reactivity of the mutant in mouse models would likely be lower than in humans, i.e. the relative dose for the same effect might need to be higher, the

5   effect in terms of protease inhibition is likely to be similar.

The data presented show as a proof-of-principle that the serpin scaffold can be used to generate specific APC inhibitors, using only very few mutations, that these inhibitors can have procoagulant

10   activities both *in vitro* and *in vivo* and as such show promise as procoagulant agents for treatment and prophylaxis of bleeding disorders such as hemophilia.

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Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	Thrombin	APC	Fold inhibition APC/thrombin
A22 WT PCI	28.21 ± 1.51	0.68 ± 0.032	0.02
A22 P1'K PCI	0.022 ± 0.0024	0.88 ± 0.074	40
A22 P2KP1'K PCI	~ 0.03*	0.28 ± 0.013	9.3
FL α$_1$AT Pitts C232S	292.76 ± 17.60	108.16 ± 7.086	0.4
FL α$_1$AT Pitts C232S P2K	0.051 ± 0.0028	64.82 ± 7.14	1,271
FL α$_1$AT Pitts C232S P1'K	0.17 ± 0.017	95.66 ± 13.70	563
FL α$_1$AT Pitts C232S P2KP1'K	no inhibition after 4 h	15.14 ± 1.68	no thrombin inhibition

Table 1

Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	Thrombin + heparin	APC + heparin	Fold inhibition APC/Thrombin
A22 WT PCI	1310.32 ± 218.72	564.47 ± 71.29	0.4
A22 P1'K PCI	0.017 ± 0.0019	321.54 ± 31.94	18914
A22 P2KP1'K PCI	0.11 ± 0.040*	146.38 ± 18.85	1331.7

Table 2

Second-order rate constants of inhibition (mM^{-1}.s^{-1})		
Variant	fXa	Fold inhibition APC/fXa
A22 WT PCI	10.31 ± 0.73	0.07
A22 P1'K PCI	0.52 ± 0.079	1.7
A22 P2KP1'K PCI	no detectable inhibition	no fXa inhibition
FL α$_1$AT Pitts C232S	41.33 ± 2.36	2.6
FL α$_1$AT Pitts C232S P2K	3.93 ± 0.31	16.5
FL α$_1$AT Pitts C232S P1'K	4.89 ± 0.16	19.6
FL α$_1$AT Pitts C232S P2KP1'K	0.12 ± 0.010	126.2

Table 3

Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	fXIa	APC	Inhibition of APC/fXIa
A22 WT PCI	8.59 ± 0.43	0.68 ± 0.032	0.08
A22 P2KP1'K PCI	0.023 ± 0.0052	0.28 ± 0.013	12.2

Table 4

Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	Thrombin	APC	Inhibition of APC/Thrombin
A22 WT PCI	28.21 ± 1.51	0.68 ± 0.032	0.02
A22 P2KP1'K PCI	~ 0.03*	0.28 ± 0.013	9.3
A22 D8 PCI (P4QP2RP1'N)	0.084 ± 0.0016	1.00 ± 0.15	11.9
A22 4.H11 PCI (P4KP2RP1'H)	0.021 ± 0.0012	0.45 ± 0.15	21.4
A22 2.B10 PCI (P4SP2LP1'K)	0.016 ± 0.00090	0.43 ± 0.0086	26.9
A22 5.E12 PCI (P4HP2RP1'V)	0.023 ± 0.0021	0.26 ± 0.035	11.3

Table 5

Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	fXIa	APC	Inhibition of APC/fXIa
FL α$_1$AT Pitts C232S	398.88 ± 13.012	108.16 ± 7.086	0.3
FL α$_1$AT Pitts C232S P2KP1'K	0.47 ± 0.037	15.14 ± 1.68	32.2

Table 6

	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'
WT	T	I	F	T	F	R	S	A	R	L
P2KP1'K	T	I	F	T	K	R	K	A	R	L
E7	T	I	S	T	H	R	R	A	R	L
E10	T	I	R	T	Q	R	V	A	R	L
E11	T	I	T	T	L	R	Y	A	R	L
D8	T	I	Q	T	R	R	N	A	R	L
H11	T	I	A	T	Q	R	Y	A	R	L

Table 7

	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'
WT	T	I	F	T	F	R	S	A	R	L
P2KP1'K	T	I	F	T	K	R	K	A	R	L
2.B10	T	I	S	T	L	R	K	A	R	L
3.B11	T	I	F	T	F	R	R	A	R	L
3.C3	T	I	V	T	R	R	I	A	R	L
3.E8	T	I	F	T	R	R	K	A	R	L
3.G10	T	I	C	T	L	R	K	A	R	L
3.G11	T	I	W	T	W	R	N	A	R	L
3.H11	T	I	W	T	W	R	N	A	R	L
4.E4	T	I	K	T	D	R	M	A	R	L
4.F7	T	I	F	T	V	R	K	A	R	L
4.F10	T	I	R	T	R	R	I	A	R	L
4.H7	T	I	G	T	I	R	R	A	R	L
4.H11	T	I	K	T	R	R	H	A	R	L
5.D2	T	I	T	T	R	R	V	A	R	L
5.E7	T	I	L	T	R	R	I	A	R	L
5.E12	T	I	H	T	R	R	V	A	R	L

Table 8

	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'
α₁AT WT	L	E	A	I	**P**	M	**S**	I	P	P
α₁AT Pitts	L	E	A	I	**P**	R	**S**	I	P	P
P2.G11	L	E	A	I	**K**	R	**S**	I	P	P
P2.F10	L	E	A	I	**R**	R	**S**	I	P	P
P2.D8	L	E	A	I	**K**	R	**S**	I	P	P
P2.G8	L	E	A	I	**R**	R	**S**	I	P	P
P2.E7	L	E	A	I	**R**	R	**S**	I	P	P
P2.D10	L	E	A	I	**R**	R	**S**	I	P	P
P2.G4	L	E	A	I	**R**	R	**S**	I	P	P
P2.F4	L	E	A	I	**R**	R	**S**	I	P	P
P1'.H8	L	E	A	I	**P**	R	**E**	I	P	P
P1'.A11	L	E	A	I	**P**	R	**R**	I	P	P
P1'.F10	L	E	A	I	**P**	R	**E**	I	P	P
P1'.F9	L	E	A	I	**P**	R	**K**	I	P	P
P1'.F4	L	E	A	I	**P**	R	**E**	I	P	P
4.G9	L	E	A	I	**T**	R	**N**	I	P	P
4.G4	L	E	A	I	**Q**	R	**K**	I	P	P
3.E5	L	E	A	I	**R**	R	**A**	I	P	P
3.B6	L	E	A	I	**S**	R	**R**	I	P	P
3.B2	L	E	A	I	**K**	R	**N**	I	P	P
3.A10	L	E	A	I	**T**	R	**Y**	I	P	P
2.H1	L	E	A	I	**R**	R	**H**	I	P	P
2.C6	L	E	A	I	**T**	R	**R**	I	P	P
1.H10	L	E	A	I	**V**	R	**R**	I	P	P
1.B11	L	E	A	I	**R**	R	**C**	I	P	P
1.A12	L	E	A	I	**K**	R	**H**	I	P	P
2.E5	L	E	A	I	**T**	R	**R**	I	P	P
3.G9	L	E	A	I	**Y**	R	**R**	I	P	P
3.F4	L	E	A	I	**A**	R	**R**	I	P	P
3.C9	L	E	A	I	**C**	R	**K**	I	P	P
2.H5	L	E	A	I	**K**	R	**N**	I	P	P
2.E7	L	E	A	I	**W**	R	**N**	I	P	P
1.B2	L	E	A	I	**S**	R	**R**	I	P	P
5.C12	L	E	A	I	**H**	R	**N**	I	P	P
5.A6	L	E	A	I	**R**	R	**N**	I	P	P
4.E1	L	E	A	I	**P**	R	**K**	I	P	P
4.C12	L	E	A	I	**N**	R	**N**	I	P	P
3.F8	L	E	A	I	**T**	R	**M**	I	P	P
3.C10	L	E	A	I	**T**	R	**H**	I	P	P
2.E8	L	E	A	I	**K**	R	**S**	I	P	P
1.H9	L	E	A	I	**T**	R	**Q**	I	P	P

Table 9

Second-order rate constants of inhibition (mM^{-1}.s^{-1})			
Variant	Thrombin	APC	Inhibition of APC/thrombin
FL α$_1$AT Pitts C232S	292.76 ± 17.60	108.16 ± 7.086	0.4
FL α$_1$AT Pitts C232S P2KP1'K	no inhibition after 4 h	15.14 ± 1.68	No thrombin inhibition
FL α$_1$AT Pitts C232S P2R	0.042 ± 0.0024	61.12 ± 6.26	1455.2
FL α$_1$AT Pitts C232S P1'R	0.68 ± 0.068	131.57 ± 13.32	193.5
FL α$_1$AT Pitts C232S P1'E	0.15 ± 0.015	2.99 ± 0.29	19.9
FL α$_1$AT Pitts C232S P2TP1'N	0.27 ± 0.047	62.37 ± 2.46	231.0
FL α$_1$AT Pitts C232S P2TP1'Y	0.023 ± 0.0014	5.70 ± 0.83	247.8
FL α$_1$AT Pitts C232S P2QP1'K	0.0038 ± 0.0013	33.41 ± 6.36	8792.1
FL α$_1$AT Pitts C232S P2KP1'H	no inhibition after 2 h	28.84 ± 3.05	No thrombin inhibition
FL α$_1$AT Pitts C232S P2KP1'N	0.015 ± 0.0026	37.80 ± 2.48	2520.0
FL α$_1$AT Pitts C232S P2RP1'C	0.034 ± 0.0094	24.55 ± 2.15	722.1

5

Table 10

Variant	PT (s)	aPTT (s)
Plasma	27.2 ± 0.8	60.3
FL α_1AT Pitts C232S P2K	27.0 ± 0.5	107.2
FL α_1AT Pitts C232S P1'K	27.1 ± 0.4	228.1
FL α_1AT Pitts C232S P2R	27.8 ± 0.4	111.6
FL α_1AT Pitts C232S P1'R	28.1 ± 0.5	287
FL α_1AT Pitts C232S P1'E	27.2 ± 0.4	84
FL α_1AT Pitts C232S P2TP1'N	29.5 ± 0.9	>300
FL α_1AT Pitts C232S P2TP1'Y	28.3 ± 0.8	185.4
FL α_1AT Pitts C232S P2QP1'K	27.9 ± 0.3	111.5
FL α_1AT Pitts C232S P2KP1'H	27.4 ± 0.9	77.8
FL α_1AT Pitts C232S P2KP1'N	27.8 ± 0.3	81.9
FL α_1AT Pitts C232S P2RP1'C	28.5 ± 0.6	ND

5

Table 11

Second-order rate constants of inhibition (mM^{-1}.s^{-1})		
Variant	fXa	Inhibition of APC/fXa
FL α_1AT Pitts C232S	41.33 ± 2.36	2.6
FL α_1AT Pitts C232S P2KP1'K	0.12 ± 0.010	126.2
FL α_1AT Pitts C232S P2R	4.79 ± 0.58	12.8
FL α_1AT Pitts C232S P2QP1'K	1.082 ± 0.15	30.9
FL α_1AT Pitts C232S P2KP1'H	0.62 ± 0.040	46.5
FL α_1AT Pitts C232S P2KP1'N	0.91 ± 0.13	41.5

Table 12

Second-order rate constants of inhibition (mM^{-1}.s^{-1})				
Variant	Thrombin	APC	fXa	aPTT (s)
FL α_1AT Pitts C232S	292.76 ± 17.60	108.16 ± 7.086	41.33 ± 2.36	>300 (55.0 ± 3.8)
FL α_1AT Pitts C232S P2KP1'K	no inhibition after 4 h	15.14 ± 1.87	0.12 ± 0.010	62.1 ± 4.2 (55.0 ± 3.8)
FL α_1AT Pitts C232S P2RP1'Q	0.0054 ± 0.0011	8.30 ± 1.11	0.13 ± 0.0067	55.1 ± 2.8 (49.3 ± 2.5)
FL α_1AT Pitts C232S P2KP1'Q	0.0029 ± 0.0015	9.00 ± 0.67	0.17 ± 0.010	53.9 ± 3.0 (49.3 ± 2.5)

Table 13

Sequences

```
  1 mqlflllclv llspqgaslh rhhpremkkr vedlhvgatv apssrrdftf dlyralasaa
 61 psqniffspv sismslamls lgagsstkmq ileglglnlq kssekelhrg fqqllqelnq
121 prdgfqlslg nalftdlvvd lqdtfvsamk tlyladtfpt nfrdsaqamk qindyvakqt
181 kgkivdllkn ldsnavvimv nyiffkakwe tsfnhkgtqe qdfyvtsetv vrvpmmsred
241 qyhylldrnl scrvvgvpyq gnatalfilp segkmqqven glsektlrkw lkmfkkrqle
301 lylpkfsieg syqlekvlps lgisnvftsh adlsgisnhs niqvsemvhk avvevdesgt
361 raaaatgtif tfrsarlnsq rlvfnrpflm fivdnnilfl gkvnrp
```

SEQ ID NO: 1 Protein C inhibitor (PCI)

Mature protein, including the propeptide, corresponds to residues 20
to 406. Signal sequence corresponds to residues 1-19. Propeptide
corresponds to residues 20-25. Residues P4, P2, P1 and P1' of the
RCL bold and underlined.


```
  1 mermlpllal gllaagfcpa vlchpnspld eenltqenqd rgthvdlgla sanvdfafsl
 61 ykqlvlkapd knvifsplsi stalaflslg ahnttlteil kglkfnltet seaeihqsfq
121 hllrtlnqss delqlsmqna mfvkeqlsll drftedakrl ygseafatdf qdsaaakkli
181 ndyvkngtrg kitdlikdld sqtmmvlvny iffkakwemp fdpqdthqsr fylskkkwvm
241 vpmmslhhlt ipyfrdeels ctvvelkytg nasalfilpd qdkmeeveam llpetlkrwr
301 dslefreige lylpkfsisr dynlndillq lgieeaftsk adlsgitgar nlavsqvvhk
361 avldvfeegt easaatavki tllsalvetr tivrfnrpfl miivptdtqn iffmskvtnp
421 kqa
```

SEQ ID NO: 2 Alpha-1-antichymotrypsin

Mature protein corresponds to residues 26 to 423. Residues P4, P2,
P1 and P1' of the RCL bold and underlined


```
  1 masrltlltl lllllagdra ssnpnatsss sqdpeslqdr qegkvattvi skmlfvepil
 61 evsslpttns ttnsatkita nttdepttqp ttepttqpti qptqpttqlp tdsptqpttg
121 sfcpgpvtlc sdleshstea vlgdalvdfs lklyhafsam kkvetnmafs pfsiaslltq
181 vllgagentk tnlesilsyp kdftcvhqal kgfttkgvts vsqifhspdl airdtfvnas
241 rtlyssprv lsnnsdanle lintwvaknt nnkisrllds lpsdtrlvll naiylsakwk
301 ttfdpkktrm epfhfknsvi kvpmmnskky pvahfidqtl kakvgqlqls hnlslvilvp
361 qnlkhrledm eqalspsvfk aimeklemsk fqptlltlpr ikvttsqdml simekleffd
421 fsydlnlcgl tedpdlqvsa mqhqtvlelt etgveaaaas aisvartllv fevqqpflfv
481 lwdqqhkfpv fmgrvydpra
```

SEQ ID NO: 3 C1-esterase inhibitor

Mature protein corresponds to residues 23-500. Residues P4, P2, P1
and P1' of the RCL bold and underlined

```
  1 mallwgllvl swsclqgpcs vfspvsamep lgrqltsgpn qeqvspltll klgnqepggq
 61 talksppgvc srdptpeqth rlarammaft adlfslvaqt stcpnlilsp lsvalalshl
121 algaqnhtlq rlqqvlhags gpclphllsr lcqdlgpgaf rlaarmylqk qfpikedfle
181 qseqlfgakp vsltgkqedd laninqwvke ategkiqefl sglpedtvll llnaihfqgf
241 wrnkfdpslt qrdsfhldeq ftvpvemmqa rtyplrwfll eqpeiqvahf pfknnmsfvv
301 lvpthfewnv sqvlanlswd tlhpplvwer ptkvrlpkly lkhqmdlvat lsqlglqelf
361 qapdlrgise qslvvsgvqh qstlelsevg veaaaatsiamsrmslssfs vnrpflffif
421 edttglplfv gsvrnpnpsa prelkeqqds pgnkdflqsl kgfprgdklf gpdlklvppm
481 eedypqfgsp k
```

           SEQ ID NO: 4 α₂-Antiplasmin

Mature protein corresponds to residues 28-491. Residues P4, P2, P1
and P1' of the RCL for inhibition of chymotrypsin in bold, residues
for the inhibition of plasmin underlined.

```
  1 mysnvigtvt sqkrkvylls llligfwdcv tchgspvdic takprdipmn pmciyrspek
 61 katedegseq kipeatnrrv welskansrf attfyqhlad skndndnifl splsistafa
121 mtklgacndt lqqlmevfkf dtisektsdq ihfffaklnc rlyrkankss klvsanrlfg
181 dksltfnety qdiselvyga klqpldfken aeqsraaink wvsnktegri tdvipseain
241 eltvlvlvnt iyfkglwksk fspentrkel fykadgescs asmmyqegkf ryrrvaegtq
301 vlelpfkgdd itmvlilpkp ekslakveke ltpevlqewl deleemmlvv hmprfriedg
361 fslkeqlqdm glvdlfspek sklpgivaeg rddlyvsdaf hkaflevnee qseaaastav
421 viagrslnpn rvtfkanrpf lvfirevpln tiifmgrvan pcvk
```

           SEQ ID NO: 5 Antithrombin (ATIII)

Mature protein corresponds to residues 33-464. Residues P4, P2, P1 and
P1' of the RCL bold and underlined

```
  1 mkhslnalli fliitsawgg skgpldqlek ggetaqsadp qweqlnnknl smpllpadfh
 61 kentvtndwi pegeedddyl dlekifsedd dyidivdsls vsptdsdvsa gnilqlfhgk
121 sriqrlniln akfafnlyrv lkdqvntfdn ifiapvgist amgmislglk getheqvhsi
181 lhfkdfvnas skyeittihn lfrklthrlf rrnfgytlrs vndlyiqkqf pilldfktkv
241 reyyfaeaqi adfsdpafis ktnnhimklt kglikdalen idpatqmmil nciyfkgswv
301 nkfpvemthn hnfrlnerev vkvsmmqtkg nflaandqel dcdilqleyv ggismlivvp
361 hkmsgmktle aqltprvver wqksmtnrtr evllpkfkle knynlveslk lmgirmlfdk
421 ngnmagisdq riaidlfkhq gtitvneegt qattvttvgf mplstqvrft vdrpflfliy
481 ehrtscllfm grvanpsrs
```

           SEQ ID NO: 6 Heparin cofactor II

Mature protein corresponds to residues 20-499. Residues P4, P2, P1
and P1' of the RCL bold and underlined

```
  1 mpssvswgil llaglcclvp vslaedpqgd aaqktdtshh dqdhptfnki tpnlaefafs
 61 lyrqlahqsn stniffspvs iatafamlsl gtkadthdei leglnfnlte ipeaqihegf
121 qellrtlnqp dsqlqlttgn glflseglkl vdkfledvkk lyhseaftvn fgdteeakkq
181 indyvekgtq gkivdlvkel drdtvfalvn yiffkgkwer pfevkdteee dfhvdqvttv
241 kvpmmkrlgm fniqhckkls swvllmkylg nataifflpd egklqhlene lthdiitkfl
301 enedrrsasl hlpklsitgt ydlksvlgql gitkvfsnga dlsgvteeap lklskavhka
361 vltidekgte aagamfleai pmsippevkf nkpfvflmie qntksplfmg kvvnptqk
```

SEQ ID NO: 7 α_1-antitrypsin (α_1AT)

Mature protein corresponds to residues 25-418. Residues P4, P2, P1 and P1' of the RCL bold and underlined

```
  1 mhlidyllll lvgllalshg qlhvehdges csnsshqqil etgegspslk iapanadfaf
 61 rfyyliaset pgkniffspl sisaayamls lgacshsrsq ileglgfnlt elsesdvhrg
121 fqhllhtlnl pghgletrvg salflshnlk flakflndtm avyeaklfht nfydtvgtiq
181 lindhvkket rgkivdlvse lkkdvlmvlv nyiyfkalwe kpfissrttp kdfyvdcntt
241 vrvpmmlqdq ehhwylhdry lpcsvlrmdy kgdatvffil pnqgkmreie evltpemlmr
301 wnnllrkrnf ykklelhlpk fsisgsyvld qilprlgftd lfskwadlsg itkqqkleas
361 ksfhkatldv deagteaaaa tsfaikffsa qtnrhilrfn rpflvvifst stqsvlflgk
421 vvdptkp
```
                         SEQ ID NO: 8 Kallistatin

Mature protein corresponds to residues 21-427. Residues P4, P2, P1 and P1' of the RCL bold and underlined

```
  1 mqmspaltcl vlglalvfge gsavhhppsy vahlasdfgv rvfqqvaqas kdrnvvfspy
 61 qvasvlamlq lttggetqqq iqaamgfkid dkgmapalrh lykelmgpwn kdeisttdai
121 fvqrdlklvq qfmphffrlf rstvkqvdfs everarfiin dwvkthtkgm isnllgkgav
181 dqltrlvlvn alyfngqwkt pfpdssthrr lfhksdgstv svpmmaqtnk fnytefttpd
241 ghyydilelp yhgdtlsmfi aapyekevpl saltnilsaq lishwkgnmt rlprllvlpk
301 fsletevdlr kplenlgmtd mfrqfqadft slsdqeplhv aqalqkvkie vnesgtvass
361 stavivsarm apeeiimdrp flfvvrhnpt gtvlfmgqvm ep
```

            SEQ ID NO: 9 Plasminogen activator inhibitor

Mature protein corresponds to residues 24-402. Residues P4, P2, P1 and P1' of the RCL bold and underlined

```
  1 mkvvpsllls vllaqvwlvp glapspqspe tpapqnqtsr vvqapkeeee deqeaseeka
 61 seeekawlma srqqlakets nfgfsllrki smrhdgnmvf spfgmslamt glmlgatgpt
121 etqikrglhl qalkptkpgl lpslfkglre tlsrnlelgl tqgsfafihk dfdvketffn
181 lskryfdtec vpmnfrnasq akrlmnhyin ketrgkipkl fdeinpetkl ilvdyilfkg
241 kwltpfdpvf tevdtfhldk yktikvpmmy gagkfastfd knfrchvlkl pyqgnatmlv
301 vlmekmgdhl aledylttdl vetwlrnmkt rnmevffpkf kldqkyemhe llrqmgirri
361 fspfadlsel satgrnlqvs rvlqrtviev dergteavag ilseitaysm ppvikvdrpf
421 hfmiyeetsg mllflgrvvn ptll
```

SEQ ID NO: 10 Protein Z dependent inhibitor

Mature protein corresponds to residues 22-444. Residues P4, P2, P1 and P1' of the RCL bold and underlined

```
  1 mnwhlplfll asvtlpsics hfnplsleel gsntgiqvfn qivksrphdn ivisphgias
 61 vlgmlqlqad grtkkqlamv mrygvngvgk ilkkinkaiv skknkdivtv anavfvknas
121 eievpfvtrn kdvfqcevrn vnfedpasac dsinawvkne trdmidnlls pdlidgvltr
181 lvlvnavyfk glwksrfqpe ntkkrtfvaa dgksyqvpml aqlsvfrcgs tsapndlwyn
241 fielpyhqes ismlialpte sstplsaiip histktidsw msimvpkrvq vilpkftava
301 qtdlkeplkv lgitdmfdss kanfakittg senlhvshil qkakievsed gtkasaatta
361 iliarssppw fivdrpflff irhnptgavl fmgqinkp
```

SEQ ID NO: 11 Protease nexin 1

Mature protein corresponds to residues residues 20-398 – isoform a. Residues P4, P2, P1 and P1' of the RCL bold and underlined

```
  1 mpssvswgil llaglcclvp vslaedpqgd aaqktdtshh dqdhptfnki tpnlaefafs
 61 lyrqlahqsn stniffspvs iatafamlsl gtkadthdei leglnfnlte ipeaqihegf
121 qellrtlnqp dsqlqlttgn glflseglkl vdkfledvkk lyhseaftvn fgdteeakkq
181 indyvekgtq gkivdlvkel drdtvfalvn yiffkqkwer pfevkdteee dfhvdqvttv
241 kvpmmkrlgm fniqhckkls swvllmkylg nataifflpd egklqhlene lthdiitkfl
301 enedrrsasl hlpklsitgt ydlksvlgql gitkvfsnga dlsgvteeap lklskavhka
361 vltidekgte aagamfleai krkippevkf nkpfvflmie qntksplfmg kvvnptqk
```

SEQ ID NO: 12 Modified Serpin in α₁AT scaffold

Mature protein corresponds to residues 25-418. Residues P4, P2, P1 and P1' of the RCL bold and underlined

Claims

1.   A modified serpin comprising an amino acid sequence having at least 70% sequence identity to residues 25-418 of SEQ ID NO: 12,

wherein the residue corresponding to residue 379 of SEQ ID NO: 12 is A; the residue corresponding to residue 381 of SEQ ID NO: 12 is K; the residue corresponding to residue 382 of SEQ ID NO: 12 is R; and the residue corresponding to residue 383 of SEQ ID NO: 12 is K; and

wherein the inhibition of activated Protein C relative to the inhibition of one or more procoagulant proteases selected from thrombin, fVIIa, fXa, fIXa and fXIa is increased for the modified serpin compared to a serpin comprising the amino acid sequence of residues 25-418 of SEQ ID NO: 7.

2.   A modified serpin according to claim 1 comprising;

(i) the amino acid sequence of residues 25-418 of SEQ ID NO: 12;

(ii) an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue C256 with S;

(iii) an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue E25 with S; or

(iv) an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue E25 with S and the replacement of C256 with S.

3.   A serpin comprising the amino acid sequence of residues 25-418 of SEQ ID NO: 12.

4.   A serpin comprising an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue E25 with S.

5.   A serpin comprising an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue C256 with S.

6.   A serpin comprising an amino acid sequence that differs from the amino acid sequence of residues 25-418 of SEQ ID NO: 12 by the replacement of residue C256 with S and the replacement of residue E25 with S.

7. A pharmaceutical composition comprising the serpin according to any one of claims 1 to 6 and a pharmaceutically acceptable excipient.

8. Use of the serpin according to any one of claims 1 to 6 for treatment of bleeding or promotion of hemostasis in an individual in need thereof.

9. Use of the serpin according to any one of claims 1 to 6 in the manufacture of a medicament for treatment of bleeding or promotion of hemostasis in an individual in need thereof.

10. The use according to claim 8 or 9, wherein the individual has a bleeding disorder.

11. The use according to claim 10, wherein the bleeding disorder is hemophilia.

12. The use according to claim 8 or 9 wherein the individual is a trauma patient.
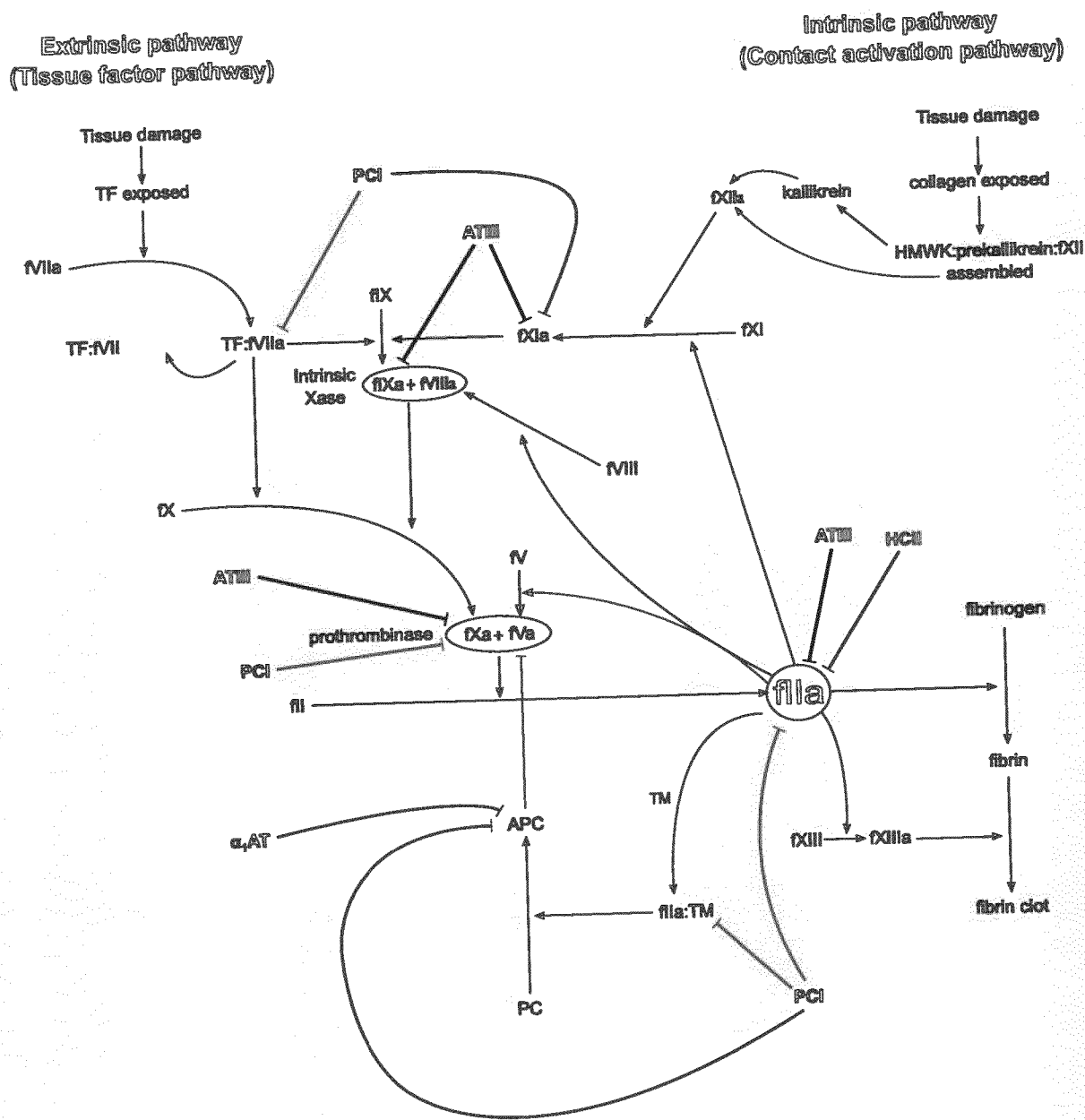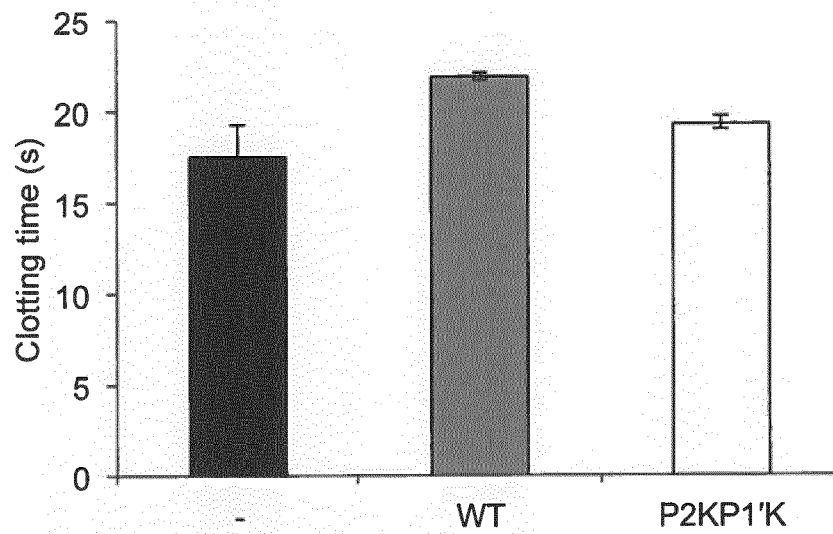
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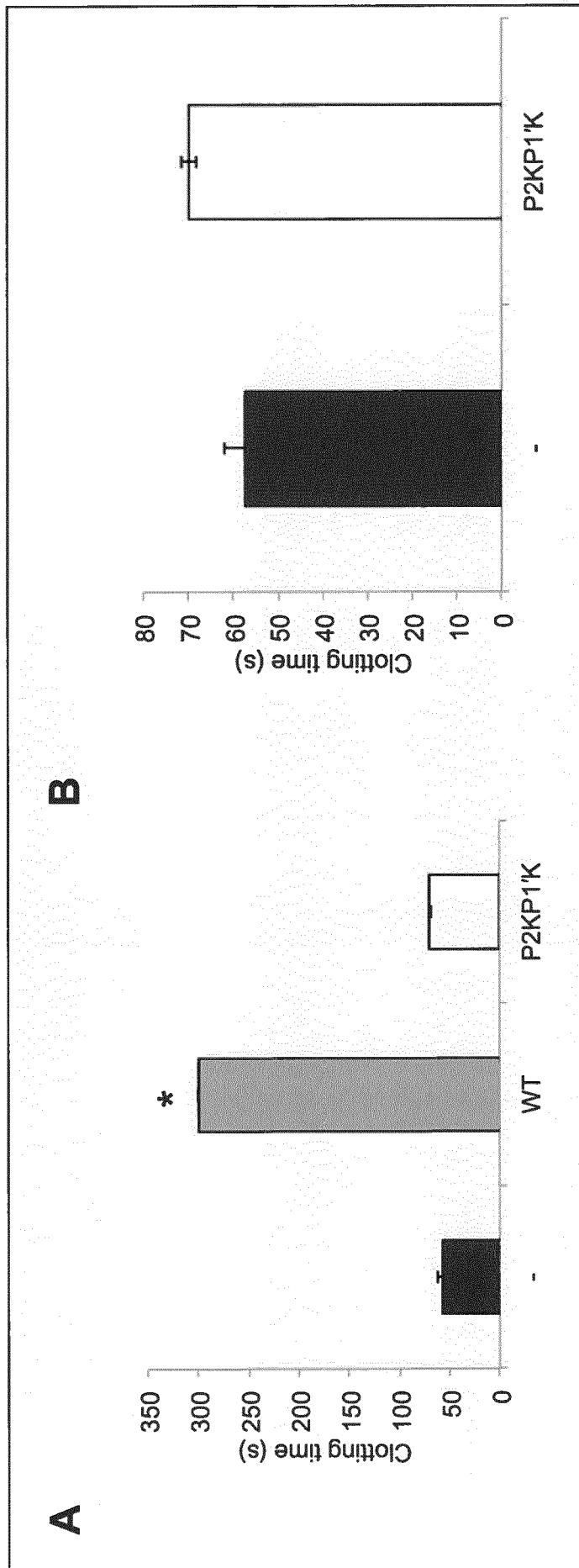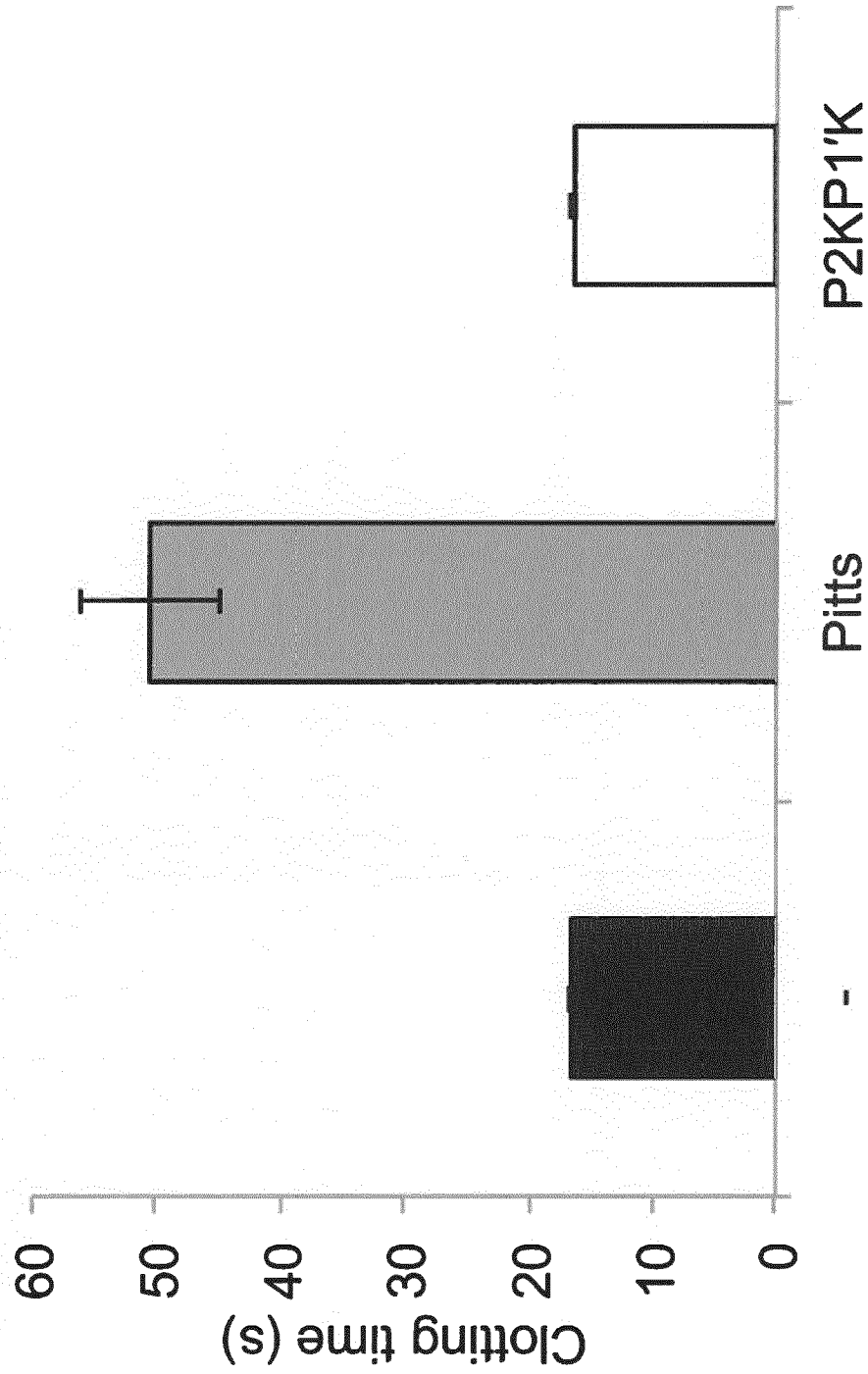
Figure 2

Figure 3

Figure 4
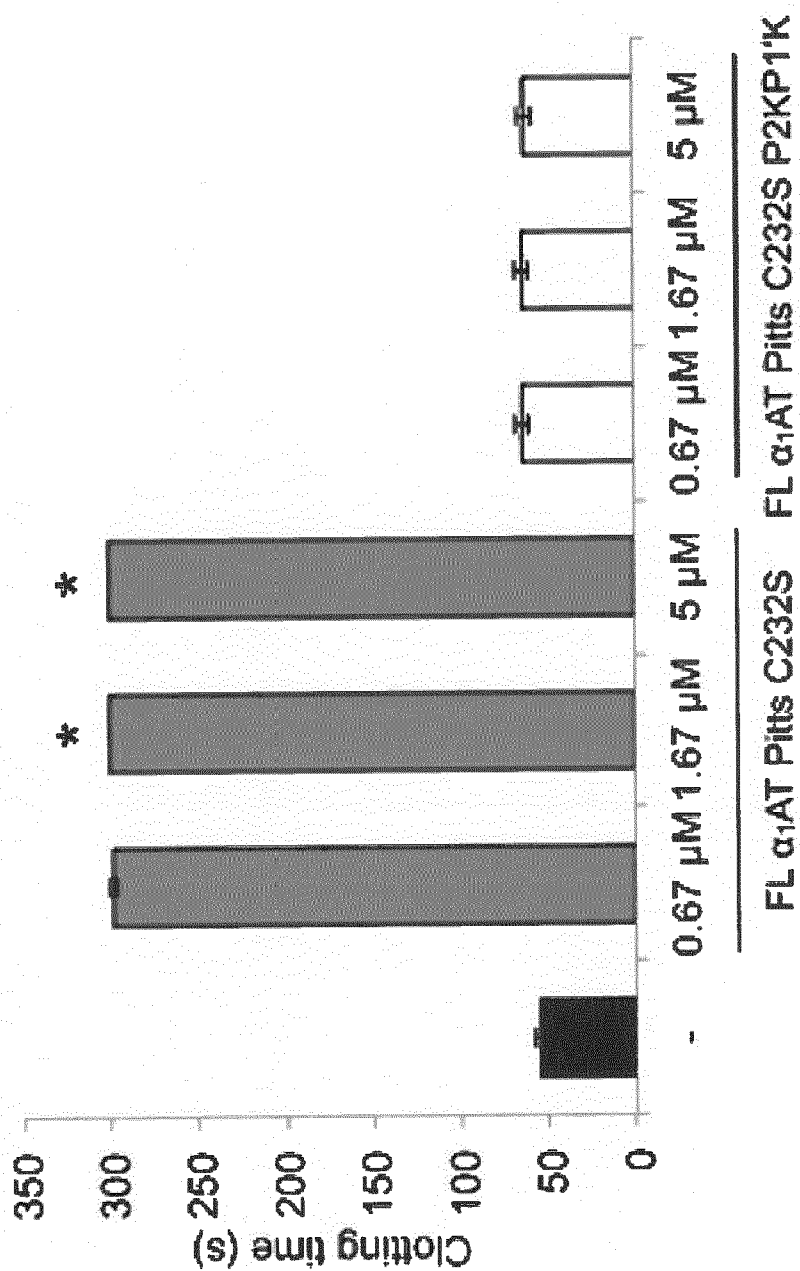
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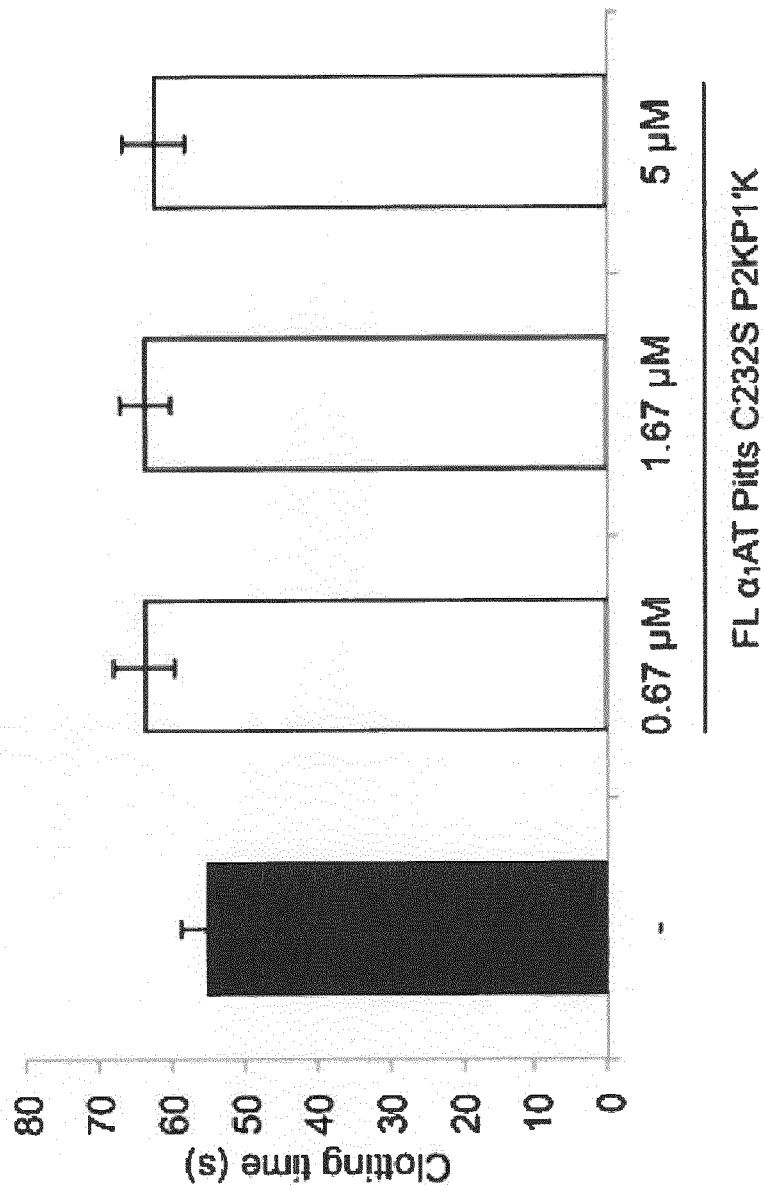
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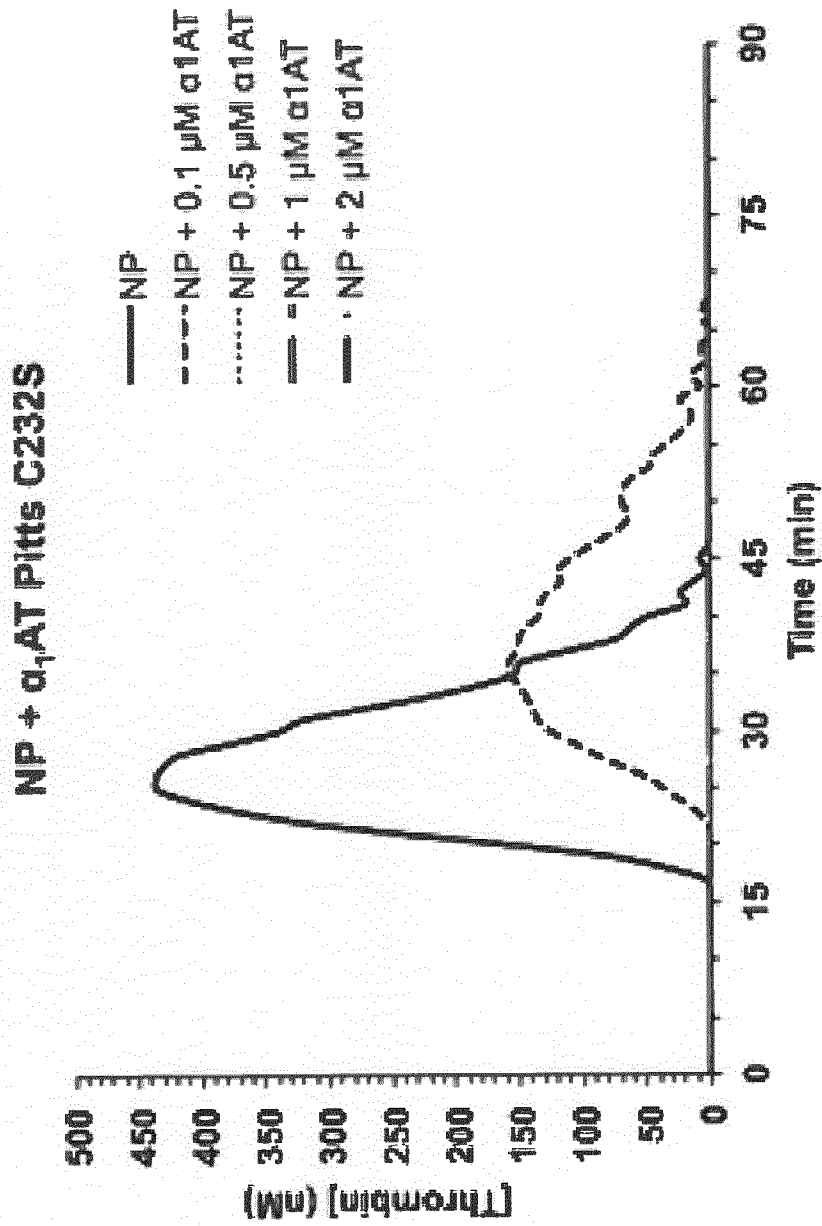
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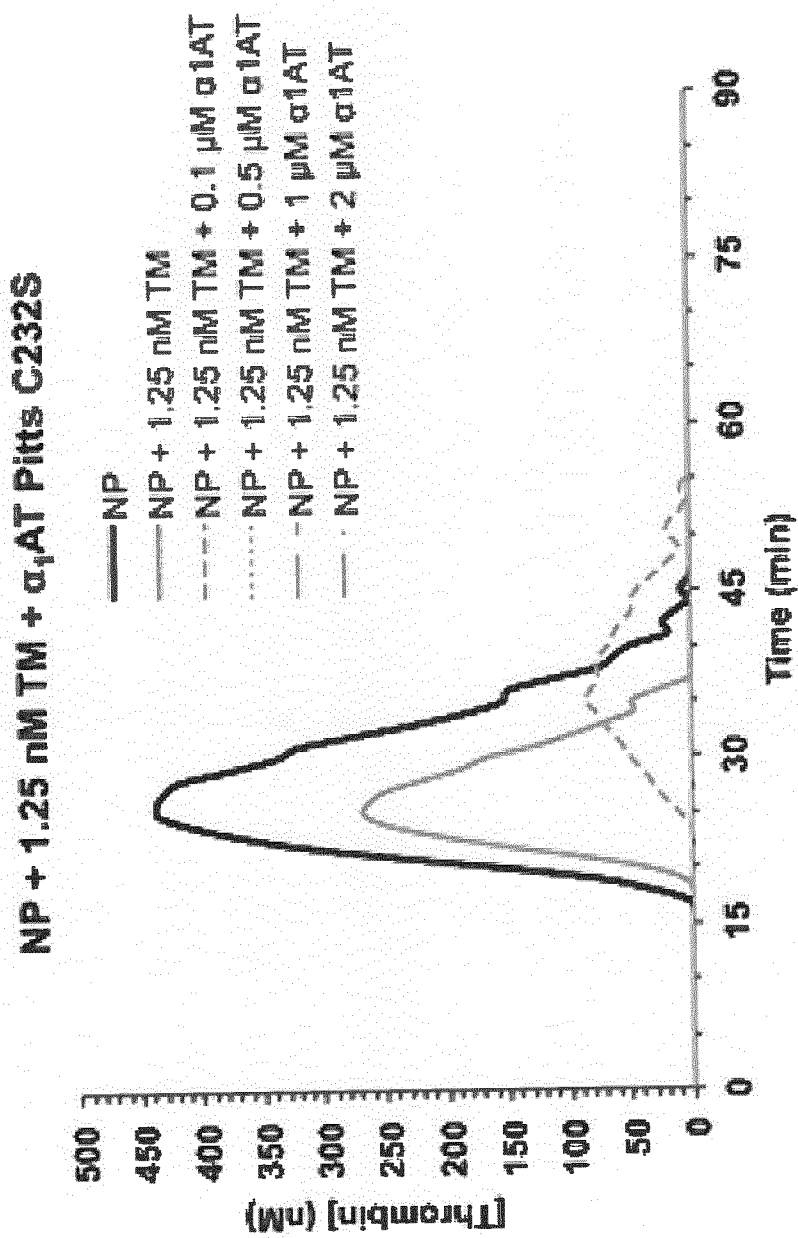
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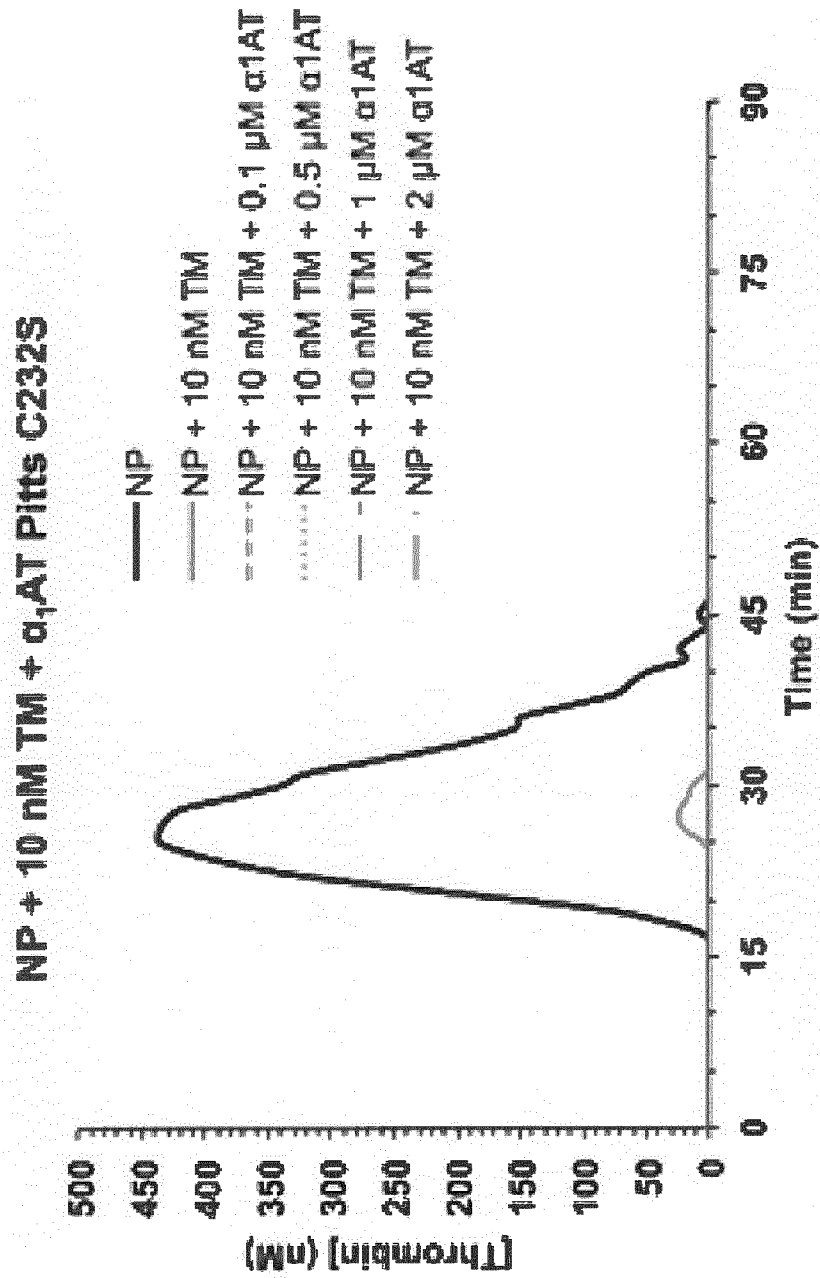
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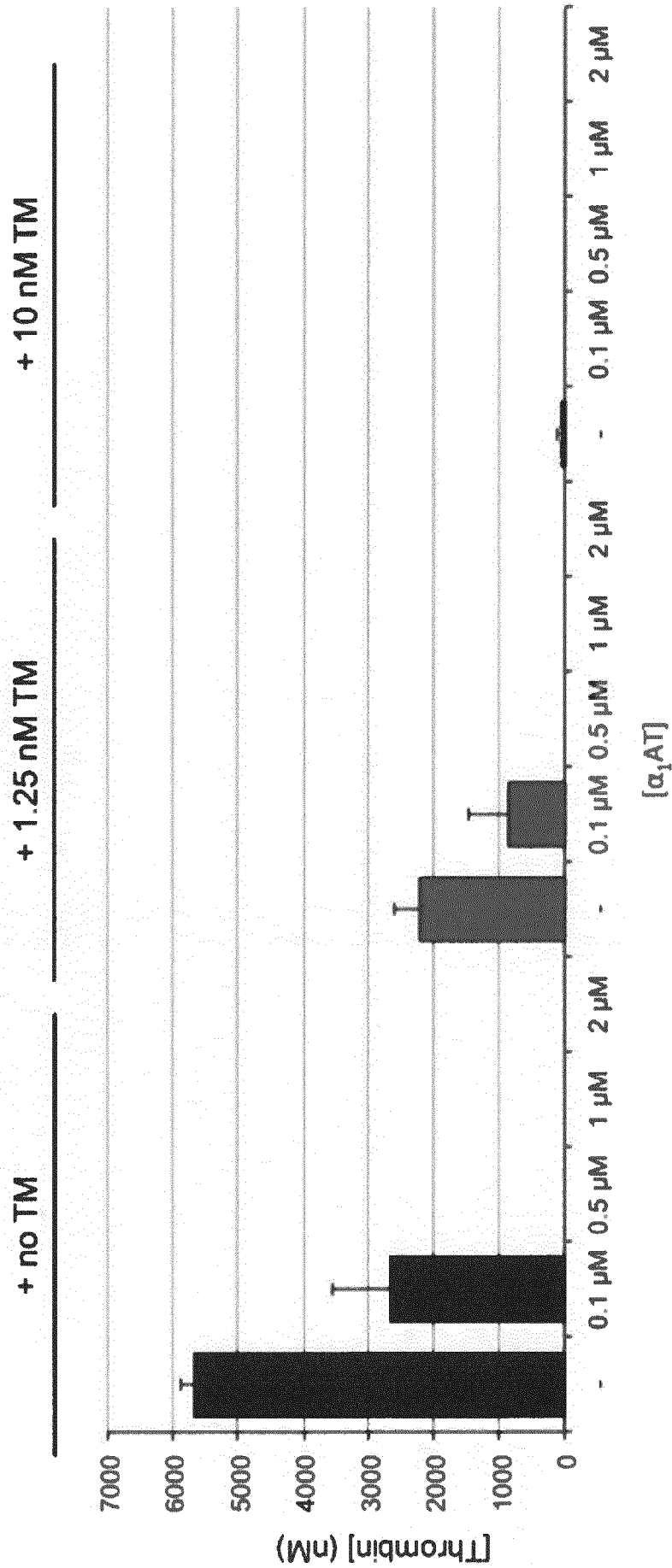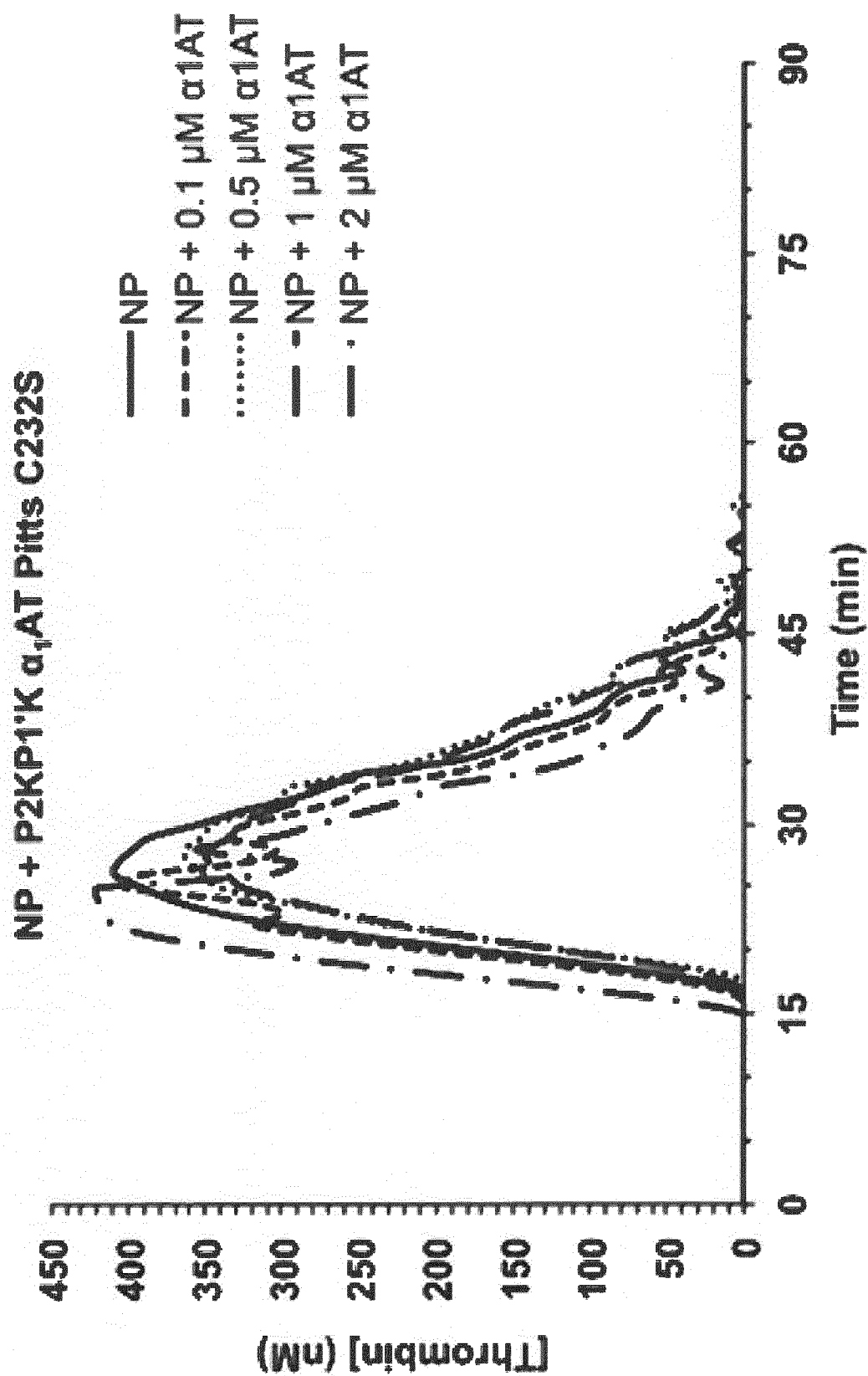
Figure 6D

Figure 7A
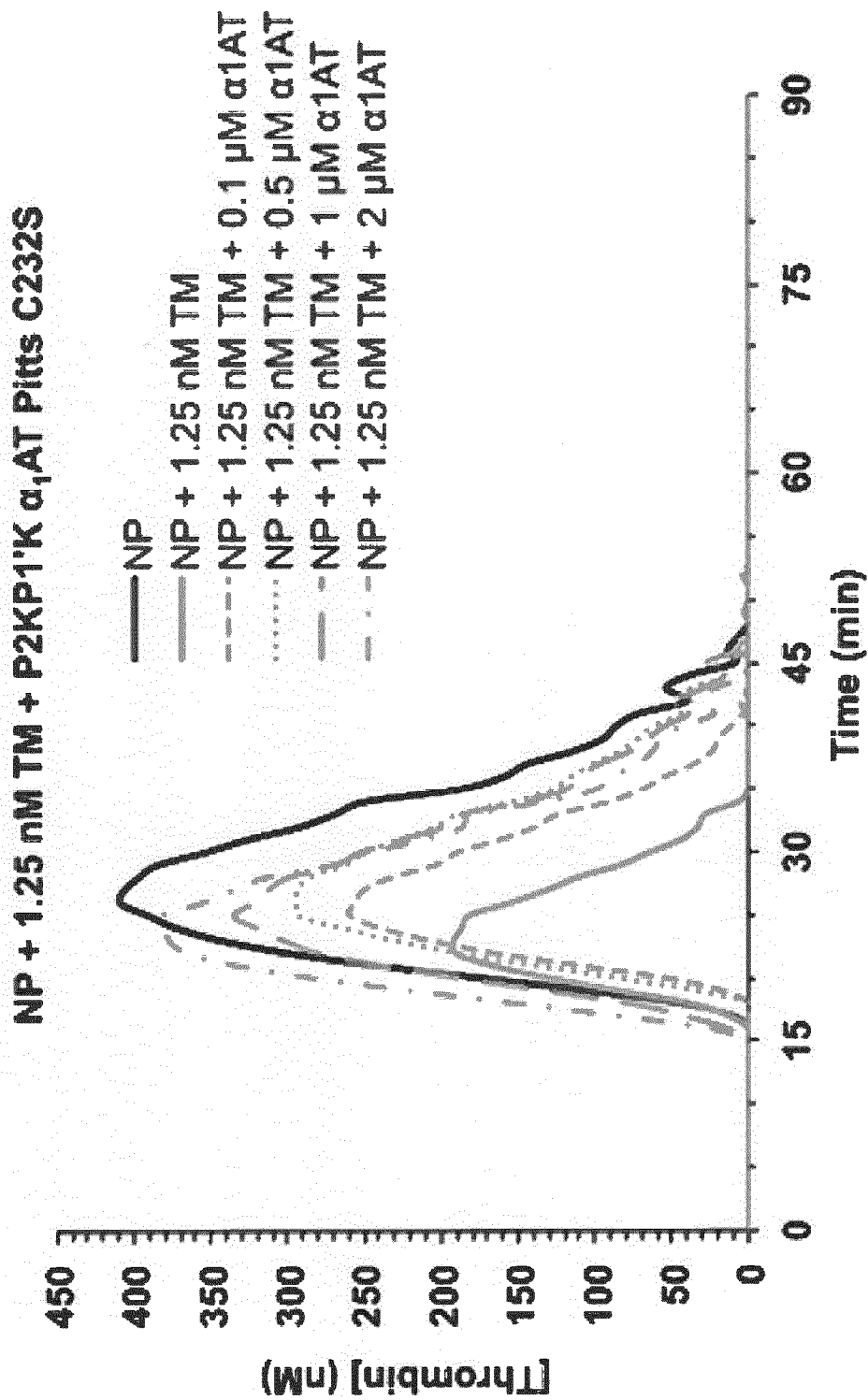
Figure 7B

Figure 7C

Figure 7D

A

HA + α₁AT Pitts C232S

B
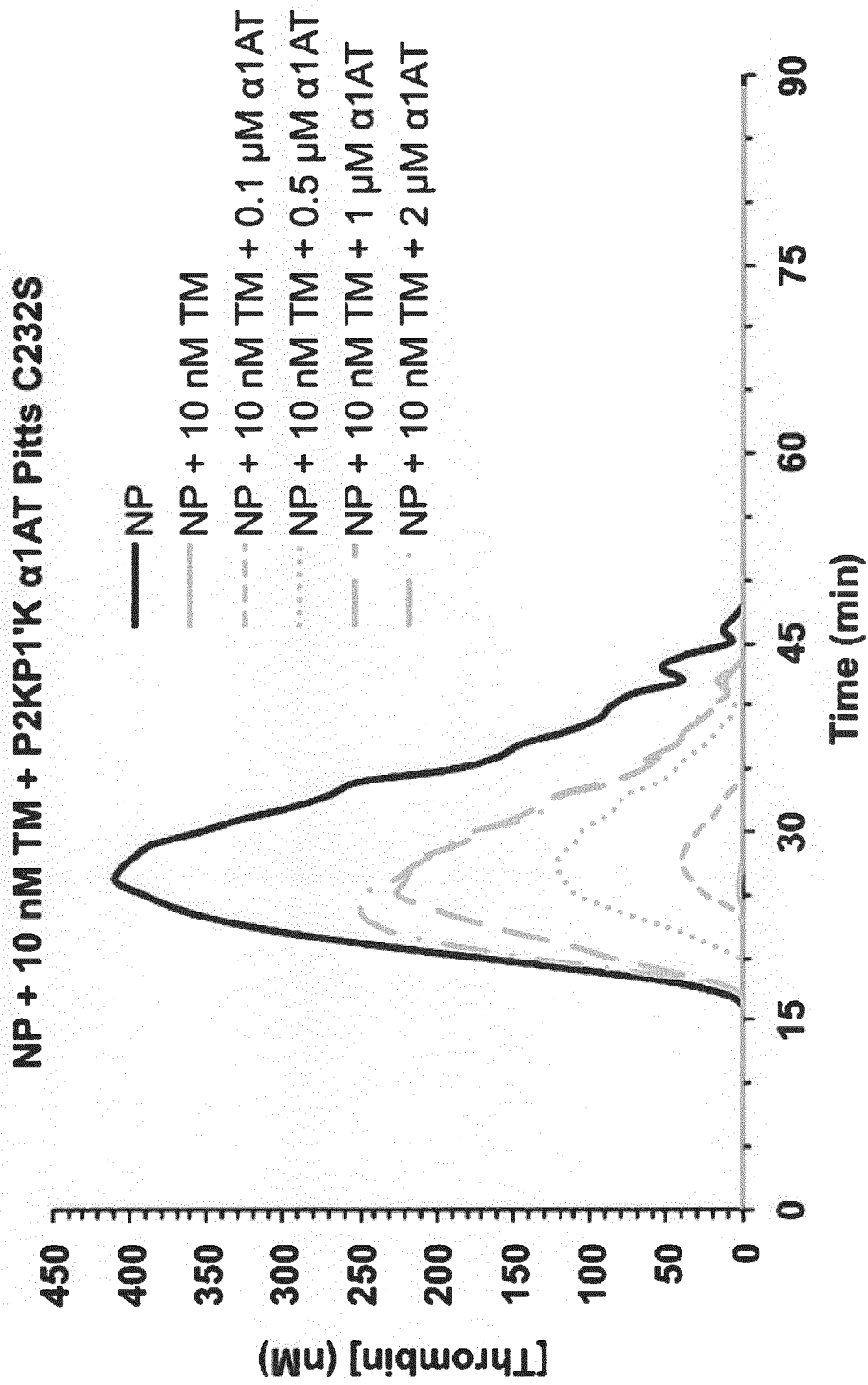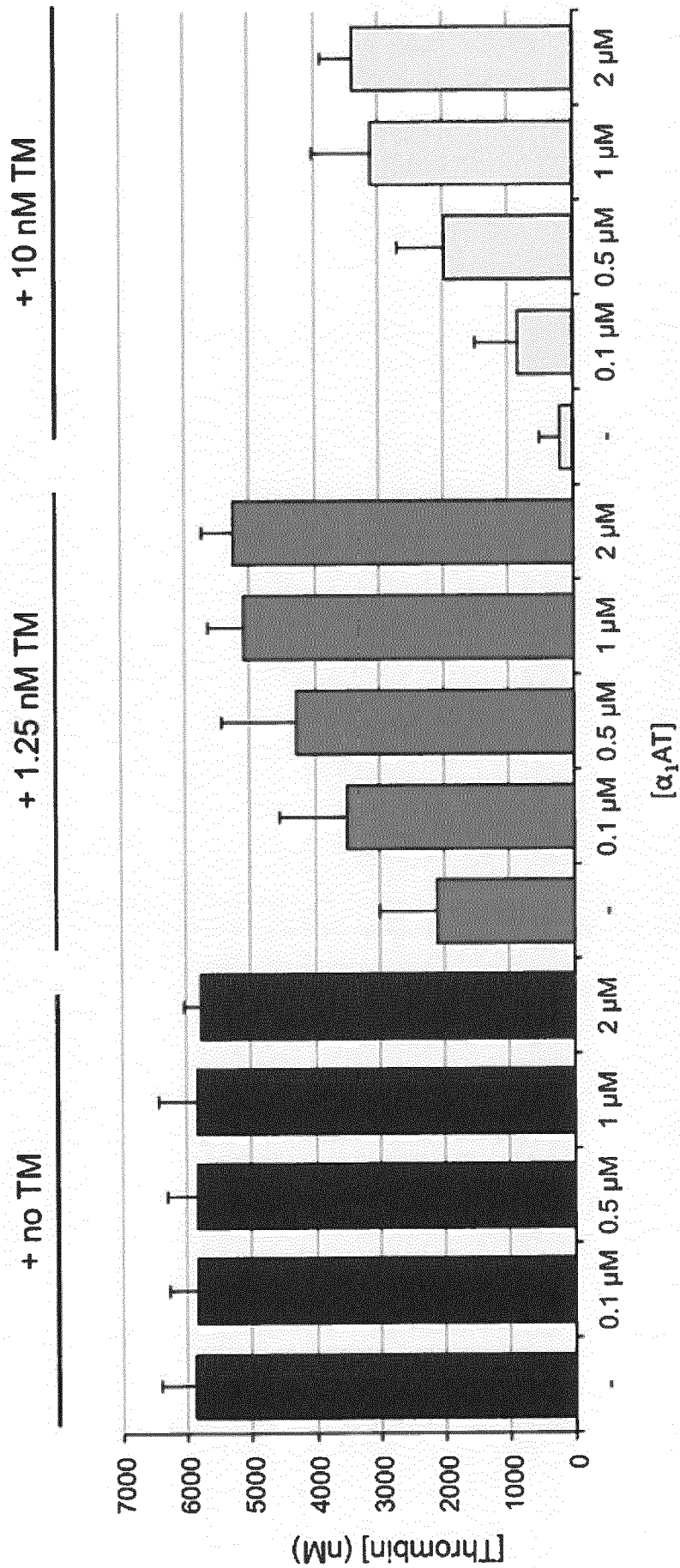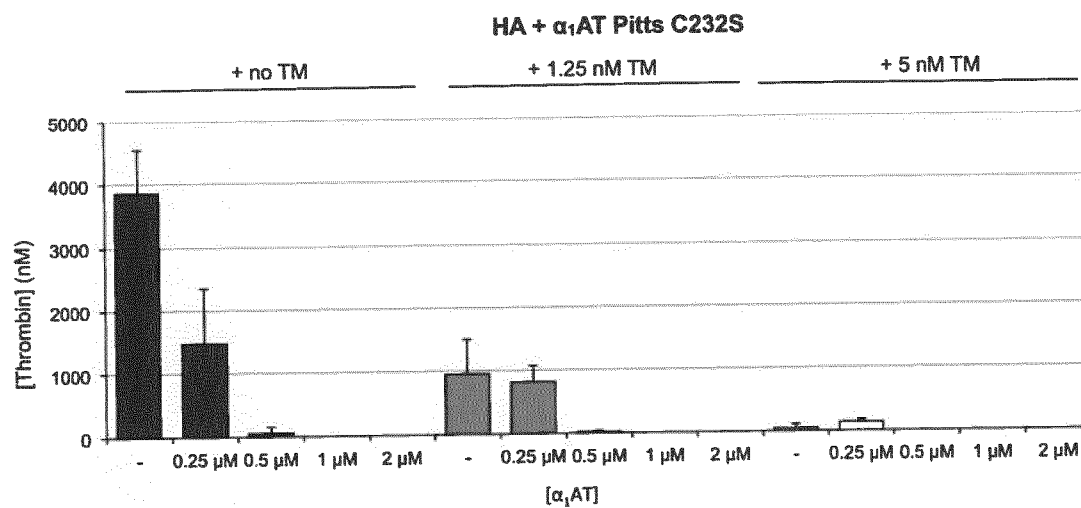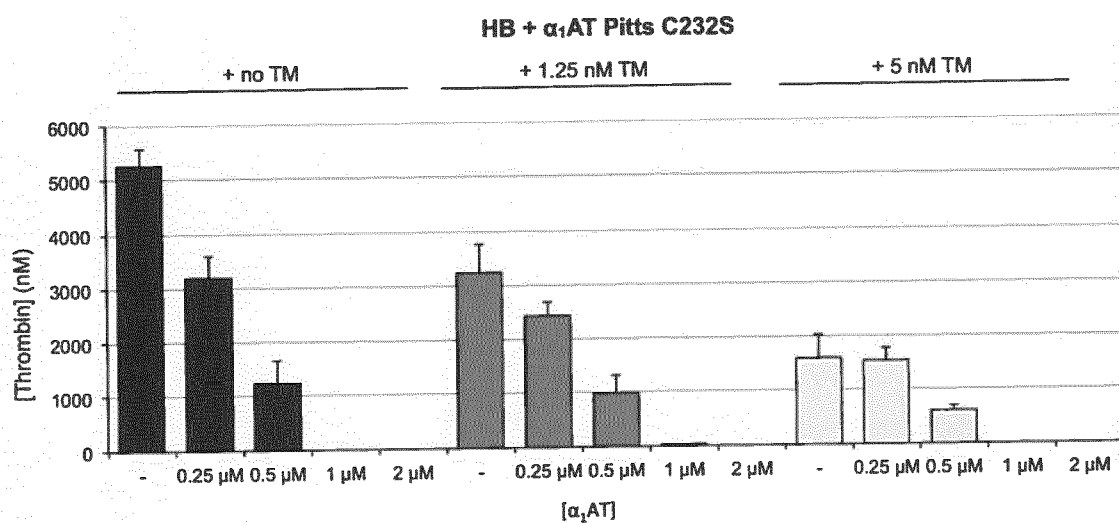
HB + α₁AT Pitts C232S
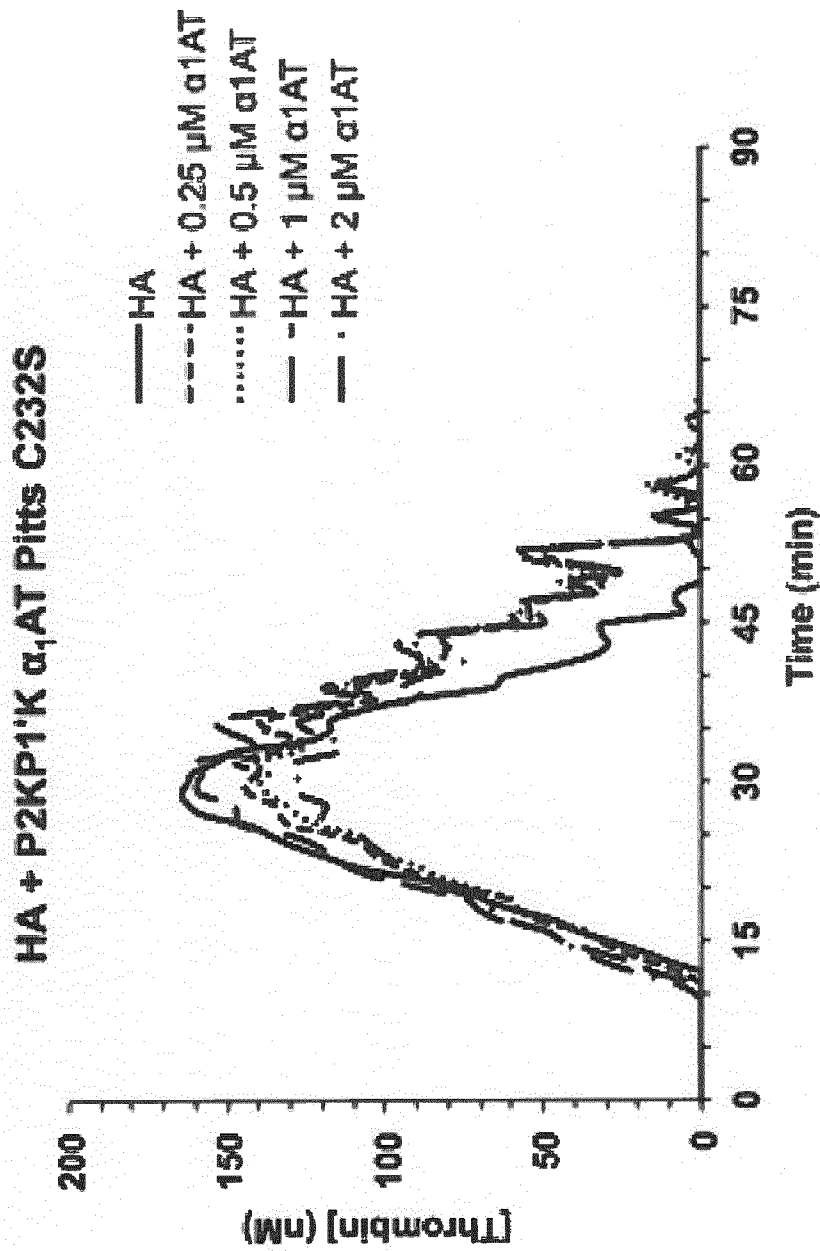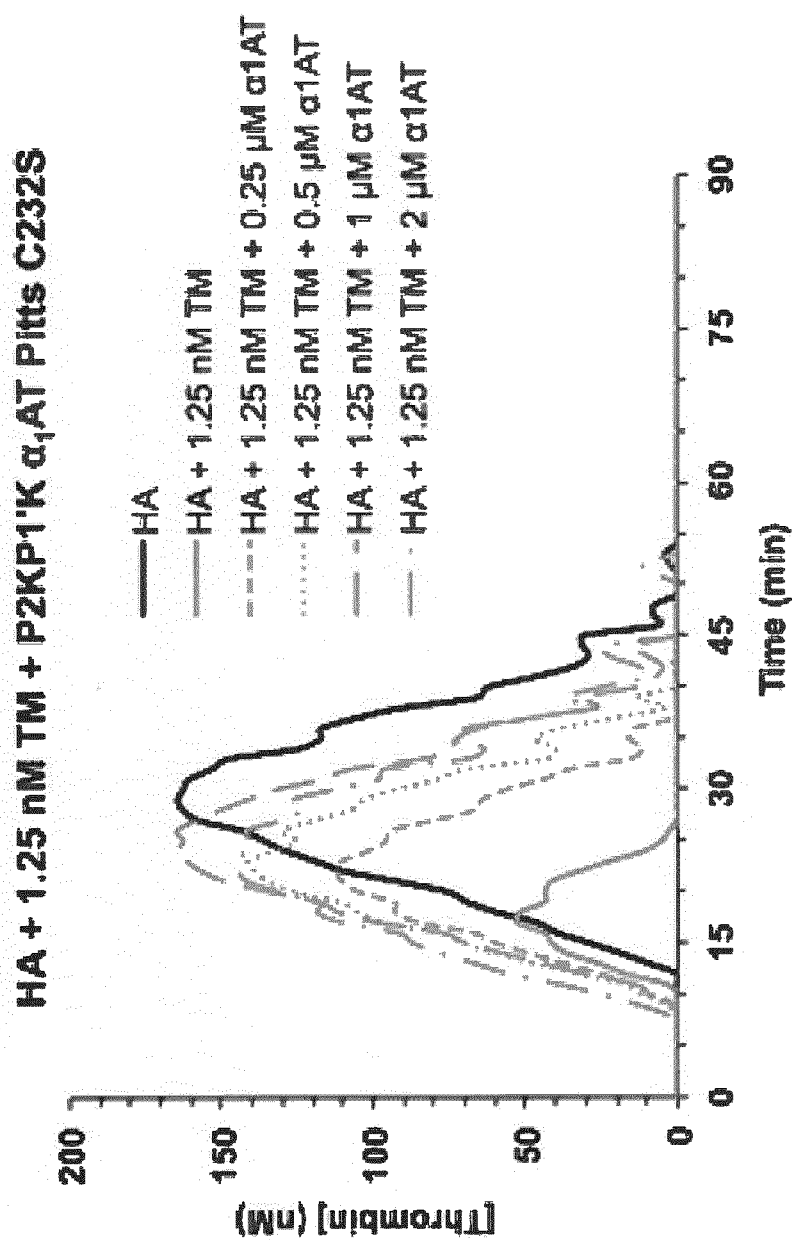
Figure 8

Figure 9A

Figure 9B
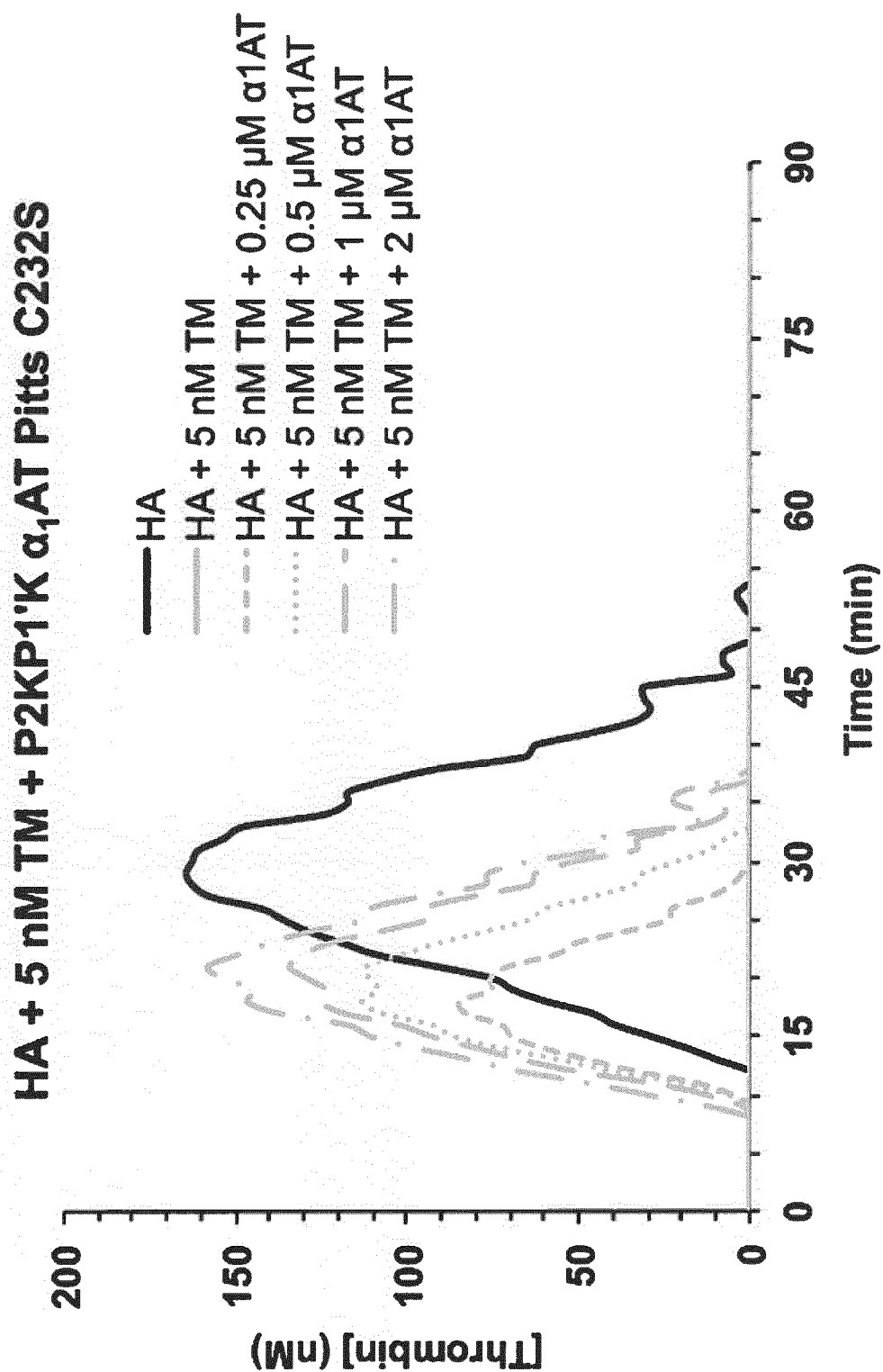
Figure 9C

Figure 9D

Figure 10A
HB + P2KP1'K α₁AT Pitts CZ32S

Legend:
- HB
- HB + 0.25 µM α1AT
- HB + 0.5 µM α1AT
- HB + 1 µM α1AT
- HB + 2 µM α1AT
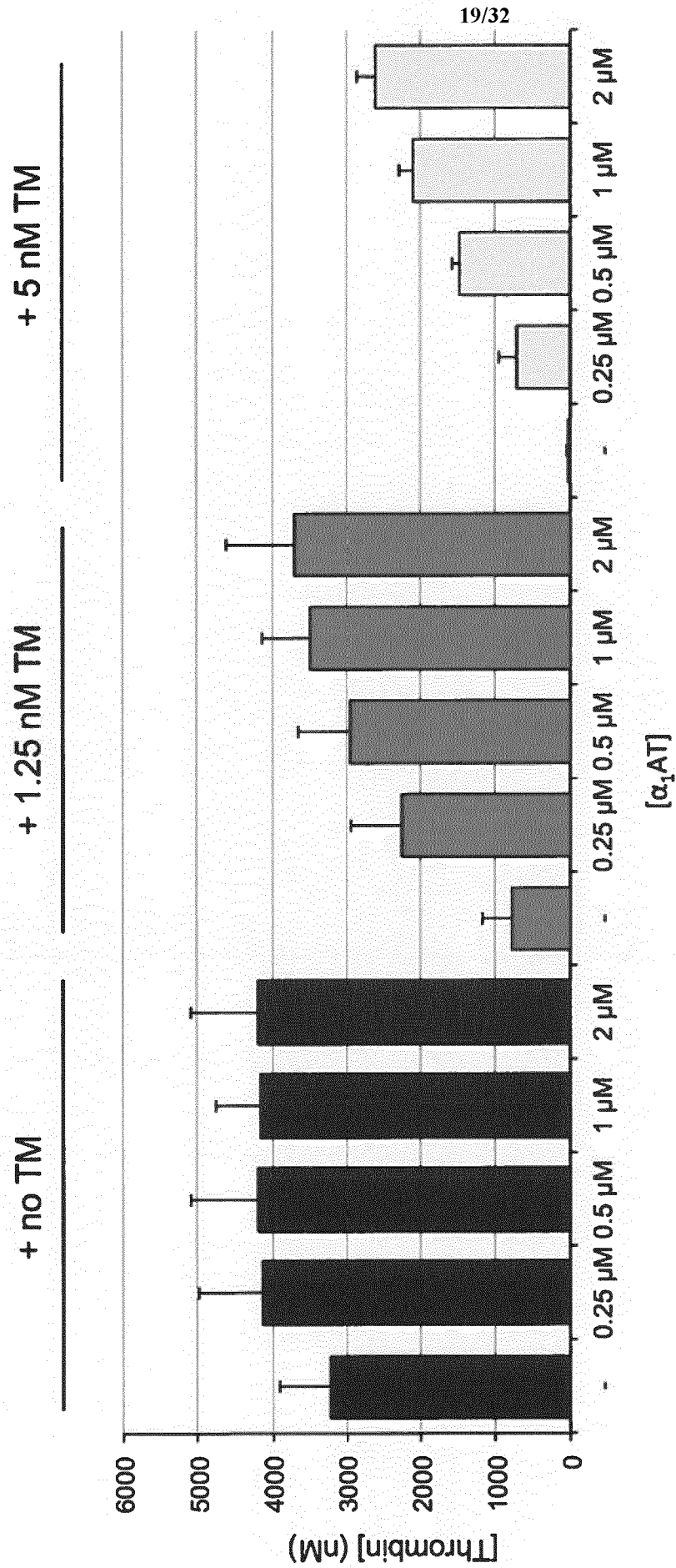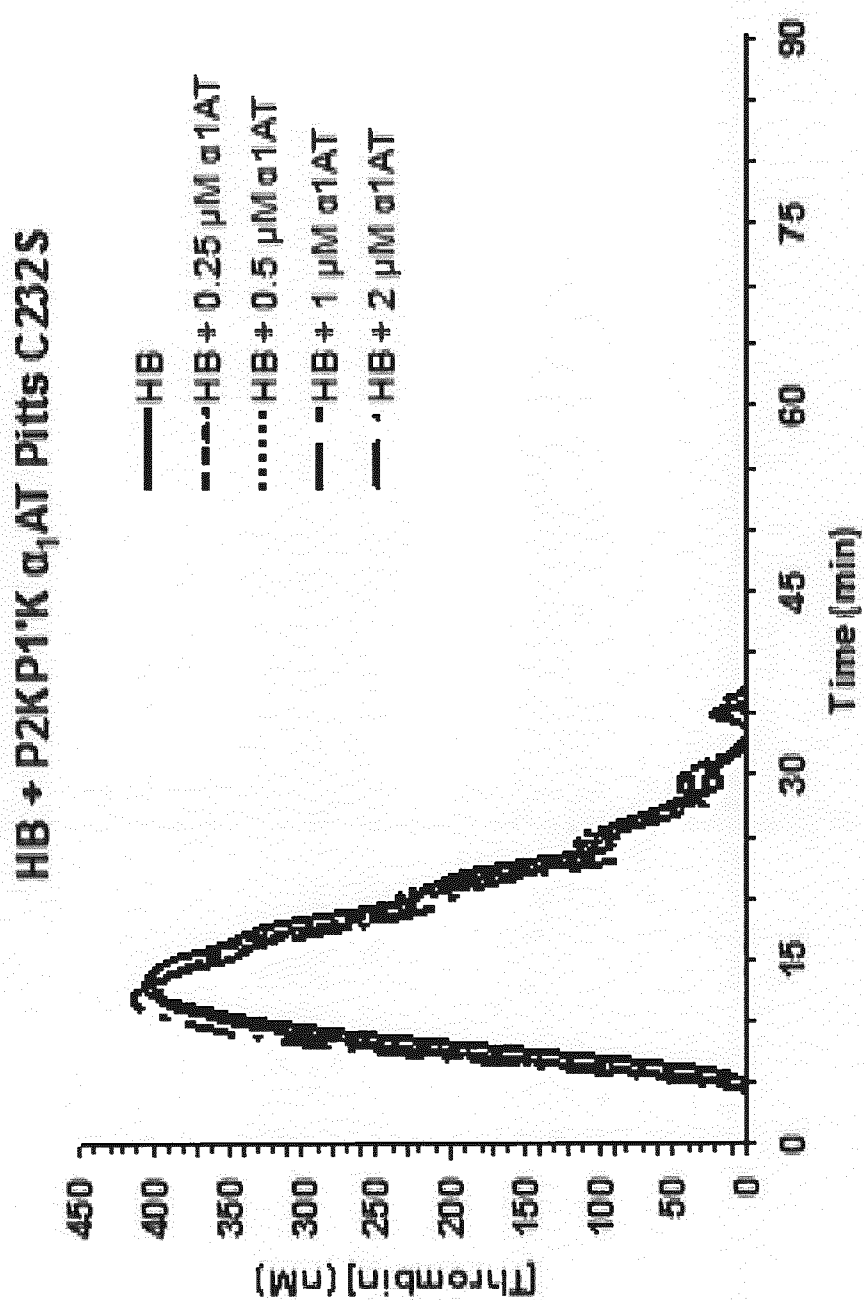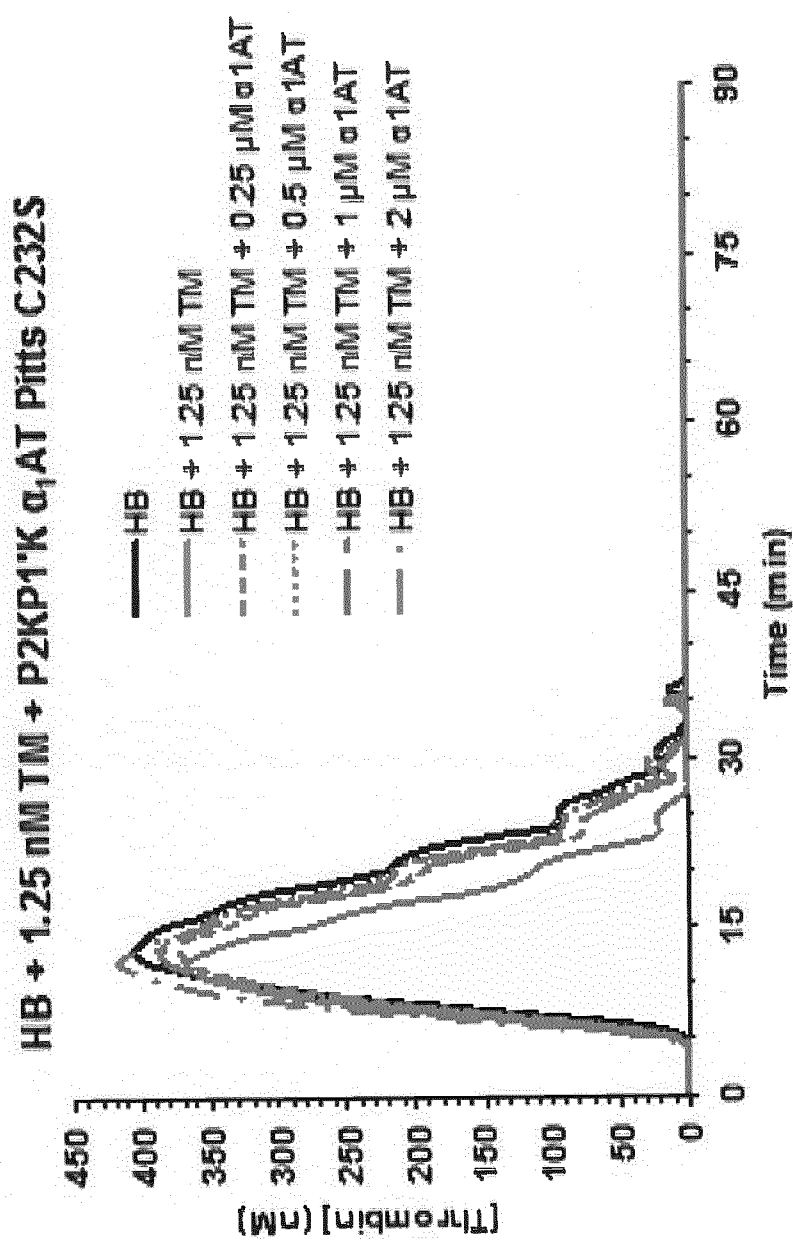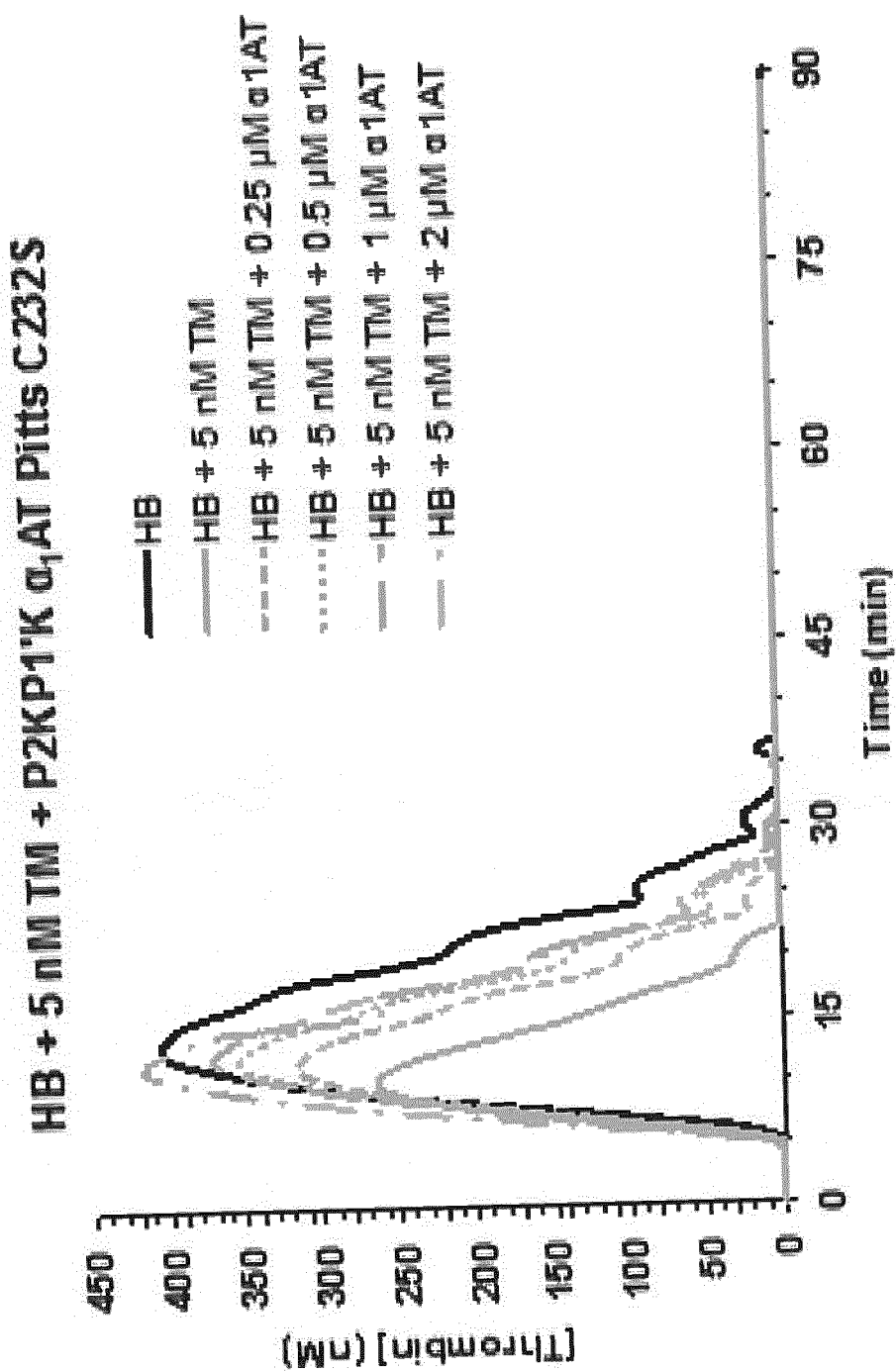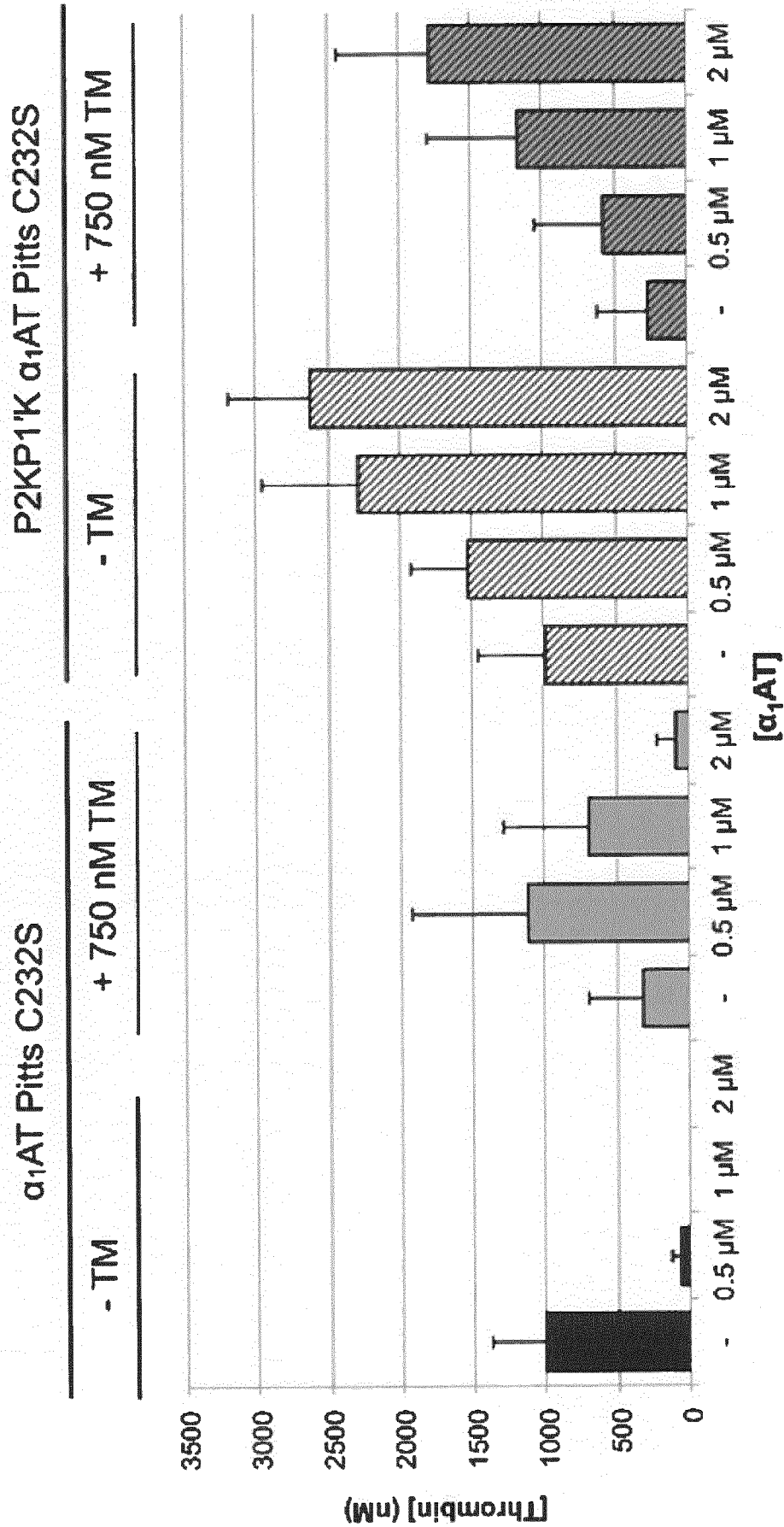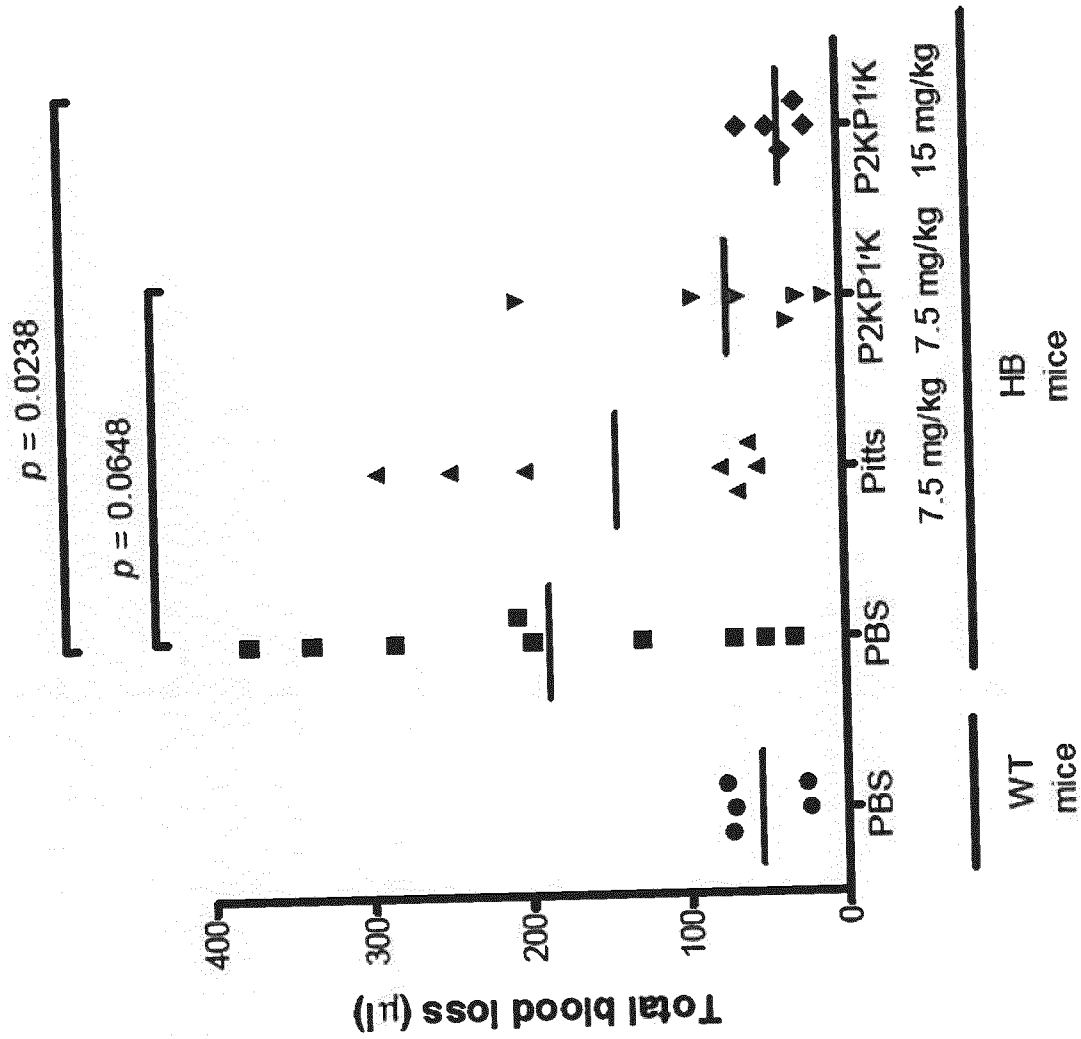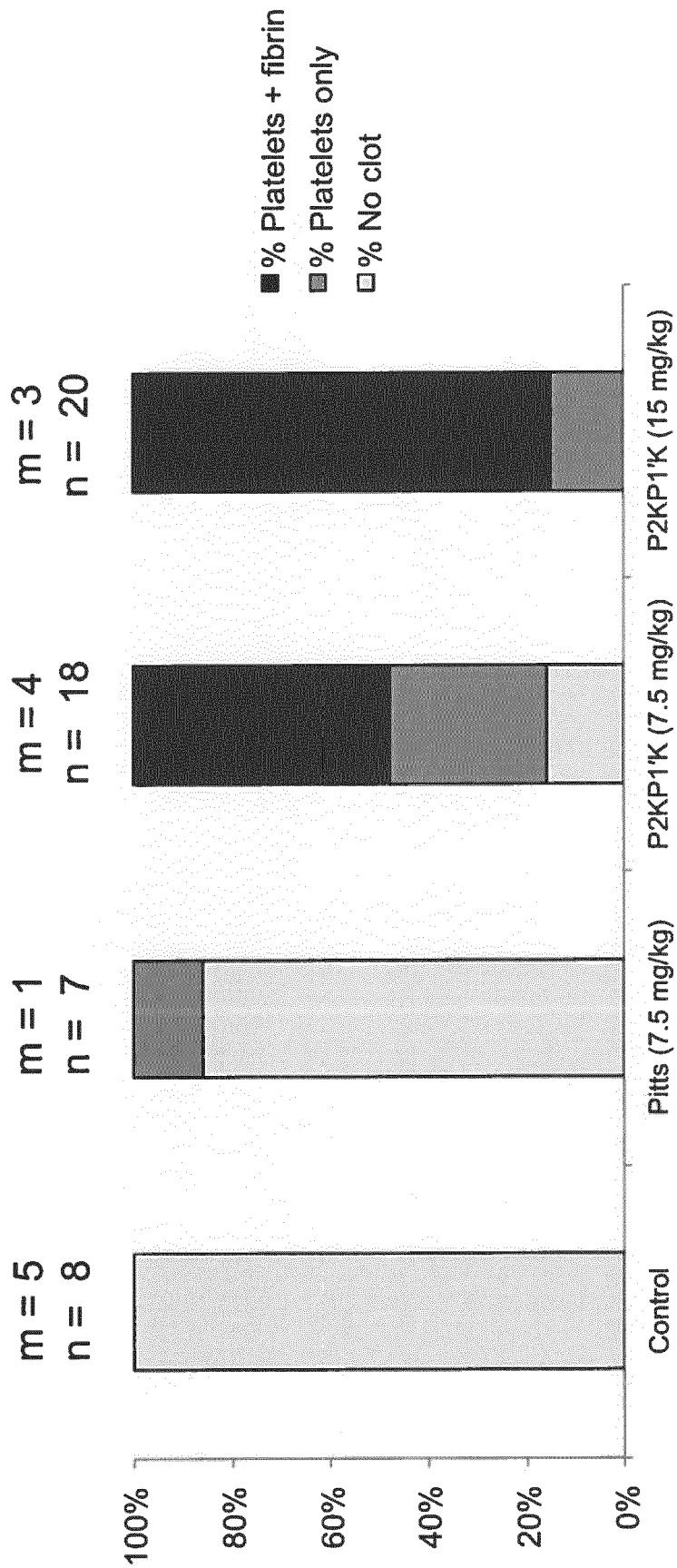
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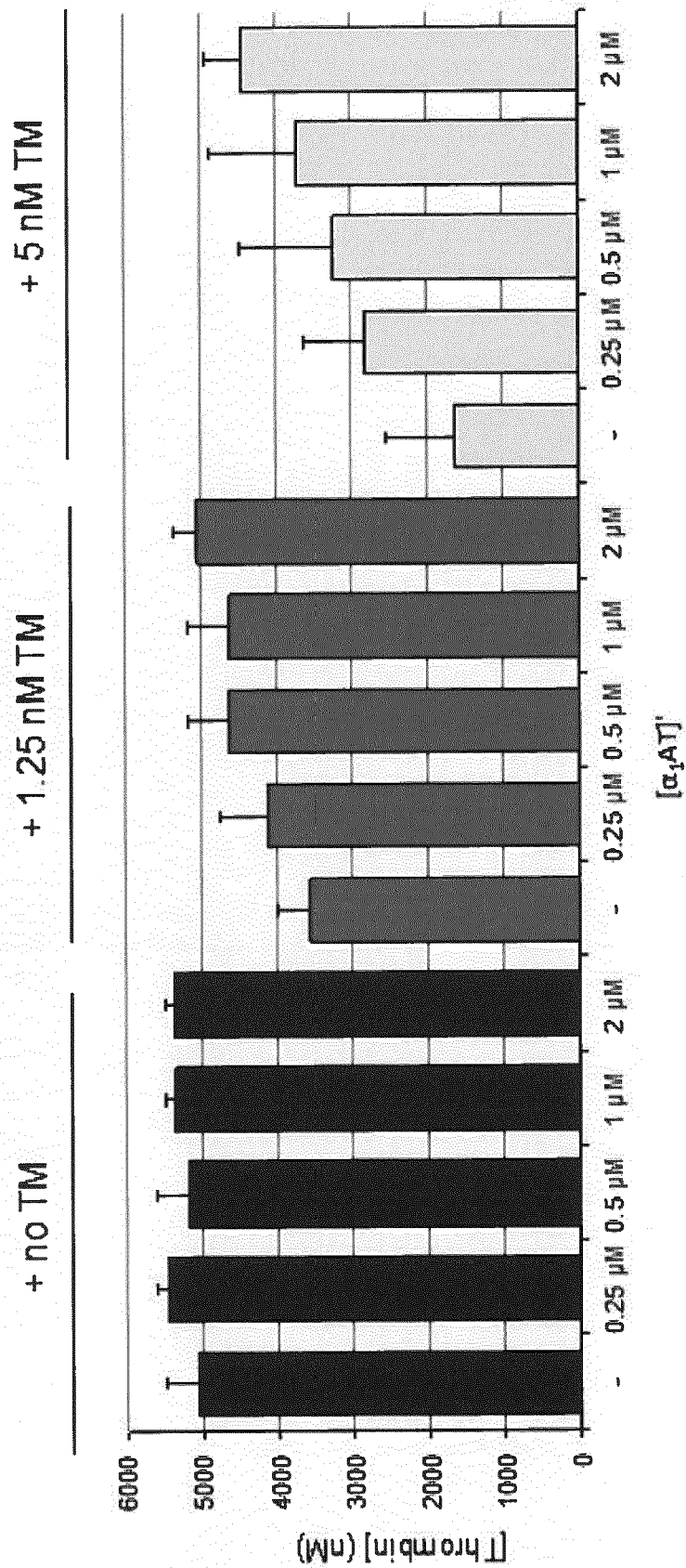
Figure 10C

Figure 10D
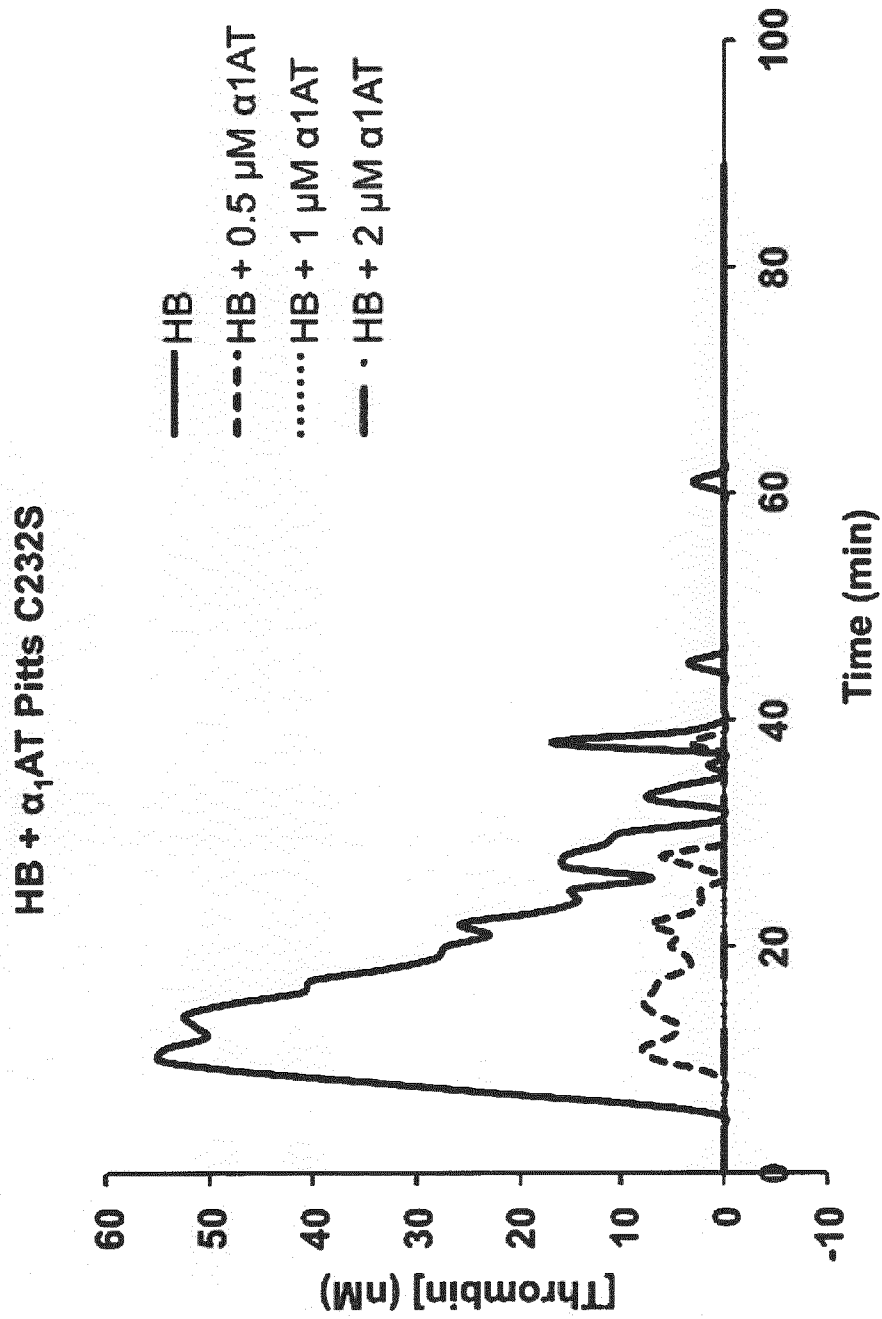
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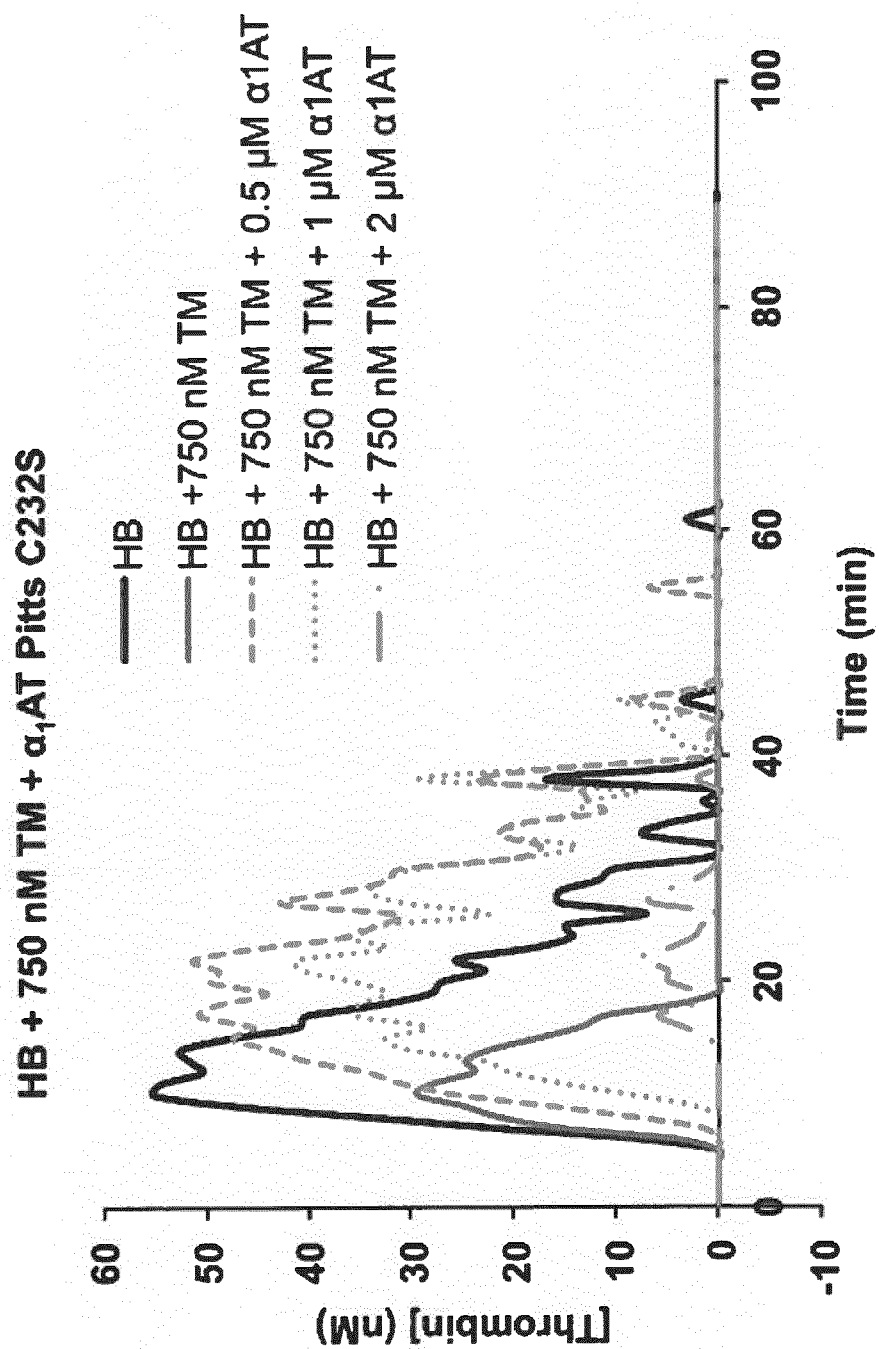
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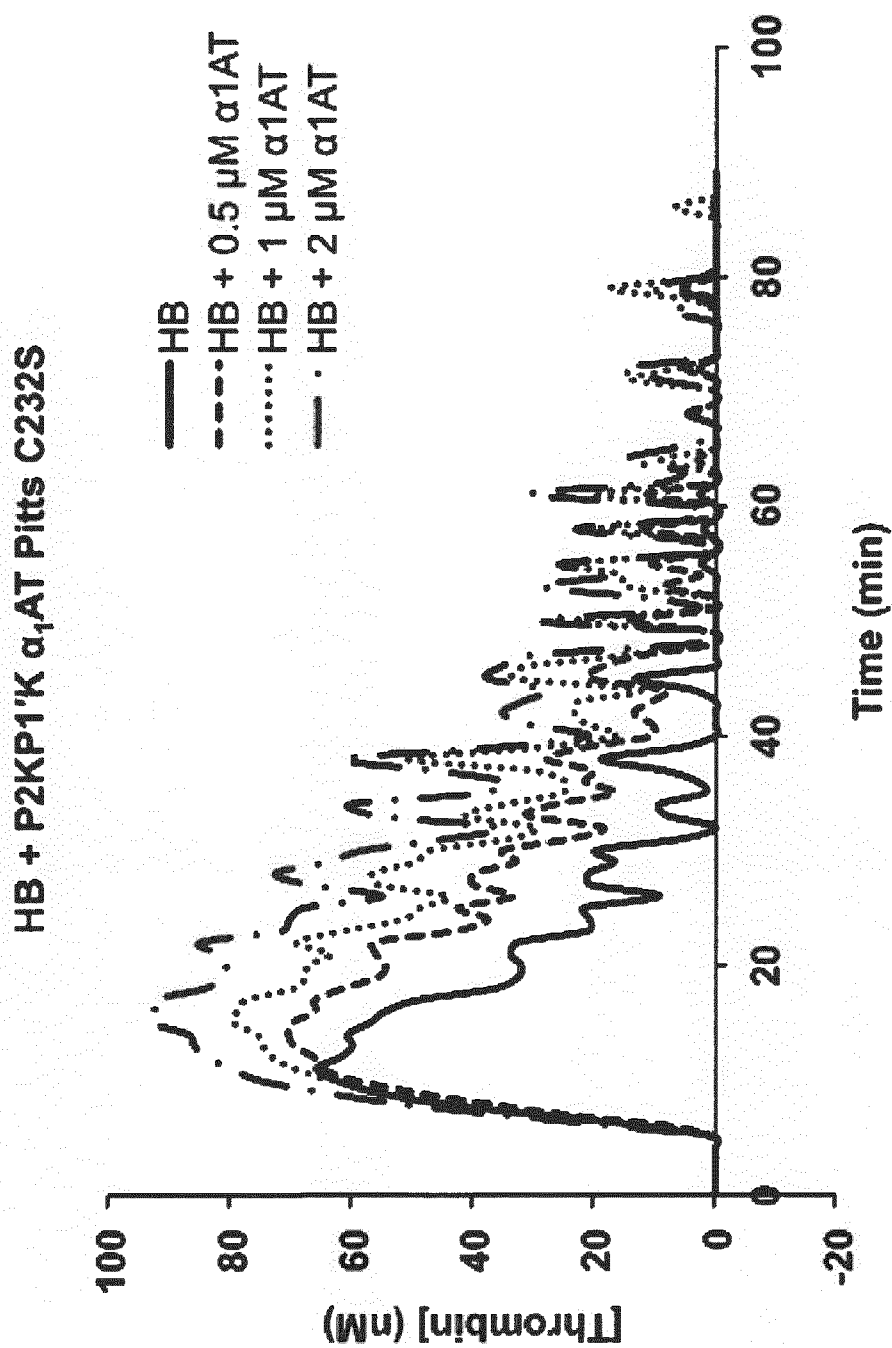
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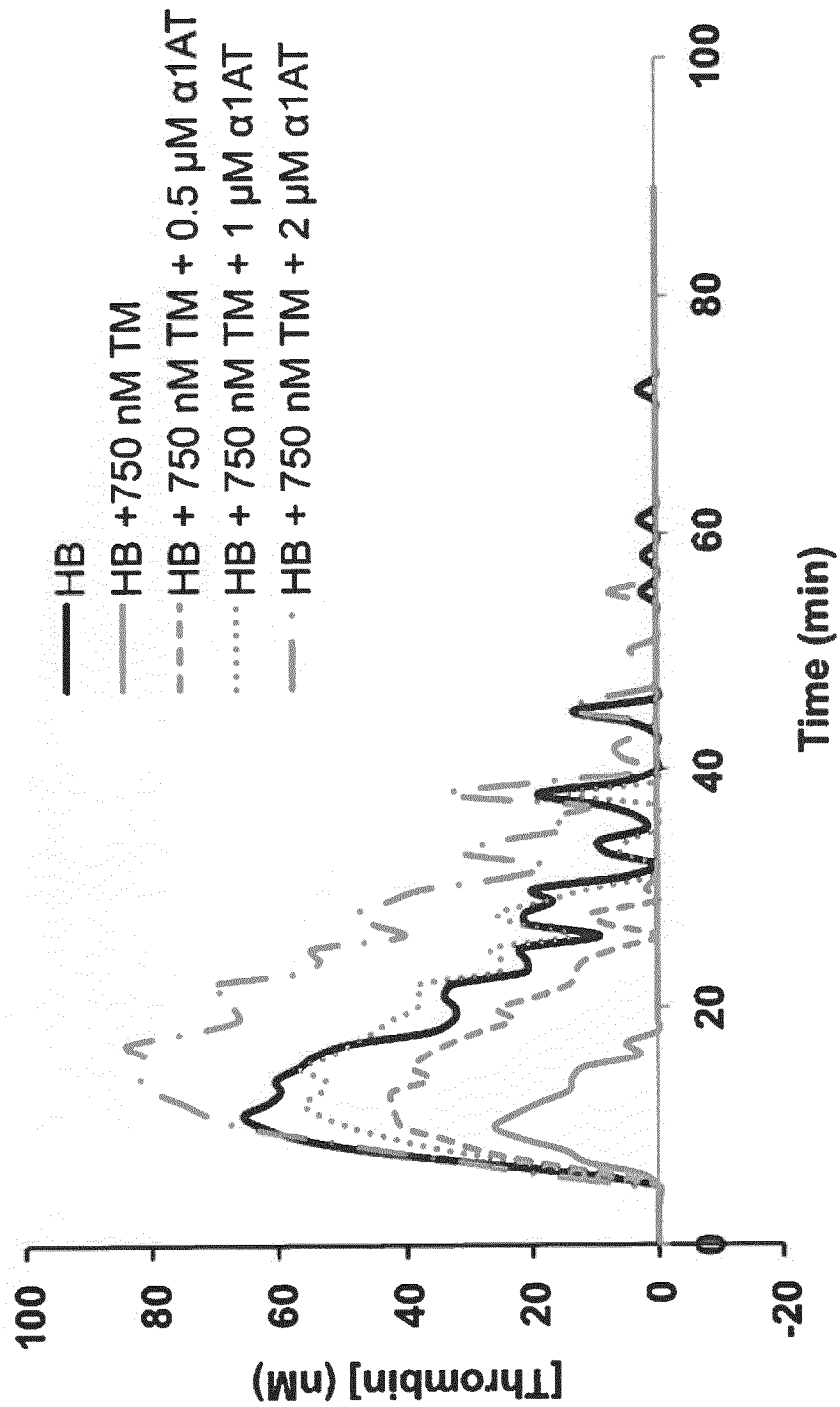
Figure 11D

Figure 11E

Figure 12

Figure 13

Figure 14

Figure 15