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<p>(54) Title: METHODS FOR TREATING THROMBOTIC DISORDERS</p>		
<p>(57) Abstract</p> <p>The present invention provides a method of treatment for patients with a variety of thrombotic disorders including, but not limited to, stroke, venous thrombosis, myocardial infarction, unstable angina, abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery. Said treatment is a combination therapy with human aPC and antiplatelet agents including, but not limited to, aspirin (ASA), clopidogrel, ReoPro® (abciximab), dipyridamole, ticlopidine and IIb/IIIa receptor antagonists. The synergy will result in the ability to reduce the dosages of the agents used in the combination therapy.</p>		

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**METHODS FOR TREATING THROMBOTIC DISORDERS**

This invention relates to medical science particularly  
the treatment of thrombotic disorders with activated human  
5 protein C in combination with antiplatelet agents.

Protein C is a serine protease and naturally occurring  
anticoagulant that plays a role in the regulation of  
hemostasis by inactivating Factors Va and VIIIa in the  
10 coagulation cascade. Human protein C circulates as a 2-  
chain zymogen which is activated *in vivo* by thrombin and  
thrombomodulin on phospholipid surfaces resulting in  
activated protein C (aPC).

Blood coagulation is a highly complex process regulated  
15 by the balance between pro-coagulant and anticoagulant  
mechanisms. This balance determines a condition of either  
normal hemostasis or abnormal pathological thrombus  
formation leading to events such as stroke, myocardial  
infarction and venous thrombosis. Two major factors control  
20 this balance, the generation of fibrin and the activation  
and subsequent aggregation of platelets. A critical factor  
controlling both processes is the generation of the enzyme  
thrombin, which occurs following activation of the clotting  
cascade. Thrombin is a pro-coagulant enzyme that aggregates  
25 platelets and converts circulating fibrinogen to insoluble  
fibrin, resulting in the formation of a blood clot.  
Thrombin also functions as a potent anticoagulant since it  
activates protein C zymogen to activated protein C, which in  
turn inhibits the generation of thrombin. Thus, through the

feedback regulation of thrombin generation, aPC functions as perhaps the most important down-regulator of blood coagulation resulting in protection against thrombosis.

The critical role of protein C in controlling  
5 hemostasis is exemplified by the increased rate of thrombosis in heterozygous deficiency, protein C resistance (e.g., due to the common Factor V Leiden mutation) and the fatal outcome of untreated homozygous protein C deficiency. Human activated protein C, both plasma-derived and  
10 recombinant, have been shown to be effective and safe antithrombotic agents in a variety of animal models of both venous and arterial thrombosis.

In current clinical practice, platelet inhibition, e.g., using aspirin (ASA), is well documented for efficacy  
15 in both prevention and treatment of thrombotic disease. Moreover, in conditions such as myocardial infarction and stroke, platelet inhibition has become the standard of care. However, the use of antiplatelet agents such as ASA increases the risk of bleeding, which limits the dose of the  
20 agent and duration of treatment. To block the effect of thrombin in fibrin formation, heparin remains the standard anticoagulant in the acute care setting. However, heparin has a narrow therapeutic index and is associated with significant bleeding risk especially in combination with  
25 antiplatelet agents.

Combination therapy with aspirin and a synthetic thrombin inhibitor, tissue plasminogen activator, or a monoclonal antiplatelet glycoprotein IIb/IIIa antibody has been studied in a canine coronary artery thrombosis model  
30 [Yasuda, et al., J Am Coll Cardiol., 16:714-22 (1990)]. Aspirin in combination with these agents prolonged the bleeding time and did not prevent reocclusion of the coronary artery. In addition, combination therapy has been proposed for aPC with thrombolytic agents such as tissue  
35 plasminogen activator, urokinase, or streptokinase [Griffin, et al., U.S. Patent No. 5,350,578]. However, these

combinations have not proved successful. Thus, there remains the need to identify an effective therapy for treating thrombotic disorders.

The present invention is the first to describe the combination of aPC with antiplatelet agents in the treatment of thrombosis. Accordingly, the present invention provides the use of aPC in combination with antiplatelet agents for the treatment of thrombotic disorders. This combination therapy results in enhanced efficacy in a variety of thrombotic disorders including, but not limited to, stroke, myocardial infarction, unstable angina, abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery. Furthermore, the combination of aPC and antiplatelet agents results in a synergy that will allow the reduction of the dosages of both aPC and the antiplatelet agents. The reduction of the dosages of the agents in combination therapy in turn results in reduced side effects such as increased bleeding liability often observed in combination anticoagulant/antiplatelet therapy.

The present invention provides a method of treating a thrombotic disorder in a patient in need thereof, which comprises administering to said patient a pharmaceutically effective amount of activated protein C in combination with an antiplatelet agent. Additionally, the invention provides a method of treating a thrombotic disorder in a patient in need thereof, which comprises administering to said patient a pharmaceutically effective amount of an antiplatelet agent and activated protein C such that an activated protein C plasma level of 10 ng/ml to less than 100 ng/ml is achieved.

For purposes of the present invention, as disclosed and claimed herein, the following terms are as defined below.

aPC or activated protein C whether recombinant or plasma derived. aPC includes and is preferably human

protein C although aPC may also include other species or derivatives having protein C proteolytic, amidolytic, esterolytic, and biological (anticoagulant or pro-fibrinolytic) activities. Examples of protein C derivatives are described by Gerlitz, *et al.*, U.S. patent No. 5,453,373, and Foster, *et al.*, U.S. patent No. 5,516,650, the entire teachings of which are hereby included by reference.

APTT - activated partial thromboplastin time.

HPC - human protein C zymogen.

10 r-hPC - recombinant human protein C zymogen, produced in prokaryotic cells, eukaryotic cells or transgenic animals.

r-aPC - recombinant human activated protein C produced by activating r-hPC *in vitro* or by direct secretion of the activated form of protein C from procaryotic cells, eukaryotic cells, or transgenic animals [Cottingham, WO97/20043] including, for example, secretion from human kidney 293 cells as a zymogen then purified and activated by techniques well known to the skilled artisan demonstrated in Yan, U.S. Patent No. 4,981,952, and, the entire teachings of which are herein incorporated by reference.

Zymogen - refers to secreted, inactive forms, whether one chain or two chains of protein C.

25 Antiplatelet agent - one or more agents alone or in combination which reduces the ability of platelets to aggregate. Agents understood and appreciated in the art include those cited in, for example, Remington, The Science and Practice of Pharmacy, Nineteenth Edition, Vol II, Pages 924-25, Mack Publishing Co., herein incorporated by reference. Such agents include but are not limited to aspirin (ASA), clopidogrel, ReoPro<sup>®</sup> (abciximab), dipyridamole, ticlopidine and IIb/IIIa antagonists.

35 Treating - describes the management and care of a patient for the purpose of combating the disease, condition, or disorder and includes the administration of a compound of the present invention to prevent the onset of the symptoms

or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

Continuous infusion - continuing substantially uninterrupted the introduction of a solution into a vein for  
5 a specified period of time.

Bolus injection - the injection of a drug in a defined quantity (called a bolus) over a period of time up to about 120 minutes.

Pharmaceutically effective amount - represents an  
10 amount of a compound of the invention that is capable of inhibiting a thrombotic disorder in mammals. The particular dose of the compound administered according to this invention will, of course, be determined by the attending physician evaluating the particular circumstances  
15 surrounding the case, including the compound administered, the particular condition being treated, and similar considerations.

Thrombotic disorder - a disorder relating to, or affected with the formation or presence of a blood clot  
20 within a blood vessel. Thrombotic disorders include, but are not limited to, stroke, myocardial infarction, unstable angina, abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery.

25 The present invention preferably relates to the treatment of thrombotic disorders with activated protein C in combination with an antiplatelet agent. The aPC used in such combination can be formulated according to known methods to prepare pharmaceutically useful compositions.  
30 The aPC will be administered parenterally to ensure its delivery into the bloodstream in an effective form by injecting the appropriate dose as continuous infusion for about 24 hours to about 144 hours.

In conjunction with treatment with an antiplatelet  
35 agent, the amount of aPC administered will be from about 4 mg/70 Kg/24 hours to 160 mg/70 kg/24 hours or an equivalent

designations of  $0.1 \text{ mg/m}^2$  to  $4 \text{ mg/m}^2$  or  $2 \text{ } \mu\text{g/kg/hr}$  to  $96 \text{ } \mu\text{g/kg/hr}$ . More preferably the amount of aPC administered will be about  $4 \text{ mg/70 Kg/24 hours}$  to  $120 \text{ mg/70 Kg/24 hours}$  or an equivalent designations of  $0.1 \text{ mg/m}^2$  to  $3 \text{ mg/m}^2$  or  $2.4 \text{ } \mu\text{g/kg/hr}$  to  $72 \text{ } \mu\text{g/kg/hr}$ . While more preferably the amount of aPC administered will be about  $4 \text{ mg/70 Kg/24 hours}$  to  $80 \text{ mg/70 kg/24 hours}$  or an equivalent designation of  $0.1 \text{ mg/m}^2$  to  $2 \text{ mg/m}^2$  or  $2.4 \text{ } \mu\text{g/kg/hr}$  to  $48 \text{ } \mu\text{g/kg/hr}$ . Even more preferably the amount of aPC administered will be about  $4 \text{ mg/70 Kg/24 hours}$  to  $60 \text{ mg/70 kg/24 hours}$  or an equivalent designations of  $0.1 \text{ mg/m}^2$  to  $1.5 \text{ mg/m}^2$  or  $2.4 \text{ } \mu\text{g/kg/hr}$  to  $36 \text{ } \mu\text{g/kg/hr}$ . Yet even more preferably the amount of aPC administered will be about  $10 \text{ mg/70 Kg/24 hours}$  to  $50 \text{ mg/70 kg/24 hours}$  or an equivalent designations of  $0.25 \text{ mg/m}^2$  to  $1.25 \text{ mg/m}^2$  or  $6 \text{ } \mu\text{g/kg/hr}$  to  $30 \text{ } \mu\text{g/kg/hr}$ . Still even more preferably the amount of aPC administered will be about  $20 \text{ mg/70 kg/24 hours}$  to  $40 \text{ mg/70 kg/24 hours}$  or an equivalent designations of  $0.5 \text{ mg/m}^2$  to  $1.0 \text{ mg/m}^2$  or  $12 \text{ } \mu\text{g/kg/hr}$  to  $24 \text{ } \mu\text{g/kg/hr}$ . The most preferable amount of aPC administered will be about  $40 \text{ mg/70 kg/24 hours}$  or an equivalent designation of  $1.0 \text{ mg/m}^2$  or  $24 \text{ } \mu\text{g/kg/hr}$ . The appropriate dose of aPC administered with an antiplatelet therapeutic will result in either an improved efficacy or reduction in dose of either agent or both.

The plasma ranges obtained from the amount of aPC administered will be  $2 \text{ ng/ml}$  to less than  $100 \text{ ng/ml}$ . The preferred plasma ranges are from about  $20 \text{ ng/ml}$  to  $80 \text{ ng/ml}$ . Most preferably plasma ranges are from about  $30 \text{ ng/ml}$  to about  $60 \text{ ng/ml}$  and still more preferably about  $50 \text{ ng/ml}$ .

Alternatively, the aPC will be administered by injecting one third of the appropriate dose per hour as a bolus injection followed by the remaining two thirds of the hourly dose as continuous infusion for one hour followed by continuous infusion of the appropriate dose for twenty-three



hours which results in the appropriate dose administered over 24 hours.

The phrase "in combination with" refers to the administration of antiplatelet agents with aPC either  
5 simultaneously, sequentially or a combination thereof. The administration of platelet inhibitors in combination with aPC will result in enhanced efficacy in a variety of thrombotic disorders including, but not limited to, stroke, venous thrombosis, myocardial infarction, unstable angina,  
10 abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery. The synergy also will result in ability to reduce the dosages of agents in combination therapy resulting in reduced side effects such as increases in bleeding liability often  
15 observed in combination anticoagulant/antiplatelet therapy.

The antiplatelet agent utilized and the appropriate dose level is understood and appreciated in the art. A skilled artisan recognizes the appropriate dose level to use for each antiplatelet agent to achieve a pharmaceutically  
20 effective amount for treating thrombotic disorders. Antiplatelet agents suitable for use under the present invention include, but are not limited to, clinically recognized and commercially available agents such as aspirin (ASA), clopidogrel, ReoPro<sup>®</sup> (abciximab), dipyridamole,  
25 ticlopidine and IIb/IIIa antagonists. The amount of the antiplatelet agent aspirin (ASA) administered in conjunction with aPC will be about 10 mg to 1000 mg, given once daily. The amount of the antiplatelet agent ticlopidine administered in conjunction with aPC will be about 50 mg to  
30 1250 mg, given two times daily (B.I.D.). The amount of the antiplatelet agent dipyridamole administered in conjunction with aPC will be about 15 mg to 500 mg, given four times daily. The amount of the antiplatelet agent clopidogrel administered in conjunction with aPC will be about 40 mg to  
35 1000 mg, given once a day. The amount of the antiplatelet

agent ReoPro<sup>®</sup> (abciximab) administered in conjunction with aPC will be about 0.025  $\mu\text{g}/\text{kg}/\text{min}$  to 1  $\mu\text{g}/\text{kg}/\text{min}$  given as an infusion for twelve hours. Alternatively, ReoPro<sup>®</sup> can be administered in conjunction with aPC as a bolus injection at about 0.05 mg/kg to 1.0 mg/kg. In addition, ReoPro<sup>®</sup> can be administered in conjunction with aPC as a bolus injection followed by an infusion for twelve hours. The amount of the IIb/IIIa antagonist utilized in combination with aPC will be about 0.1 mg/kg to about 100 mg/kg depending on the specific agent employed [see for example, Fisher, et al., US Patent NO. 5,618,843, the entire teaching of which is herein incorporated by reference]. One skilled in the art will be able to determine the appropriate dose level to use to achieve a pharmaceutically effective amount.

The aPC combined with the antiplatelet agents improves the antithrombotic effect of an antiplatelet agent alone. Thus, this combination therapy may reduce the therapeutic doses of aPC as well as reduce doses of antiplatelet agents required for therapeutic treatment of thrombosis, thereby avoiding the complications such as bleeding tendency, toxicity and general side effects of high doses of antiplatelet agents.

#### Preparation 1

##### Preparation of Human Protein C

Recombinant human protein C (r-hPC) was produced in Human Kidney 293 cells by techniques well known to the skilled artisan such as those set forth in Yan, U.S. Patent No. 4,981,952, the entire teaching of which is herein incorporated by reference. The gene encoding human protein C is disclosed and claimed in Bang, et al., U.S. Patent No. 4,775,624, the entire teaching of which is incorporated herein by reference. The plasmid used to express human protein C in 293 cells was plasmid pLPC which is disclosed in Bang, et al., U.S. Patent No. 4,992,373, the entire

teaching of which is incorporated herein by reference. The construction of plasmid pLPC is also described in European Patent Publication No. 0 445 939, and in Grinnell, *et al.*, 1987, Bio/Technology 5:1189-1192, the teachings of which are also incorporated herein by reference. Briefly, the plasmid was transfected into 293 cells, then stable transformants were identified, subcultured and grown in serum-free media. After fermentation, cell-free medium was obtained by microfiltration.

10 The human protein C was separated from the culture fluid by an adaptation of the techniques of Yan, U.S. Patent No. 4,981,952, the entire teaching of which is herein incorporated by reference. The clarified medium was made 4 mM in EDTA before it was absorbed to an anion exchange resin (Fast-Flow Q, Pharmacia). After washing with 4 column 15 volumes of 20 mM Tris, 200 mM NaCl, pH 7.4 and 2 column volumes of 20 mM Tris, 150 mM NaCl, pH 7.4, the bound recombinant human protein C zymogen was eluted with 20 mM Tris, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.4. The eluted protein 20 was greater than 95% pure after elution as judged by SDS-polyacrylamide gel electrophoresis.

Further purification of the protein was accomplished by making the protein 3 M in NaCl followed by adsorption to a hydrophobic interaction resin (Toyopearl Phenyl 650 M, 25 TosoHaas) equilibrated in 20 mM Tris, 3 M NaCl, 10 mM CaCl<sub>2</sub>, pH 7.4. After washing with 2 column volumes of equilibration buffer without CaCl<sub>2</sub>, the recombinant human protein C was eluted with 20 mM Tris, pH 7.4.

The eluted protein was prepared for activation by 30 removal of residual calcium. The recombinant human protein C was passed over a metal affinity column (Chelex-100, Bio-Rad) to remove calcium and again bound to an anion exchanger (Fast Flow Q, Pharmacia). Both of these columns were arranged in series and equilibrated in 20 mM Tris, 150 mM 35 NaCl, 5 mM EDTA, pH 6.5. Following loading of the protein, the Chelex-100 column was washed with one column volume of

the same buffer before disconnecting it from the series. The anion exchange column was washed with 3 column volumes of equilibration buffer before eluting the protein with 0.4 M NaCl, 20 mM Tris-acetate, pH 6.5. Protein concentrations of recombinant human protein C and recombinant activated protein C solutions were measured by UV 280 nm extinction  $E^{0.1\%}=1.81$  or 1.85, respectively.

### Preparation 2

#### 10           Activation of Recombinant Human Protein C

Bovine thrombin was coupled to Activated CH-Sepharose 4B (Pharmacia) in the presence of 50 mM HEPES, pH 7.5 at 4°C. The coupling reaction was done on resin already packed into a column using approximately 5000 units thrombin/ml resin. The thrombin solution was circulated through the column for approximately 3 hours before adding MEA to a concentration of 0.6 ml/l of circulating solution. The MEA-containing solution was circulated for an additional 10-12 hours to assure complete blockage of the unreacted amines on the resin. Following blocking, the thrombin-coupled resin was washed with 10 column volumes of 1 M NaCl, 20 mM Tris, pH 6.5 to remove all non-specifically bound protein, and was used in activation reactions after equilibrating in activation buffer.

25           Purified r-hPC was made 5 mM in EDTA (to chelate any residual calcium) and diluted to a concentration of 2 mg/ml with 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5. This material was passed through a thrombin column equilibrated at 37°C with 50 mM NaCl and either 20 mM Tris pH 7.4 or 20 mM Tris-acetate pH 6.5. The flow rate was adjusted to allow for approximately 20 min. of contact time between the r-hPC and thrombin resin. The effluent was collected and immediately assayed for amidolytic activity. If the material did not have a specific activity (amidolytic) comparable to an established standard of aPC, it was recycled over the thrombin column to activate the r-hPC to

completion. This was followed by 1:1 dilution of the material with 20 mM buffer as above, with a pH of anywhere between 7.4 or 6.0 (lower pH being preferable to prevent autodegradation) to keep the aPC at lower concentrations while it awaited the next processing step.

Removal of leached thrombin from the aPC material was accomplished by binding the aPC to an anion exchange resin (Fast Flow Q, Pharmacia) equilibrated in activation buffer (either 20 mM Tris, pH 7.4 or preferably 20 mM Tris-acetate, pH 6.5) with 150 mM NaCl. Thrombin passes through the column and elutes during a 2-6 column volume wash with 20 mM equilibration buffer. Bound aPC is eluted with a step gradient using 0.4 M NaCl in either 5 mM Tris-acetate, pH 6.5 or 20 mM Tris, pH 7.4. Higher volume washes of the column facilitated more complete removal of the dodecapeptide. The material eluted from this column was stored either in a frozen solution (-20°C) or as a lyophilized powder.

The amidolytic activity (AU) of aPC was determined by release of p-nitroaniline from the synthetic substrate H-D-Phe-Pip-Arg-p-nitroanilide (S-2238) purchased from Kabi Vitrum using a Beckman DU-7400 diode array spectrophotometer. One unit of activated protein C was defined as the amount of enzyme required for the release of 1  $\mu$ mol of p-nitroaniline in 1 min. at 25°C, pH 7.4, using an extinction coefficient for p-nitroaniline at 405 nm of 9620  $M^{-1}cm^{-1}$ .

The anticoagulant activity of activated protein C was determined by measuring the prolongation of the clotting time in the activated partial thromboplastin time (APTT) clotting assay. A standard curve was prepared in dilution buffer (1 mg/ml radioimmunoassay grade BSA, 20 mM Tris, pH 7.4, 150 mM NaCl, 0.02%  $NaN_3$ ) ranging in protein C concentration from 125-1000 ng/ml, while samples were prepared at several dilutions in this concentration range. To each sample cuvette, 50  $\mu$ l of cold horse plasma and 50  $\mu$ l

of reconstituted activated partial thromboplastin time reagent (APTT Reagent, Sigma) were added and incubated at 37 °C for 5 min. After incubation, 50  $\mu$ l of the appropriate samples or standards were added to each cuvette. Dilution buffer was used in place of sample or standard to determine basal clotting time. The timer of the fibrometer (CoA Screener Hemostasis Analyzer, American Labor) was started upon the addition of 50  $\mu$ l 37°C 30 mM CaCl<sub>2</sub> to each sample or standard. Activated protein C concentration in samples are calculated from the linear regression equation of the standard curve. Clotting times reported here are the average of a minimum of three replicates, including standard curve samples.

15

### Preparation 3

#### Formulation of Activated Protein C

A stable lyophilized formulation of activated protein C was prepared by a process which comprises lyophilizing a solution comprising about 2.5 mg/mL activated protein C, about 15 mg/mL sucrose, about 20 mg/mL NaCl, and a sodium citrate buffer having a pH greater than 5.5 but less than 6.5. Additionally, the stable lyophilized formulation of activated protein C comprises lyophilizing a solution comprising about 5 mg/mL activated protein C, about 30 mg/mL sucrose, about 38 mg/mL NaCl, and a citrate buffer having a pH greater than 5.5 but less than 6.5.

The ratio of aPC:salt:bulking agent (w:w:w) is an important factor in a formulation suitable for the freeze drying process. The ratio varies depending on the concentration of aPC, salt selection and concentration and bulking agent selection and concentration. Particularly, a ratio of about 1 part activated protein C to about 7.6 parts salt to about 6 parts bulking agent is preferred.

A unit dosage formulation of activated protein C suitable for administration by continuous infusion was prepared by mixing activated protein C, NaCl, sucrose, and

sodium citrate buffer. After mixing, 4 mL of the solution was transferred to a unit dosage receptacle and lyophilized. The unit dosage receptacle containing about 5 mg to about 20 mg of activated protein C, suitable for administering a dosage of about 0.01 mg/kg/hr to about 0.05 mg/kg/hr to patients in need thereof, was sealed and stored until use.

#### Example 1

##### Guinea Pig AV shunt model of thrombosis

10 Because platelet inhibition has proven clinical efficacy and aPC plays a critical role in controlling hemostasis, the possible synergy between aPC and ASA was examined in the Guinea Pig arterial/venous (AV) shunt thrombosis model. In this model of thrombosis, aPC has been shown to be an effective antithrombotic agent, causing dose-dependent inhibition of thrombus formation.

To examine the effect of aPC and aspirin, Guinea pigs (approximately 500 g) were anesthetized with 20 mg/kg Rompun and 125 mg/kg Ketaset, and a shunt was inserted connecting the right carotid artery and the left jugular vein. The shunt contained a cotton thread which stimulated thrombus formation. aPC was administered to inhibit thrombus formation with a bolus plus infusion dosing regimen (0.5 mg/kg bolus plus 3 mg/kg/hr). Additionally, whole blood aPTT was performed before and during the dosing regimen and circulating aPC plasma levels were measured by immunocapture amidolytic assay. Aspirin was administered at 10 mg/kg, intravenously, and in studies with heparin, a dose of 30 units/kg bolus followed by an infusion of 40 units/kg/hr was used. Bolus dosing was performed via cannulation of the left jugular vein and blood sampling was performed via cannulation of the right jugular vein. Blood samples were drawn at consecutive time points into 3.8% Na<sub>3</sub> citrate containing 300 mM Benzamidine HCl (9 volumes blood to 1 volume citrate/benzamidine solution). Blood volume was replaced in the animal with saline after each draw. Plasma

was separated by centrifugation and frozen at  $-20^{\circ}\text{C}$ . Plasma concentration of aPC was determined by an immunocapture amidolytic assay.

To examine potential antithrombotic synergism between  
5 aPC and the anti-platelet agent ASA, the dose of ASA was  
chosen to give near completed inhibition of platelet cyclo-  
oxygenase, as measured by inhibition of thromboxane B2  
release. As shown in Table 1, a dose of 10 mg/kg ASA  
effectively inhibited platelet cyclo-oxygenase, however it  
10 had no significant effect on thrombus weight. Using this  
dose of ASA, which caused no effect on thrombus weight,  
experiments in conjunction with aPC were performed. For  
comparative purposes, heparin, the standard clinical  
anticoagulant, was also compared. As shown in Table 2, the  
15 dose of aPC and heparin were chosen to each give  
approximately the same antithrombotic effect in the model  
(45.9 and 43.3% inhibition, respectively). The combination  
of ASA and aPC resulted in a significantly greater  
inhibition of thrombus formation than was observed with aPC  
20 alone ( $p=0.01$ ). In contrast, the combination of the  
anticoagulant heparin with ASA showed no significantly  
greater effect than that observed with heparin alone. These  
data demonstrate a synergy between anti-platelet agents and  
aPC, and suggest that: (a) patients on either ASA or other  
25 antiplatelet agents therapy will achieve increased efficacy  
by combination therapy with aPC, and (b) therapeutic doses  
of aPC may be reduced in presence of anti-platelet therapy.



Table 1

The effect of ASA on platelet Thromboxane B2 release and thrombus weight in the Guinea Pig AV-Shunt model. The data shows that despite near maximal inhibition of Thromboxane B2 release, ASA had no effect on thrombus weight.

Treatment	Thromboxane B2 Release	Thrombus Weight
Control (PBS)	638 ng/ml	38.3 ± 1.1 mg (n=10)
ASA (10 mg/kg)	10 ng/ml	36.4 ± 1.3 mg (n=8)

10

Table 2

Antithrombotic synergism between aPC, but not heparin, and ASA.

Treatment	Thrombus Weight* (minus ASA)	Thrombus Weight* (plus ASA)
Control	100 ± 3	95 ± 3
aPC	46 ± 5	27 ± 5 (p=0.01)
Heparin	43 ± 5	39 ± 6

\* % of control

15

Example 2

The Effect of aPC and a IIb/IIIa antagonist in the Guinea Pig AV Shunt Model of Thrombosis

To examine the effect of combination therapy of aPC and a representative synthetic IIb/IIIa receptor antagonist, the guinea pig AV shunt model of thrombosis as described in Example 1, was utilized. 2-([6-carboxy-n-hexyl]carboxamidyl)-5-amidino benzofuran trifluoroacetate prepared as described in Fisher, et al., US Patent NO. 5,618,843 was used. The combination of aPC and the IIb/IIIa antagonist resulted in a significantly greater inhibition of thrombus formation than was observed with aPC alone (Table 3). These

data demonstrate a synergy between aPC and a synthetic IIb/IIIa antagonist.

TABLE 3

5           Antithrombotic synergism between aPC and a IIb/IIIa antagonist.

Treatment	Thrombus Weight* (minus IIb/IIIa antagonist)	Thrombus Weight* (plus IIb/IIIa antagonist)
Control	100 ± 3	82 ± 3
aPC	46 ± 5	9 ± 1

\* % of control

10

Example 3

The Effect of aPC and abciximab in the  
Treatment of Thrombotic Disorders

Abciximab (ReoPro<sup>®</sup>) is an agent which inhibits platelet aggregation by binding to IIb/IIIa receptors on the platelet cell surface. Combination therapy with aPC and abciximab is effective in inhibiting thrombosis by down-regulating the blood coagulation process and inhibiting platelet aggregation. Abciximab is administered as a bolus injection at about 0.05 mg/kg to about 1.0 mg/kg followed by a continuous infusion of about 0.025 ug/kg/min to about 1 ug/kg/min for 12 hours. aPC is administered as a bolus injection followed by a continuous infusion or as a continuous infusion of about 2  $\mu$ g/kg/hr to about 96  $\mu$ g/kg/hr for about 24 to about 144 hours.

25           This combination therapy results in a synergy that is safer and more efficacious and reduces the dosages of both aPC and abciximab necessary to treat thrombotic disorders.

We claim:

1. A method of treating a thrombotic disorder in a patient in need thereof, which comprises, administering to  
5 said patient a pharmaceutically effective amount of activated protein C in combination with an antiplatelet agent.
2. The method according to Claim 1, wherein the  
10 patient is suffering from acute thrombotic stroke, venous thrombosis, myocardial infarction, unstable angina, abrupt closure following angioplasty or stent placement, or thrombosis as a result of peripheral vascular surgery.
- 15 3. The method according to Claim 2, wherein the amount of activated protein C administered is about 2  $\mu\text{g}/\text{kg}/\text{hr}$  to about 96  $\mu\text{g}/\text{kg}/\text{hr}$ .
- 20 4. The method of Claim 3 wherein the activated protein C is administered by continuous infusion for about 24 to about 144 hours.
- 25 5. The method according to Claim 3, wherein said antiplatelet agent is selected from a group consisting of aspirin (ASA), clopidogrel, abciximab, dipyridamole, ticlopidine, IIb/IIIa receptor antagonists or a combination thereof.
- 30 6. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA).

7. A method according to Claim 5, wherein said antiplatelet agent is ticlopidine.

5 8. A method according to Claim 5, wherein said antiplatelet agent is clopidogrel.

9. A method according to Claim 5, wherein said antiplatelet agent is abciximab.

10

10. A method according to Claim 5, wherein said antiplatelet agent is dipyridamole.

11. A method according to Claim 5, wherein said  
15 antiplatelet agent is a IIb/IIIa receptor antagonist.

12. A method of treating a thrombotic disorder in a patient in need thereof, which comprises administering to said patient a pharmaceutically effective amount of an  
20 antiplatelet agent and activated protein C such that an activated protein C plasma level of 10 ng/ml to less than 100 ng/ml is achieved.

13. The method of Claim 12, wherein said patient is  
25 suffering from acute thrombotic stroke, venous thrombosis, myocardial infarction, unstable angina, abrupt closure following angioplasty or stent placement, or thrombosis as a result of peripheral vascular surgery.

14. The method of Claim 12 wherein the activated protein C is administered by continuous infusion for about 24 to about 144 hours.

5 15. The method of Claim 12 wherein the activated protein C is administered first as a bolus then as a continuous infusion.

10 16. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA) and clopidogrel.

17. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA) and ticlopidine.

15 18. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA) and dipyridamole.

19. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA) and abciximab.

20

20. A method according to Claim 5, wherein said antiplatelet agent is aspirin (ASA) and IIb/IIIa antagonists.

25 21. Activated protein C in combination with an antiplatelet agent for use as a medicament for the treatment of thrombotic disorders.

22. The use of Claim 21, wherein the thrombotic disorder is acute thrombotic stroke, venous thrombosis, myocardial infarction, unstable angina, abrupt closure  
5 following angioplasty or stent placement, or thrombosis as a result of peripheral vascular surgery.

23. The use of claim 22, wherein the dosage of activated protein C is about 2  $\mu\text{g}/\text{kg}/\text{hr}$  to about  
10 96  $\mu\text{g}/\text{kg}/\text{hr}$ .

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11071

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC(6) : A61K 38/48, A01N 37/36  
 US CL : 424/94.64, 514/165  
 According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**  
 Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 424/94.64, 514/165

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 93/09807 A1 (SCRIPPS RESEARCH INSTITUTE) 27 May 1993, especially pages 8-9, page 11, pages 19-21, pages 27-28, Table 1, and pages 70-72.	1-7, 10, 12, 17, 21-23 ----- 8-9, 11, 13-16, 18-20
X -- Y	POPMA et al. Adjuncts to Thrombolysis for Myocardial Reperfusion. Ann. Intern. Med. 01 July 1991, Vol. 115, No. 1, pages 34-44, see entire document, especially Table 1.	1-7, 11-13, 17, 20-23 ----- 8-10, 14-16, 18-19
Y	REMINGTON. J.P. The Science and Practice of Pharmacy. Easton, PA: Mack Publishing Co. 1995, Nineteenth Ed., Vol. II, Chapter 54, pages 924-925, especially page 925.	1-23

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 21 AUGUST 1998	Date of mailing of the international search report 02 OCT 1998
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11071

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRUBER et al. Generation of Both Plasmin and Activated Protein C During Thrombolytic Therapy with Streptokinase. Blood. 15 November 1993, Vol. 82, No. 10, Suppl. 1, page 212a, Abstract No. 835.	1-23
Y	ESMON, C.T. The Regulation of Natural Anticoagulant Pathways. Science. 13 March 1987, Vol. 235, pages 1348-1352, see entire document.	1-23



**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US98/11071

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

**APS, INDEX BIOSCIENCE AND PHARMACOLOGY**

search terms: antiplatelet factor#, clopidogrel, ticlopidine, dipyridamole, aspirin, IIb/IIIa antagonist#, abciximab, activated protein c, apc, thrombo?