# Targeting activated protein C to treat hemophilia

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**Purpose of review**
Hemophilia is a debilitating disease, marked by frequent, painful bleeding events, joint deterioration and early death. All current treatments consist of i.v. infusions of replacement factor or other procoagulant factors, and are incompletely effective, due in part to the short half-lives of the proteins. An alternative approach is to rebalance hemostasis by inhibiting natural anticoagulant mechanisms. In this article, we explain why activated protein C (APC) is an appropriate and safe target for the treatment of hemophilia.

**Recent findings**
A serpin (serine protease inhibitor) was engineered to specifically inhibit APC and was found to rescue hemostasis in a hemophilia mouse model, even after a severe tail clip injury. However, APC is also anti-inflammatory and has cytoprotective activities, raising safety concerns over the use of an APC inhibitor to treat hemophilia. We summarize the molecular basis of the anticoagulant and signaling activities of APC to assess the potential impact of targeting APC.

**Summary**
We conclude that the signaling and anticoagulant functions of APC are in spatially and kinetically distinct compartments, and that it is possible to specifically inhibit the anticoagulant activity of APC. Targeting APC with a serpin is remarkably effective and may be safe for long-term prophylactic use in the treatment of hemophilia.

**Keywords**
activated protein C, endothelial protein C receptor, hemostasis, protease activated receptor-1, serpin

## INTRODUCTION

Hemostasis is a dynamic process involving procoagulant and anticoagulant factors operating simultaneously to detect and repair damage to the vascular endothelium, and to prevent inappropriate clot formation (Fig. 1). Perturbations affecting this balance cause bleeding or thrombosis, because of insufficient or excessive formation of the enzyme thrombin. Hemophilia is a debilitating bleeding disorder caused by deficiency or defects in factor (f) VIII (hemophilia A) or fIX (hemophilia B). Treatment usually consists of prophylactic or on-demand infusions of the missing factor, but a fraction of patients develop inhibitory antibodies and require the use of 'bypassing' agents. Both replacement and bypassing treatments work by bolstering procoagulant forces, but rebalancing can also be achieved by suppression of anticoagulants. In this review, we describe our reasons for targeting activated protein C (APC) to rebalance hemostasis in hemophilia; the remarkable efficacy of an engineered APC-specific inhibitor; and how compartmentalization of APC activities should allay concerns over safety.

## THE RATIONALE FOR TARGETING ACTIVATED PROTEIN C IN HEMOPHILIA

APC is activated from its precursor zymogen protein C by thrombin bound to the cofactor thrombomodulin on the surface of endothelial cells [1]. This event is accelerated 20-fold by the binding of protein C to the same cell surface via an interaction between its γ-carboxyglutamic acid (Gla) domain

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**Curr Opin Hematol** 2017, 24:446–452

DOI:10.1097/MOH.0000000000000364

## KEY POINTS

- APC is a powerful anticoagulant that limits thrombin production.
- Inhibition of APC restores normal hemostasis in hemophilia models.
- The anti-inflammatory and cytoprotective activities attributed to APC are distinct from its anticoagulant function.
- APC can be safely targeted to treat hemophilia.

and endothelial protein C receptor (EPCR) [2] (Fig. 2). Once formed, APC exerts its anticoagulant activity by proteolytically inactivating the cofactors

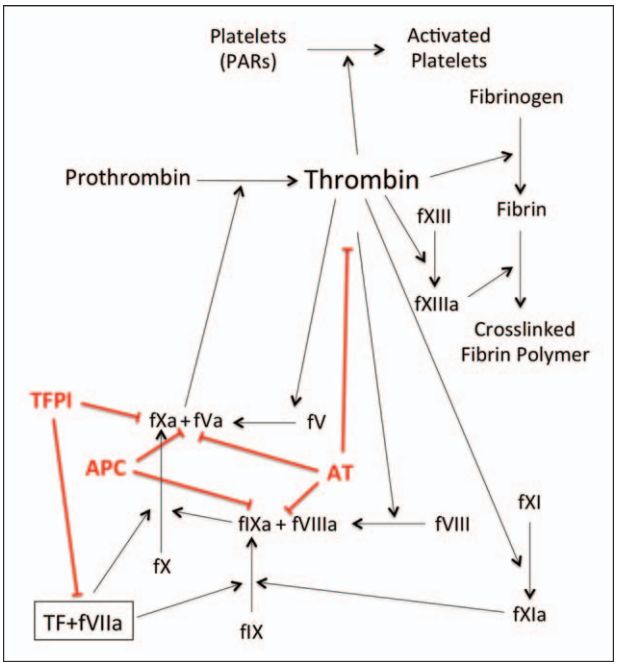


**FIGURE 1.** Schematic of the hemostatic network and the major endogenous anticoagulants. Stable hemostatic clots are formed by the action of thrombin on platelets and fibrinogen. Too little thrombin leads to bleeding and too much thrombin causes thrombosis. Clotting is initiated by the extrinsic Xase complex (TF-fVIIa) on subendothelial cells, rapidly producing fXa to feed the prothrombinase complex (fXa-fVa). The extrinsic Xase complex is inhibited by TFPI, and further fXa generation relies on the intrinsic Xase complex (fIXa-fVIIIa, deficient in hemophilia). The serpin antithrombin (AT) inhibits fXa and thrombin, and APC breaks down the intrinsic Xase and prothrombinase complexes. Treating hemophilia aims to improve thrombin generation by either replacing the missing factor or augmenting the concentration of other clotting factors. Alternative strategies include inhibition of TFPI and APC, or lowering the concentration of AT. TF, tissue factor; TFPI, tissue factor pathway inhibitor.

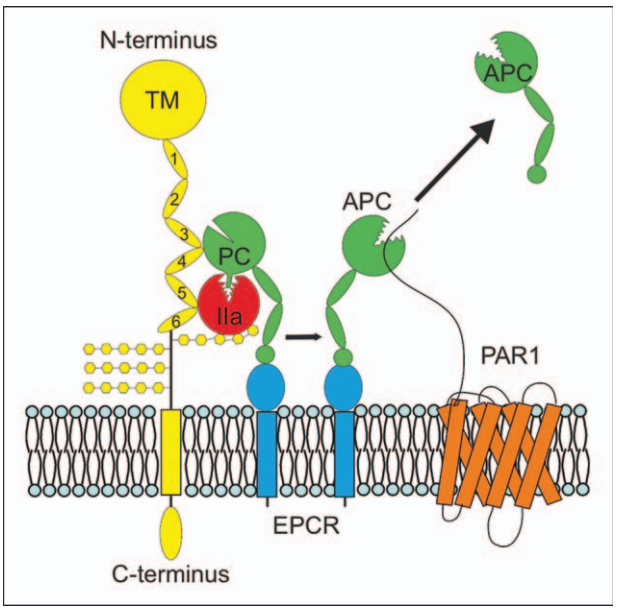


**FIGURE 2.** APC generation and signaling on endothelial cells. Thrombin (IIa) activates protein C (PC) after binding to thrombomodulin (TM) on endothelial cells (blue lipid bilayer). Thrombin interacts with TM via EGF-like domains 5 and 6, sometimes with the aid of chondroitin sulfate (hexagons). PC binds to endothelial protein C receptor (EPCR) via its Gla domain (green circle), thereby approximating PC to thrombin. Once formed, APC can cleave PAR1 to elicit anti-inflammatory and cytoprotective effects. However, APC dissociates rapidly and is replaced by the more abundant PC. APC, activated protein C; PAR1, protease activated receptor-1.

of the intrinsic Xase (fVIIIa) and prothrombinase (fVa) complexes (Fig. 1). Its potency is well established, but was recently demonstrated anew in a family with an unexplained bleeding disorder [3]. We found a c.1611C>A mutation in the thrombomodulin gene (p.Cys537Stop), causing a truncation within the C-terminal transmembrane helix and resulting in highly elevated plasma levels of soluble thrombomodulin. The elevation in circulating thrombomodulin caused systemic protein C activation, and resulted in trauma-induced and some spontaneous bleeding in affected family members. However, one carrier of the thrombomodulin mutation had no history of bleeding, and sequencing revealed that he was also a carrier of the $fV_{Leiden}$ mutation, which causes APC resistance.

The Leiden mutation, c.1691G>A (Arg506Gln), is present in 5% of whites and is the most common cause of thrombophilia [4]. The mutation is at the primary APC cleavage site in fVa and confers partial APC resistance by slowing the rate of inactivation by seven-fold (three-fold when the cofactor protein S is present) [5]. It is sufficiently common that $fV_{Leiden}$ has been found coincidentally in hemophilia

patients, and in some cases the bleeding severity appears milder than what would be expected based solely on factor levels (for review, see [6]). These clinical observations were supported by the rescue of hemostasis in hemophilia mice crossed with $fV_{Leiden}$ mice; however, the effect appeared to be limited to small vessels [7]. The magnitude of the effect of $fV_{Leiden}$ in ameliorating the clinical manifestations of hemophilia is somewhat controversial. However, observations are limited to $fV_{Leiden}$ heterozygotes in whom half of the fVa generated will be inactivated by APC at the normal rate, and half at a rate only 3–7-fold slower. Overall, the data suggest that if fVa can be protected from degradation by APC, normal hemostasis can occur in the absence of the intrinsic Xase complex (i.e. in hemophilia).

## AN ACTIVATED PROTEIN C-SPECIFIC SERPIN NORMALIZES BLEEDING IN HEMOPHILIA MODELS

APC is a trypsin-like serine protease, and its principal physiological inhibitor is the serpin (serine protease inhibitor) protein C inhibitor (PCI). However, PCI is highly promiscuous and is a better inhibitor of procoagulant proteases, such as thrombin, fXa, fXIa, fVIIa-TF, than of the anticoagulant APC [8]. Indeed, adding PCI to plasma to a final concentration of 5 μM extends the activated partial thromboplastin time (aPTT) from 1 min to unclottable after 5 min [9]. The challenge, therefore, was to alter the specificity of PCI so as to reduce the inhibition of procoagulant proteases while maintaining or improving inhibition of APC. The serpin mechanism of protease inhibition is well established (Fig. 3a) [10], and the principal determinant of serpin specificity is the amino acid composition of the reactive center loop (RCL). The RCL of the serpin acts as a pseudo-substrate for serine proteases by forming a recognition complex before hydrolysis begins. In the commonly used nomenclature, the bond that is targeted by a protease is between the P1 and P1′ residues, and amino acids to either side are numbered sequentially (with a ′ toward the C-terminus). Proteases typically utilize the sequences from P4 to P3′ to recognize their substrates, with specificity primarily determined by the residue at the P1 position. All clotting proteases, including APC, require a P1 arginine in their substrates, so to confer our desired specificity profile we mutated residues on either side of P1, guided by published structures of thrombin and APC [11▪] (Fig. 3b).

The active sites of thrombin and APC were most different at the S2 and S1′ sites (where P2 and P1′ side chains fit), with APC predicted to be more accommodating of large positively charged side chains. We found that mutating P2 and P1′ to lysine conferred a high degree of specificity for APC over thrombin, and when the mutated PCI was added to plasma at 5 μM the aPTT was unaffected. However, the mutated PCI was not an attractive drug candidate because of its low rate of APC inhibition ($280\,M^{-1}s^{-1}$, a two-fold reduction relative to wild-type) and short half-life (24 h).

We therefore decided to switch the template serpin to $\alpha_1$-antitrypsin (α1AT) which has a 5–7 day half-life. The P1 Arg 'Pittsburgh' variant has been reported in several people, most of whom had associated bleeding due to the change in specificity from neutrophil elastase to coagulation factors [12,13]. The Pittsburgh variant of α1AT also happens to be an excellent inhibitor of APC, with a rate constant of $\sim 1 \times 10^5\,M^{-1}s^{-1}$. When the flanking Lys mutations were made at the P2 and P1′ positions, a similar gain in specificity was obtained with no increase in aPTT observed when spiked into plasma. Importantly, when the mutated α1AT (SerpinPC) was added to normal or hemophilia plasma (in the presence of thrombomodulin to activate the protein C system), it rescued thrombin generation in a dose-dependent manner. The effect of SerpinPC on hemostasis was assessed in a hemophilia mouse model, monitoring clot formation by intravital microscopy after laser-induced injury and blood loss after tail clip. With both challenges, we observed correction of hemostasis. These results demonstrated that inhibition of the protein C anticoagulant pathway by an APC-specific serpin is effective at restoring hemostasis *in vivo* in the absence of the intrinsic Xase complex, and may be useful in the treatment of hemophilia.

## ACTIVATED PROTEIN C SIGNALING IS DEPENDENT ON ENDOTHELIAL PROTEIN C RECEPTOR AND PROTEASE ACTIVATED RECEPTOR-1

In addition to its anticoagulant function, APC also exerts important anti-inflammatory, antiproliferative and cytoprotective activities that have been demonstrated *in vitro* and in animal models. Indeed, recombinant APC was approved as a treatment for sepsis (marketed by Eli Lilly as Xigris), although it was later withdrawn after a placebo-controlled clinical trial (PROWESS-SHOCK) failed to demonstrate efficacy [14]. It is therefore important to consider whether inhibition of APC might have unwanted proinflammatory effects by reviewing what is known about how APC exerts its signaling activities.

As mentioned above, EPCR binding to protein C accelerates the formation of APC, thereby exerting an anticoagulant function. However, EPCR is

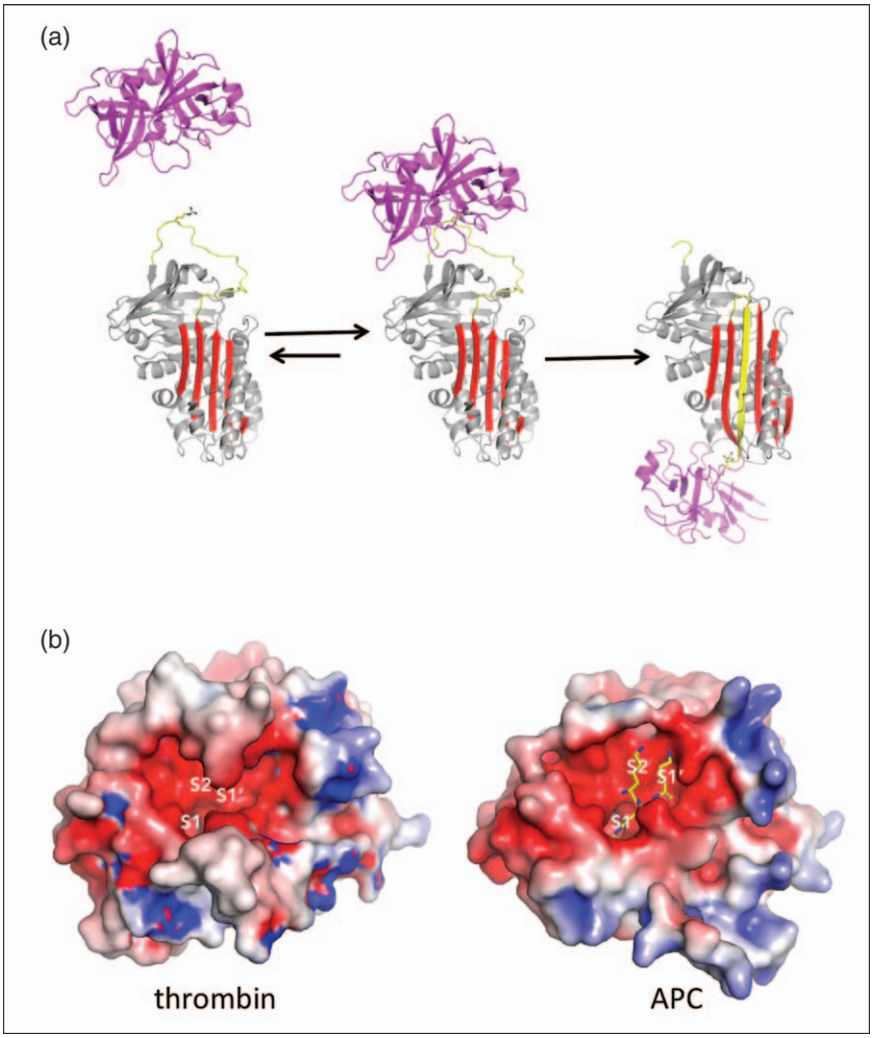


**FIGURE 3.** The serpin mechanism of protease inhibition requires reactive center loop accommodation into the protease active site. (a) Native serpins are composed of two major features required for protease inhibition, β-sheet A (red) and the reactive center loop (RCL, yellow). Inhibition of a protease (magenta) depends on recognition of the P1 residue (sticks) and surrounding RCL residues as a substrate (middle). At the acyl-enzyme stage of hydrolysis, the covalently attached protease is flung to the opposite pole of the serpin through incorporation of the RCL into β-sheet A (right). (b) The structures of thrombin (left) and APC are shown in the standard orientation with their surfaces colored according to electrostatics (red negative, blue positive). The subsites that interact with the P2, P1 and P1′ residues of a serpin (S, indicated) are different between the two proteases. This was exploited by engineering a serpin with Lys-Arg-Lys sequence (yellow sticks) to confer specificity for APC over thrombin. APC, activated protein C.

absolutely essential for all of the signaling activities attributed to APC, by localizing APC to the endothelial cell surface where it can cleave and activate the G-protein coupled receptor, protease activated receptor 1 (PAR-1) (Fig. 2). The dependence of APC signaling on both EPCR and PAR-1 was shown *in vitro* [15] and in mouse models [16]. APC cleavage of PAR-1 produces anti-inflammatory and cytoprotective effect, but paradoxically thrombin cleavage of PAR-1 (25 000-times faster than APC) has proinflammatory, proapoptotic effects and reduces endothelial barrier function (reviewed in [17]). Since thrombin, as the protein C activator, is necessarily present at the time APC is formed on endothelial cells, it is unclear which PAR-1 signaling pathway is dominant with endogenous proteins in the normal physiological setting. This issue was partially resolved by some recent studies by Ray Rezaie's group, demonstrating that occupancy of EPCR by protein C or APC is sufficient to elicit protective signaling, even if cleavage of PAR-1 is mediated by thrombin [18,19,20▪▪]. It was concluded that in the physiological setting where EPCR is occupied by protein C or APC, PAR-1 cleavage by either thrombin or APC will elicit anti-inflammatory and cytoprotective effects.

 

## PROTEIN C DEFICIENCY

Protein C deficiency in humans is associated with a 10-fold increase in risk of early and recurrent venous thrombosis, and homozygous deficiency causes neonatal purpura fulminans, a life-threatening condition characterized by microvascular thrombosis. Similar effects are seen in animal models, with full deficiency in mice leading to neonatal death caused by disseminated intravascular coagulation (DIC) [21]. The importance of the protein C levels in thrombosis is therefore well established. To investigate the importance of endogenous protein C in inflammation, partial knockout mice were made with levels ranging from 1 to 18% of normal [22,23]. Mice with levels below 3% were prone to DIC, similar to complete knockouts. Surviving mice with low protein C levels were found to be highly susceptible to lipopolysaccharide (LPS) challenge, exhibiting increased inflammatory markers and signs of DIC. In contrast, mice with levels approximately 18% of normal were protected from low-dose LPS challenge. It is interesting to note that warfarin treatment typically reduces the amount of correctly processed Gla domain to about 20% for vitamin K-dependent coagulation factors, including protein C, and its use has not been associated with any inflammatory disease.

## SELECTIVE INHIBITON OF ACTIVATED PROTEIN C ACTIVITIES

To investigate the relative importance of the anticoagulant and anti-inflammatory effects of protein C/APC in mice, anti-APC monoclonal antibodies were generated by the Esmon group [24]. MAPC1591 was found to bind specifically to APC over protein C and inhibit only its anticoagulant function, presumably by sterically interfering with binding to its substrate fVa. MAPC1591 did not, however, inhibit endothelial cell binding, consistent with an epitope on the catalytic domain of APC. In contrast, MPC1609 did not distinguish between protein C and APC, and completely blocked binding to endothelial cells, suggesting an epitope on the Gla domain. Both antibodies inhibited the anticoagulant activity of APC in a one-stage coagulation assay. However, only MPC1609 blocked the cytoprotective effect of APC *in vitro*, consistent with previous studies showing the dependence of EPCR binding for APC signaling. These antibodies were then given to mice at a dose of 10 mg/kg, followed by administration of a sublethal dose of LPS. All MPC1609-treated mice died within a 3-day window, whereas all of the MAPC1591 and vehicle-treated mice survived. Serum interlukin-6 (IL-6) and kidney function markers [blood urea nitrogen (BUN) and creatinine] were elevated with MPC1609 treatment, whereas none were elevated in MAPC1591-treated group. Indeed, IL-6, creatinine and BUN levels were even lower in the MAPC1591 group than vehicle-treated controls, indicating a possible protective effect of inhibiting the anticoagulant activity of APC in this model. Thrombin-antithrombin complex levels were higher than control in the MAPC1591-treated animals, confirming effective inhibition of APC anticoagulant activity. Similar results were obtained with these antibodies in mouse models of gram-negative pneumosepsis [25] and renal ischemia reperfusion injury [26▪]. The conclusion from these studies was that the anticoagulant function of endogenous APC is not required for its anti-inflammatory or cytoprotective activities. Treatment of hemophilia by inhibiting APC should therefore be safe, provided that the inhibitor selectively targets its anticoagulant function.

## COMPARTMENTALIZATION OF ACTIVATED PROTEIN C ACTIVITIES

The signaling activities of APC are dependent on binding to EPCR on endothelial cells, and the anticoagulant activity of APC requires release from EPCR and diffusion to the site where clotting is occurring. If PAR-1 cleavage by APC (and not thrombin) is essential for its signaling activities, administration of an APC inhibitor would, in theory, inhibit both its anticoagulant and anti-inflammatory functions. However, provided that APC inhibition is slower than its release from EPCR on which it was formed, then only its anticoagulant function can be affected.

Protein C and APC bind to EPCR with identical affinity (Kd of ~30 nM) [27], and as protein C circulates at a higher concentration than APC (70 nM vs. 40pM) [28], once APC dissociates from EPCR it will not reassociate, limiting its activity to anticoagulation. So, we can think of APC as existing in two compartments: the EPCR-bound signaling pool and the circulating anticoagulant pool. The key to ensuring selective inhibition of the anticoagulant pool of APC is to tailor the rate of inhibition so that it is slower than the rate of dissociation from EPCR. The dissociation rate of APC from EPCR is reported to be $0.09\ s^{-1}$ [29], giving newly formed APC a residence half-life of less than 8 s. SerpinPC inhibits APC with a second-order rate constant of approximately $15\,000\ M^{-1}\ s^{-1}$, and would require a concentration of 5 μM (equivalent to a dose of more than 10 mg/kg) to achieve a $t_{1/2}$ of inhibition of 8 s. Therefore, provided that efficacy can be demonstrated in hemophilia at doses below 10 mg/kg, there should be no effect on APC-mediated signaling. The LPS

studies using MAPC1591 were conducted at a dose of 10 mg/kg (about 1 μM peak concentration), and antibodies typically bind their targets with rate constants of approximately $10^5\,M^{-1}\,s^{-1}$, which translates to a $t_{1/2}$ of inhibition of 7 s. It may, therefore, be possible to safely exceed doses of an inhibitor that would theoretically inhibit APC before it dissociates from EPCR. This makes sense because cleavage of PAR1 must occur faster than dissociation of APC from EPCR. Indeed, a dose of 20 mg/kg MAPC1591 was 'safe' in the renal ischemia reperfusion model [26▪]. On the basis of studies from the Rezaie group, it is also possible that inhibition of the proteolytic activity of APC will have no effect on signaling, even at infinitely high doses of an inhibitor, as protective signaling can be achieved by thrombin cleavage of PAR-1 so long as protein C/APC is bound to EPCR.

## CONCLUSION

Hemophilia treatment has remained essentially unchanged for over 100 years; blood transfusions became plasma infusions became factor infusions. Safety was greatly improved with the introduction of recombinant factors, but efficacy was still limited by short half-lives. Recently, there has been a surge in innovation in the hemophilia space, including gene therapy, an fVIIIa-mimicking bispecific antibody, a small interfering RNA molecule against antithrombin and antibodies against tissue-factor pathway inhibitor. The latter two approaches aim to rebalance hemostasis by reducing intrinsic anticoagulant activity. Why then has no APC inhibitor been developed? One explanation is the fear of affecting its anti-inflammatory activities. It may also have been unclear how effective an APC-inhibitor would be in treating hemophilia, as reports of amelioration of clinical severity with coinheritance of $fV_{Leiden}$ are mixed, and in mouse models $fV_{Leiden}$ only partially rescues hemostasis. We created an APC-specific serpin (SerpinPC) and found that it rescued hemostasis in an intravital microscopy experiment and in the more severe tail-clip model using hemophilia B mice. We have since produced mammalian-expressed SerpinPC and have demonstrated efficacy at very low doses in hemophilia A mice (unpublished data). The safety concerns, however, persist, and although the effect of long-term administration of an APC inhibitor on inflammation is difficult to predict, it is clear that the anticoagulant and anti-inflammatory functions of APC are executed by two separate pools, circulating and EPCR-bound. The template of SerpinPC, $\alpha_1$AT, does not bind to endothelial cells, and does not affect the binding of protein C/APC to EPCR. SerpinPC is therefore unlikely affect the anti-inflammatory functions of APC, even at high doses, but this will need to be tested in LPS-challenged mice.

### Acknowledgements

*S.G.I.P. was supported by a Wellcome Trust studentship.*

### Financial support and sponsorship

*S.G.I.P. is currently supported by a grant from Cambridge University Hospital Trust.*

### Conflicts of interest

*J.A.H., T.P.B. and S.G.I.P. have shares in ApcinteX Ltd, a company founded to develop SerpinPC.*

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