Apixaban suppresses the release of TF-positive microvesicles and restrains cancer cell proliferation through directly inhibiting TF-fVIIa activity

Apixaban inhibits fVIIa and prevents $TF^{+}MV$ release

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Summary

What is known on this topic

- Cancer cells release tissue factor-positive microvesicles (TF^+MV) in response to PAR2 activation
- Apixaban and Rivaroxaban competitively inhibit fXa activity

What this paper adds

- Apixaban and Rivaroxaban reduce the release of TF^+MV through the activation of PAR2 by fXa
- Cellular TF-fVIIa complex also induces auto-activation of PAR2 on cancer cells, resulting in TF^+MV release and cell proliferation
- Apixaban competitively inhibits the proteolytic activity of fVIIa with similar kinetics to that of fXa
- Apixaban reduces cancer cell proliferation by inhibiting TF-fVIIa activity

Summary

The activation of protease activated receptor (PAR)-2 by factor Xa (fXa) promotes the release of tissue factor-positive microvesicles (TF^+MV), and contributes to proliferation in cancer cells. This study examined the ability of direct oral anticoagulants (DOAC), Apixaban and Rivaroxaban, to inhibit the release of TF^+MV from two cell lines (MDA-MB-231 & AsPC-1) as well as cell proliferation.

Activation of the cells with fXa (10 nM) enhanced the release of TF^+MV but was suppressed in the presence of either DOAC. These microvesicles were found to contain fVIIa, but not fXa. Incubation of cell lines with Apixaban (1.8 µM) but not Rivaroxaban (1.8 µM), in the absence of fXa decreased the release of TF^+MV below that of resting cells, in a PAR2 dependent manner. Furthermore, incubation with Apixaban reduced the proliferation rate in both cells lines. Incubation of purified fVIIa with Apixaban but not Rivaroxaban resulted in complete inhibition of fVIIa proteolytic activity as measured using two fVIIa chromogenic substrates. Pre-incubation of the cells with an inhibitory anti-fVIIa antibody, with Apixaban or the blocking of PAR2 suppressed the release of TF^+MV to a comparable level, and reduced cell proliferation but the effect was not cumulative.

This study has established that the activation of PAR2 by TF-fVIIa complex is the principal mediator in augmenting the release of TF^+MV as well as cancer cell proliferation. Importantly, for the first time we have shown that Apixaban selectively inhibits the proteolytic activity of fVIIa as well as the signalling arising from the TF-fVIIa complex.

Keywords : Direct oral anticoagulants, Factor VIIa, Microvesicles, Protease-activated receptor-2, Tissue factor

Introduction

The bidirectional association between coagulation and cancer has been established [1]. Advanced malignancies are known to cause a wide range of thrombotic diseases including venous thrombosis, pulmonary embolism and disseminated intravascular coagulation [2] but patient survival may be improved by the administration of anticoagulants [3,4]. Tissue factor (TF) has recently been shown to be an interlinking molecule between the coagulation and cancer and the correlation between levels of TF expression and a poor overall prognosis has been demonstrated in a number of different cancer types [5-7]. Furthermore, cancer cells are capable of releasing large quantities of TF-positive microvesicles (TF^+MV) following activation [8,9]. These TF^+MV circulate within the bloodstream and are suggested to participate in widespread thrombosis [10-13] as well as causing damage to the vascular endothelium [14], although a direct elevation of TF^+MV does not explain the cancer-associated thrombosis across different tumour types. A possible trigger for the activation of cancer cells is likely to occur when the cells come into contact with blood components. Coagulation proteases are known to cleave a family of receptors called protease activated receptors (PAR) on the surface of cells. Among these receptors, PAR2 is known to be cleaved by factor Xa alone, and also by the TF-fVIIa-fXa ternary complex [15,16]. PAR2 has also been shown to be a target for the proteolytic activity of TF-fVIIa [15]. The upregulation of PAR2 protein in cancer cells has also been liked to aggressive cancer phenotypes [17-20]. The activation of PAR2 on the surface of various cancer cell types leads to the incorporation of TF within microvesicles [21] and also can promote increased cellular proliferation, invasion and migration, as well as the expression of IL-8 and VEGF [16,17,22,23]. More recently, a number of studies have demonstrated the induction of microvesicle release through TF-mediated PAR2 activation and have studied the underlying mechanisms [24-28].

Direct Oral Anticoagulants (DOAC) are a recent group of anticoagulants with a fast onset of action and have the advantage of not needing regular monitoring by blood testing

[29]. Apixaban and Rivaroxaban are two such compounds which function by directly inhibiting the proteolytic activity of fXa, acting on both the circulating and clot-bound fXa [30,31]. In this study, we report the contribution of TF-fVIIa as a major activator of PAR2 in cancer cells, resulting in the release of TF^{+}MV and enhancement of cancer cell proliferation. In addition, we have identified a novel and surprising property of Apixaban in suppressing this mechanism by directly inhibiting the proteolytic activity of fVIIa.

Material and methods

Cell culture and determination of cell numbers

Cell lines were selected on the basis TF expression and microvesicle release, and not on the basis of tissue of origin [8]. MDA-MB-231 breast cancer cell line (ATCC, Teddington, UK) were cultured in DMEM and AsPC-1 pancreatic cancer cell line (ATCC) were cultured in RPMI-1640. All media were supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) to ensure the lack of any functional enzymes. Human dermal blood primary endothelial cells (HDAEC), devoid of endogenous TF were cultured in MV media containing 5% (v/v) FCS and growth supplements (PromoCell, Heidelberg, Germany). Unless otherwise stated, the cells were adapted to serum-free medium prior to use in the experiments. Cell numbers were determined using the crystal violet staining procedure and interpreted from a standard curve as previously described [32,33].

Preparation of test reagents

Apixaban and Rivaroxaban were obtained as pure compounds from Bistrol Myers Squibb (New York, USA) and Bayer (Leverkusen, Germany) respectively and used at the approximate therapeutic range (0.18-1.8 µM). The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate-buffered saline (PBS) pH 7.4, to 4 mg/ml working stock solutions. Appropriate controls using similarly diluted DMSO were used alongside, as controls. Protease activated receptor 2-agonist peptide (PAR2-AP; SLIGKV) was synthesised (Severn Biotech Ltd, Kidderminster, UK) and used at a final concentration

of 20 µM. Coagulation factors Xa and VIIa (Enzyme Research Laboratories, Swansea, UK) were diluted stepwise to the required concentrations. Blocking antibodies against PAR2 (SAM11) and PAR1 (ATAP2) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and were incubated with the cells at 20 µg/ml to block PAR2 and PAR1 respectively [34]. An inhibitory polyclonal rabbit anti-human fVIIa antibody was obtained from Abcam (Cambridge, UK) and incubated with the cells at 20 µg/ml, which was optimised beforehand.

Preparation of the TF-containing microvesicles

To generate the TF-containing microvesicles, MDA-MB-231 and AsPC-1 cells were propagated in 25 cm^2 flasks. The cells were washed with PBS and adapted to respective serum-free medium, for 2 h. The released microvesicles were then prepared from the conditioned media by ultracentrifugation according to described procedures [35]. The microvesicles were then washed with PBS and collected again. In other experiments, the cells were activated by incubation with fXa (10 nM) or PAR2-activating peptide (SLIGKV; 20 µM) for 30 min prior to collection. In variations of the experiments, the cells were supplemented with combinations of PAR2-blocking antibody (SAM11; 20 µg/ml) or PAR1-blocking (ATAP2; 20 µg/ml) or an IgG isotype (20 µg/ml), together with Apixaban (0-1.8 µM), Rivaroxaban (0-1.8 µM), or the DMSO vehicle control.

Analysis of the TF-containing microvesicles

The functional density of the released of microvesicles was determined using the Zymuphen MP-assay kit (Hyphen BioMed/Quadratech, Epsom, UK) and the microvesicle density determined from the standards provided by the kit. The released microvesicle-associated TF antigen was measured using the Quantikine TF-ELISA kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The procoagulant activity of the purified microvesicles was measured using calibrated automated thrombogram (CAT) assay. The CAT analysis was performed in normal plasma and repeated in fVII-deficient plasma (Hyphen BioMed/Quadratech, Epsom, UK). In some experiments, the microvesicles were

pre-incubated with an inhibitory mouse anti-human TF antibody (HTF-1; 20 µg/ml) or a control mouse isotype IgG (20 µg/ml; New England Biolabs, Hitchin, UK). For comparison, plasma was activated with Innovin thromboplastin reagent (Dade Behring, Deerfield, USA) at a range of 0-10 U/ml (1 U/ml = 1.3 ng/ml), and coagulation was also confirmed using a thrombin calibrator (Stago, Reading, UK). The 'lag time' to the onset of thrombin generation for each sample was compared with those of the Innovin standard to determine the relative TF activity within the microvesicle samples.

Analysis of the fVIIa and fXa antigen and activity

The presence of fX and fVII antigen was detected by western blot analysis. The samples were separated by 12% (w/v) SDS-PAGE, transferred onto nitrocellulose membranes, blocked with TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20). The membranes were then probed with a mouse monoclonal anti-fX antibody (156106), or a rabbit polyclonal anti-fVII antibody (R&D Systems), both diluted 1:2000 (v/v) in TBST. The membranes were developed with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Santa Cruz) respectively, diluted 1:4000 (v/v) and bands were visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega, Southampton, UK). Factor VII/VIIa antigen levels were determined using the Assaymax FVII-ELISA kit (Assaypro/Universal Biologicals Ltd., Cambridge, UK) according to the manufacturers' instructions.

Microvesicle and cell surface TF-fVIIa activity was measured by modification of previously described procedures [36,37]. Microvesicles were used directly and cells (5×10^4) were washed with PBS prior to assaying. The samples were incubated with fVIIa (10 nM; Enzyme Research Labs, Swansea, UK) in HEPES-buffered saline (HBS) pH 7.4, containing 1% (w/v) bovine serum albumin (BSA) and 5 mM $CaCl_2$, together with fX (100 nM) and the fXa substrate (0.2 mM; Hyphen) diluted in the same buffer (200 µl). The samples were incubated for 60 min to develop the colour. Aliquots (150 µl) were then transferred to a 96-well plate containing 2% (v/v) acetic acid (50 µl) and the absorptions measured immediately

at 410 nm. The amount of fXa generated was determined using a standard curve prepared using fXa (0-20 nM; Enzyme Research Labs). To detect microvesicle-associated fVIIa activity, in some experiments fVIIa was omitted from the above procedure. The absorption measurements were compared to a set of controls prepared using a range of fVIIa (0-10 nM) which were supplemented with recombinant TF (10 U/ml) and analysed for fXa-generation potential as above. The activity of fVIIa was also measured directly using Pefachrome® FVIIa substrate (LOXO, Dossenheim, Germany), as well as a novel and specific chromogenic substrate for factor VIIa (NH2-Asn-Leu-Thr-Arg-pNA) both of which were used at a range of 0.2-1.6 mM final concentration. Microvesicle-associated fVIIa activity and the ability of the Apixaban (1.8 µM) and Rivaroxaban (1.8 µM) to inhibit the purified fVIIa (50 nM) and microvesicle-associated fVIIa was examined.

Analysis of the expression of fVII and IL-8 mRNA

The expression of fVII mRNA and IL-8 mRNA was measured by RT-PCR as previously described [31]. Total RNA was extracted using the Ribozol solution (VWR, Lutterworth, UK). The expression of fVII mRNA or IL-8 was measured by GoTaq® 1-Step RT-qPCR System (Promega, Southampton, UK) using QuantiTect primers for fVII, IL-8 and β-actin (Qiagene, Manchester, UK) and relative amounts determined using as a reference.

Suppression of the expression of fVII and PAR2 by siRNA knock down

In order to suppress the expression of fVII and PAR2, cells were transfected with a specific set of Silencer®-siRNA (5 pmol; Life Technologies, Paisley, UK) to suppress the expression of fVII and PAR2 respectively, or transfected with a comparable set of control siRNA (5 pmol; Life Technologies) prior to activation. The concentration of each siRNA was optimised by examining the expression of each protein separately beforehand.

Approximation of binding of Apixaban to fVIIa

The crystal structures of fVIIa (4ylq), fXa (2P16) and Apixaban (GG2) were obtained on Brookhaven format (PDB) and used to estimate the location and efficiency of binding using the Autodock 4v2.6. The Autodock graphical interface AutoDockTools 1.5.6 was used, the

polar hydrogens were retained and partial charges added to the proteins using the Gasteiger charges. The search space was limited to an area of 20 × 20 × 20 Å, centred around the hydroxyl group of Ser195 in the enzymatic site of fVIIa and fXa. For each enzyme, 25 × ligand orientations (poses) were examined and ranked according to the scoring-function.

Statistical analysis

All data represent the calculated mean values from the number of experiments stated in each figure legend ± the calculated standard error of the mean. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA). Significance was determined using one-way ANOVA (analysis of variance) and Tukey's honesty significance test or where appropriate, by paired t-test.

Results

Cancer cell-derived microvesicles contain functional TF and fVIIa

Throughout the study, microvesicles were purified from the serum-free conditioned media, from resting MDA-MB-231 and AsPC-1 cell lines due to high expression of TF and microvesicle release, and was irrespective of tissue of origin. Measurement of procoagulant activity by CAT analysis showed that the microvesicles contained functional TF which was inhibited on pre-incubation with HTF-1 antibody (Figure 1A-C). Moreover, the qualitative analysis of the microvesicles showed that these possessed endogenous fXa-generation potential without the need for additional fVIIa (Figure 1D), and were also functional when examined in fVII-deficient plasma (Figure 1E-G) but not in fX-deficient plasma (not shown). Examination of the fVIIa by western blot indicated the presence of active fVIIa in both cell lines, exhibiting multiple bands (Figure 1H) which have previously been attributed to the presence of glycosylation variants [38,39]. The extrahepatic expression of fVII has been demonstrated in a number of cell lines from various tissues [40,41]. Moreover, the examination of MDA-MB-231 cell line with a monoclonal antibody, indicated the low level expression of the zymogen form of fVII [42]. However, our previous [43] and current

examination of cell lines including MDA-MB-231 cells demonstrates the presence of heavy and light chains of the activated form of fVIIa. The expression of fVIIa in MDA-MB-231 cell line was also corroborated by analysing the expression of fVII mRNA in the cells by RT-PCR [43] but was lower in AsPC-1 cell line. Moreover, no detectable endogenous fXa antigen was present. Furthermore, incubation of the cells, or the derived microvesicles with the fXa-chromogenic substrate indicated the lack of endogenous fXa activity (<50 pM fXa activity).

Apixaban directly inhibits the proteolytic activity of fVIIa

Incubation of purified fXa (10 nM) with either Apixaban (1.8 µM) or Rivaroxaban (1.8 µM) resulted in maximal inhibition of fXa activity as measured using the fXa-chromogenic substrate (Figure 2A). In addition, following supplementation of the cells with Apixaban or Rivaroxaban, the purified microvesicles were washed with PBS and purified again by ultracentrifugation. To ensure the removal of residual DOAC, the resultant microvesicles were incubated with fXa (10 nM) and the activity measured using a fXa-chromogenic substrate. The washed microvesicles did not appear to possess any anti-fXa activity against purified fXa, precluding any Apixaban or Rivaroxaban carry over by the microvesicles (Figure 2B). Incubation of Perfachrome substrate with either fVIIa or fXa resulted in the colour production (Figure 2C). In contrast, the designed chromogenic substrate (NH2-Asn-Leu-Thr-Arg-pNA; 0.4 mM) was activated by purified fVIIa but not purified fXa. Importantly, Apixaban (1.8 µM) but not Rivaroxaban (1.8 µM) was capable of inhibiting purified fVIIa (5 nM) (Figure 2D). This level of inhibition was comparable to that achieved following pre-incubation of the purified fVIIa with an inhibitory polyclonal anti-fVII antibody. Moreover, the inclusion of TF (1 U/ml) did not alter the inhibitory influence of Apixaban towards fVIIa (Figure 2E). The rate of reaction for the purified fVIIa (50 nM) was examined using the Perfachrome substrate (0.4-1.6 mM), by measuring the rate of change in absorption (410 nm) over 60 min. These analyses were carried out in the presence and absence of Apixaban (Figure 2F) which indicated competitive inhibition of fVIIa by Apixaban (Figure 2G), with a calculated Ki of 4.4 nM. Finally, incubation of microvesicles purified from the conditioned

media of MDA-MB-231 cells with Apixaban but not Rivaroxaban eliminated the microvesicle-associated fVIIa activity which was measured using the chromogenic substrate (NH2-Asn-Leu-Thr-Arg-pNA; 0.4 mM) (Figure 2H).

Apixaban and Rivaroxaban prevent the release of TF^+MV in response to fXa

Incubation of MDA-MB-231 and AsPC-1 cells with fXa (10 nM) resulted in the rapid release of microvesicles from these cell lines (Figure 3A and 3B) which also contained amplified levels of TF antigen (Figure 3C and 3D) and TF activity (Figure 3E and 3F). Moreover, inclusion of higher concentrations of Apixaban (1.8 µM) or Rivaroxaban (1.8 µM) were effective in reducing the release of microvesicles and the associated TF antigen and activity to comparable levels, with the exception of TF antigen released from MDA-MB-231 cells. This could arise as a consequence of very high incorporation of TF into these microvesicles and is in agreement with our previous findings [8]. Additionally, PAR2 activation can induce both microvesicle release and incorporation of TF into the microvesicles [21,44]. Therefore, the lower thrombin generation may be attributed to reductions in both of these processes.

Apixaban but not Rivaroxaban suppress the release of TF^+MV from non-activated cells

In addition to suppressing the cellular responses to fXa, the influence of DOAC on the release of TF^+MV from MDA-MB-231 and AsPC-1 cells under resting conditions was examined. Interestingly, Apixaban (1.8 µM) but not Rivaroxaban (1.8 µM) was capable of reducing the release of microvesicles from both cells lines to levels below that observed in resting cells (Figure 4A and 4B). This reduction was also reflected in the levels of microvesicle-associated TF antigen (Figure 4C and 4D).

Apixaban reduces the rate of cell proliferation in non-activated cells

In order to assess the influence of DOAC on cell proliferation, MDA-MB-231 cells, AsPC-1 cells and HDBEC (2×10^4), were placed in respective complete media, and were then supplemented with Apixaban (1.8 µM), Rivaroxaban (1.8 µM) or the DMSO vehicle with a further addition on day 2. Cell numbers were then determined after 4 days using the crystal

violet staining procedure. Incubation of MDA-MB-231 and AsPC-1 cells with 1.8 µM Apixaban reduced cell proliferation by 28% and 19% respectively (Figure 5A and 5B), but had no detectable influence on primary endothelial cells (Figure 5C). Our data are in line with supplementation experiments carried out using Apixaban, used at 5-50 fold higher than the therapeutic levels [45]. To further assess the involvement of endogenous fVIIa and PAR2, the expression of these proteins was suppressed in MDA-MB-231 cells by siRNA-mediated knock down. The quantification of the cell numbers at 48 h showed a substantial reduction in cell numbers following the suppression of fVII and PAR2 in these cells, compared to the cells treated with the control siRNA (Figure 5D).

Apixaban reduces TF^+MV release through preventing PAR2 activation

In order to determine if the release of TF^+MV and the increased rate of proliferation was in response to the activation of PAR2 or PAR1, the cells were pre-incubated with the blocking antibodies against PAR2 (SAM11) or PAR1 (ATAP2) in the presence and absence of the DOAC. Any alterations in the release of TF^+MV were then analysed as above. Inhibition of PAR2 using SAM11 antibody reduced the release of TF^+MV from both cell lines in response to fXa (10 nM). This reduction was comparable in magnitude to those achieved on supplementation with Apixaban or Rivaroxaban (Figure 6A and 6B). In contrast, incubation with Apixaban, or Rivaroxaban did not prevent the enhancement of the TF^+MV release following the activation of cells with PAR2-AP, from either MDA-MB-231 (Figure 6C) or AsPC-1 cells (Figure 6D), and did not affect the induction of cell proliferation by PAR2-AP activation (Figure 6E). In the absence of fXa, only Apixaban reduced the release of TF^+MV to levels attainable by blocking PAR2, but combinations of Apixaban together with SAM11 antibody presented no further inhibitory potential, when tested in MDA-MB-231 cells (Figure 6F). To further examine the contribution of PAR2 to pro-metastatic cell function, sets of MDA-MB-231 cells were incubated with Apixaban (1.8 µM), PAR2-blocking antibody (SAM11; 20 µg/ml) or used untreated. Total RNA was the extracted from the cells and the expression of IL-8 mRNA was measured using β-actin as a reference. The incubation of

cells with Apixaban resulted in 40% reduction in IL-8 expression compared to the untreated sample cells, while the inhibition of PAR2 reduced IL-8 expression by 56% (Figure 6G). Additionally, inhibition of PAR2 in these cells resulted in 47% decrease in cell proliferation (Figure 6H) and in agreement with our previous findings [43].

Apixaban functions through preventing the activation of PAR2 by TF-fVIIa complex

Since fXa appears to be absent from either of the cell lines or the microvesicles derived form them, the hypothesis that the auto-activation of PAR2 in cell lines was mainly induced by TF-fVIIa complex rather than fXa was investigated. Furthermore, the possibility that Apixaban may be influencing TF-fVIIa directly was also examined. Supplementation of cells with fVIIa (5 nM) or fXa (10 nM) induced the release of microvesicles to a similar level (Figure 7A and 7B). However, the release of TF within the microvesicles was significantly amplified following incubation of cells with fVIIa (5 nM) (Figure 7C and 7D). Furthermore, the inclusion of Apixaban, but not by Rivaroxaban abolished the release of microvesicles from the cell lines, in response to fVIIa (5 nM) (Figure 7E and 7F), as well as preventing the incorporation of TF into the released microvesicles (Figure 7G and 7H). Finally, pre-incubation of the MDA-MB-231 and AsPC-1 cells with an inhibitory anti-fVIIa antibody suppressed the rate of cell proliferation by 30% and 20% respectively.

Apixaban is estimated to bind to fVIIa and fXa with similar affinity and orientation

The examination of binding of Apixaban to fVIIa showed a suitable binding orientation in which the catalytic site may be blocked (Figure 8). Furthermore, the calculated binding energy for the interaction of Apixaban with fVIIa and fXa were calculated to be comparable (-9.78 kcal/mol and -11.66 kcal/mol, respectively). In addition, the ligand binding efficiencies were also determined to be of the same order of magnitude (-0.29 and -0.34, respectively).

Discussion

The activation of PAR1 and PAR2 is known to induce the release of microvesicles from

cells. However, the incorporation of TF within the microvesicles is mediated by PAR2 but not PAR1 [21]. Proteolytic activation of PAR2 during coagulation, is attained through the action of fXa and TF-fVIIa complex [15]. However, the expression and activation of fVII and fX by the cancer cell lines needs clarification. Furthermore, the contributions of each of these enzymes to the auto-activation of PAR2 and the release of $TF^{+}MV$ has not been examined previously. Examination of these properties in two cell lines indicated that the auto-activation of PAR2 in these cell lines was mediated through TF-fVIIa complex, although supplementation with fXa further enhanced this process. In contrast, since no fXa was detected in the cells, it may be assumed that fXa does not play a major role in the maintenance of accelerated proliferation in these cancer cells. This finding also suggests that the expression and activation of fVII/fVIIa by cancer cells may be an additional mediator in the release of procoagulant microvesicles which needs to be taken into consideration as a risk factor, in conjunction to the expression of TF [17-20,22,23]. In fact, the released microvesicles from these cells appeared to contain both TF and fVIIa, and were procoagulant even if fVIIa was omitted from the assay, or when using fVII-deficient plasma. Moreover, the cell-derived microvesicles showed proteolytic activity towards a fVIIa-specific chromogenic substrate which was inhibited in the presence of an inhibitory antibody towards fVIIa. In circulation, TF-$fVII^{+}MV$ may interact with the surface of various cells [36] where they can potentiate PAR2 activation and the further release of microvesicles from the activated cells. Consequently, the TF-fVIIa complex associated with microvesicles amplifies the exposure and release of further procoagulant material into the bloodstream. Ultimately, changes in vascular structure through inflammatory responses and cellular apoptosis may constitute an indirect mechanism through which the TF-$fVII^{+}MV$ may promote thrombosis associated with cancer.

The comparison of the effectiveness of the DOAC identified a remarkable property of Apixaban as an inhibitor of the proteolytic activity of fVIIa, as well as the PAR2 signalling by TF-fVIIa complex. This ability was specifically dependent on fVIIa, was comparable to pre-

incubation of the cells with fVIIa-inhibitory antibody, and was distinct to that of inhibiting fXa in plasma [46-49]. Furthermore, the inhibitory potential of Apixaban was not replicated on inclusion of Rivaroxaban despite the higher sensitivity of coagulation mechanism to the latter [50]. In fact the estimated Ki value for Apixaban towards human fVIIa (4.4 nM) was in line but higher than the published values against human fXa; 0.08 nM [51] and 0.74 nM [52]. Moreover, the estimated Ki value is close to that reported for the synthetic fVIIa inhibitor, BMS-593214 (5 nM) [53]. However, due to dissimilar analytical procedures used and also sensitivity to tissue factor preparations [52,54], we expect these differences to be much greater and therefore emphasis the need for verification, and urge caution at this point. In contrast, the lower circulating concentration of fVIIa compared to fXa suggests that Apixaban may be effective in blocking the action of TF-fVIIa complex *in vivo* directly, as well as by preventing downstream mechanisms [55]. In either case, the rate of TF^{+}MV release was not further suppressed following blocking of PAR2 with SAM11 antibody. Therefore, the underlying mechanism appears to involve PAR2 signalling and requires the presence of TF-fVIIa complex. Protease activated receptors are G-protein coupled receptors which act as sensors for the presence of active proteases, in particular the coagulation mechanism [56,57]. The discussions on the multiple roles of PAR2 activation, together with the multiple second messengers, the pathways involved and the cellular outcomes has been thoroughly delineated previously [58,59] and is therefore beyond the scope of this study.

As stated above, the inhibitory function of Apixaban towards fVIIa was not detectable with Rivaroxaban. The differential action of various DOAC when examined with different cell lines has been demonstrated previously [60]. We have not examined this feature in any of the other fXa-blocking DOAC which include Edoxaban, Darexaban, Otamixaban, Betrixaban, Letaxaban and Eribaxaban. Also despite the inhibition studies, at this point it is not possible to confirm any direct molecular interaction between Apixaban and fVIIa. However, the dissimilar specificity of Apixaban and Rivaroxaban can prove to be a useful tool in deciphering the mechanisms of TF-fVIIa signalling and unscrambling these from the cellular

outcomes of fXa action.

In conclusion, this study has shown that the upregulation of the release of $TF^{+}MV$ from cancer cells is mediated through auto-activation of PAR2 by the TF-fVIIa complex, on the surface of these cells. This in turn enhances the rate of proliferation in cancer cells. Importantly, we have for the first time shown that Apixaban selectively inhibits the proteolytic activity of fVIIa and therefore, may prove to be a useful agent in controlling cancer cell proliferation as well as the associated risk of thrombosis.

Authors' contributions

The study was designed by SF, AM and CE, and the experimental work carried out by SF and YM. The data were evaluated by SF, YM, AM and CE and the manuscript was prepared by SF and CE.

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Conflict of interest

AM reports: Research Support: Bayer, Bristol-Myers Squibb, Consultant and/or Honoraria: Bristol-Myers Squibb, Bayer Commissioned Talks: Bayer, Scientific Advisory Board: Bristol-Myers Squibb, Bayer. SF,YM and CE do not report any conflict of interest

References

1. Vaeki A. Trousseau's syndrome: multiple definitions and multiple mechanisms. Blood 2007;110:1723-1729.

2. Khorana AA, Francis CW, Culakova E, Kuderer NM, Lyman GH. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. J Thromb Haemost 2007;5:632-634.

3. Zacharski LR, Henderson WG, Rickles FR, Forman WB, Cornell CJ Jr, Forcier RJ, Edwards RL, Headley E, Kim SH, O'Donnell JF, et al. Effect of warfarin anticoagulation on survival in carcinoma of the lung, colon, head and neck, and prostate. Final report of VA Cooperative Study #75. Cancer. 1984;53(10):2046-2052.

4. Altinbas M, Coskun HS, Er O, Ozkan M, Eser B, Unal A, Cetin M, Soyuer S. A randomized clinical trial of combination chemotherapy with and without low-molecular-weight heparin in small cell lung cancer. J Thromb Haemost 2004;2:1266-1271.

5. Seto S, Onodera H, Kaido T, Yoshikawa A, Ishigami S, Arii S, Imamura M. Tissue factor expression in human colorectal carcinoma: correlation with hepatic metastasis and impact on prognosis. Cancer 2000;88:295-301.

6. Khorana AA, Ahrendt SA, Ryan CK, Francis CW, Hruban RH, Hu YC, Hostetter G, Harvey J, Taubman MB. Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. Clin Cancer Res 2007;13:2870-2875.

7. Kaido T, Oe H, Yoshikawa A, Mori A, Arii S, Imamura M. Tissue factor is a useful prognostic factor of recurrence in hepatocellular carcinoma in 5-year survivors. Hepatogastroenterology 2005;52:1383-1387.

8. Ettelaie C, Collier MEW, Featherby S, Benelhaj NE, Greenman J, Maraveyas A. Analysis of the potential of cancer cell lines to release tissue factor-containing microvesicles:

correlation with tissue factor and PAR2 expression. Thrombosis Journal 2016;14:2.

9. Schaffner F, Ruf W. Tissue factor and protease-activated receptor signaling in cancer. Semin Thromb Hemost 2008;34:147-153.

10. Hron G, Kollars M, Weber H, Sagaster V, Quehenberger P, Eichinger S, Kyrle PA, Weltermann A. Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb Haemost 2007;97:119-123.

11. Thaler J, Koder S, Kornek G, Pabinger I, Ay C. Microparticle-associated tissue factor activity in patients with metastatic pancreatic cancer and its effect on fibrin clot formation. Transl Res 2014;163:145-150.

12. Auwerda JJ, Yuana Y, Osanto S, de Maat MP, Sonneveld P, Bertina RM, Leebeek FW. Microparticle-associated tissue factor activity and venous thrombosis in multiple myeloma. Thromb Haemost 2011;105:14-20.

13. Hisada Y, Ay C, Auriemma AC, Cooley BC, Mackman N. Human pancreatic tumors grown in mice release tissue factor-positive microvesicles that increase venous clot size. J Thromb Haemost. 2017;15(11):2208-2217.

14. Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, Mallat Z. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. Circulation 2001;104:2649-2652.

15. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci U S A 2000;97:5255-5260.

16. Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. Proc Natl Acad Sci U S A 2001;98:7742-7747.

17. Wojtukiewicz MZ, Hempel D, Sierko E, Tucker SC, Honn KV. Protease-activated

receptors (PARs)-biology and role in cancer invasion and metastasis. Cancer Metastasis Rev 2015;34:775-796.

18. Schaffner F, Ruf W. Tissue Factor and PAR2 Signaling in the Tumor Microenvironment. Arterioscler Thromb Vasc Biol 2009;29:1999-2004.

19. Sedda S, Marafini I, Caruso R, Pallone F, Monteleone G. Proteinase activated-receptors-associated signaling in the control of gastric cancer. World J Gastroenterol 2014;20:11977-11984.

20. Ohta T, Shimizu K, Yi S, Takamura H, Amaya K, Kitagawa H, Kayahara M, Ninomiya I, Fushida S, Fujimura T, Nishimura G, Miwa K. Protease-activated receptor-2 expression and the role of trypsin in cell proliferation in human pancreatic cancers. Int J Oncol 2003;23:61-66.

21. Collier MEW, Ettelaie C. Regulation of the incorporation of tissue factor into microparticles by serine phosphorylation of the cytoplasmic domain of tissue factor. J Biol Chem 2011;286:11977-11984.

22. Hjortoe GM, Petersen LC, Albrektsen T, Sorensen BB, Norby PL, Mandal SK, Pendurthi UR, Rao LV. Tissue factor-factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated by PAR-2 and results in increased cell migration. Blood 2004;103:3029-3037.

23. Su S, Li Y, Luo Y, Sheng Y, Su Y, Padia RN, Pan ZK, Dong Z, Huang S. Proteinase-Activated Receptor 2 Expression in Breast Cancer and Its Role in Breast Cancer Cell Migration. Oncogene 2009;28:3047-3057.

24. Lima LG, Monteiro RQ. Activation of blood coagulation in cancer: implications for tumour progression. Biosci Rep. 2013;33(5).

25. Monteiro RQ, Lima LG, Gonçalves NP, DE Souza MR, Leal AC, Demasi MA, Sogayar MC, Carneiro-Lobo TC. Hypoxia regulates the expression of tissue factor pathway

signaling elements in a rat glioma model. Oncol Lett. 2016;12(1):315-322.

26. Rondon AMR, de Almeida VH, Gomes T, Verçoza BRF, Carvalho RS, König S, Rodrigues JCF, Mermelstein CDS, Versteeg HH, Monteiro RQ. Tissue factor mediates microvesicles shedding from MDA-MB-231 breast cancer cells. Biochem Biophys Res Commun. 2018;502(1):137-144.

27. Das K, Prasad R, Singh A, Bhattacharya A, Roy A, Mallik S, Mukherjee A, Sen P. Protease-activated receptor 2 promotes actomyosin dependent transforming microvesicles generation from human breast cancer. Mol Carcinog. 2018;57(12):1707-1722.

28. Grover SP, Mackman N. Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis. Arterioscler Thromb Vasc Biol. 2018;38(4):709-725.Ciurus T, Sobczak S, Cichocka-Radwan A, Lelonek M. New oral anticoagulants – a practical guide. Kardiochir Torakochirurgia Pol 2015;12:111-118.

29. Ciurus T, Sobczak S, Cichocka-Radwan A, Lelonek M. New oral anticoagulants – a practical guide. Kardiochir Torakochirurgia Pol 2015;12:111-118.

30. Frost C, Wang J, Nepal S, Schuster A, Barrett YC, Mosqueda-Garcia R, Reeves RA, LaCreta F. Apixaban, an oral, direct factor Xa inhibitor: single dose safety, pharmacokinetics, pharmacodynamics and food effect in healthy subjects. Br J Clin Pharmacol 2013;75:476-487.

31. Mueck W, Stampfuss J, Kubitza D, Becka M. Clinical pharmacokinetic and pharmacodynamic profile of rivaroxaban. Clin Pharmacokinet 2014;53:1-16.

32. Ettelaie C, Collier ME, Mei MP, Xiao YP, Maraveyas A. Enhanced binding of tissue factor-microparticles to collagen-IV and fibronectin leads to increased tissue factor activity in vitro. Thromb Haemost 2013;109:61-71.

33. Bonnekoh B, Wevers A, Jugert F, Merk H, Mahrle F. Colorimetric growth assay for

epidermal cell cultures by their crystal violet binding capacity. Arch Dermatol Res 1989;281:487-490.

34. Collier ME, Ettelaie C. Induction of endothelial cell proliferation by recombinant and microparticle-tissue factor involves beta1-integrin and extracellular signal regulated kinase activation. Arterioscler Thromb Vasc Biol. 2010;30(9):1810-1817.

35. Ettelaie C, Collier MEW, Maraveyas A, Ettelaie R. Characterization of physical properties of tissue factor–containing microvesicles and a comparison of ultracentrifuge-based recovery procedures. J Extracell Vesicles 2014;3:10.

36. Collier ME, Mei PM, Xiao YP, Maraveyas A, Ettelaie C. The uptake of tumour cell-derived microparticles by microvascular endothelial cells results in the recycling of tissue factor to the cell surface with enhanced activity, in vitro. Thromb Haemost 2013;110:966-976.

37. Ettelaie C, Collier ME, Featherby S, Greenman J, Maraveyas A. Peptidyl-prolyl isomerase 1 (Pin1) preserves the phosphorylation state of tissue factor and prolongs its release within microvesicles. Biochim Biophys Acta 2017;1865:12-24.

38. Broze GJ Jr, Hickman S, Miletich JP. Monoclonal anti-human factor VII antibodies. Detection in plasma of a second protein antigenically and genetically related to factor VII. J Clin Invest. 1985;76(3):937-946.

39. Böhm E, Seyfried BK, Dockal M, Graninger M, Hasslacher M, Neurath M, Konetschny C, Matthiessen P, Mitterer A, Scheiflinger F. Differences in N-glycosylation of recombinant human coagulation factor VII derived from BHK, CHO, and HEK293 cells. BMC Biotechnol. 2015;15:87.

40. Tang JQ, Fan Q, Wu WH, Jia ZC, Li H, Yang YM, Liu YC, Wan YL. Extrahepatic synthesis of coagulation factor VII by colorectal cancer cells promotes tumor invasion and metastasis. Chin Med J (Engl). 2010;123(24):3559-3565.

41. Koizume S, Jin MS, Miyagi E, Hirahara F, Nakamura Y, Piao JH, Asai A, Yoshida A, Tsuchiya E, Ruf W, Miyagi Y. Activation of cancer cell migration and invasion by ectopic synthesis of coagulation factor VII. Cancer Res. 2006;66(19):9453-9460.

42. Koizume S, Ito S, Yoshioka Y, Kanayama T, Nakamura Y, Yoshihara M, Yamada R, Ochiya T, Ruf W, Miyagi E, Hirahara F, Miyagi Y. High-level secretion of tissue factor-rich extracellular vesicles from ovarian cancer cells mediated by filamin-A and protease-activated receptors. Thromb Haemost. 2016;115(2):299-310.

43. Madkhali Y, Featherby S, Collier MEW, Maraveyas A, Greenman J, Ettelaie C. The ratio of factor VIIa:tissue factor content within microvesicles determines the differential influence on endothelial cells. THOpen 2019; in press.

44. Collier MEW, Ettelaie C, Goult BT, Maraveyas A, Goodall AH. Investigation of the Filamin A-Dependent Mechanisms of Tissue Factor Incorporation into Microvesicles. Thromb Haemost. 2017;117(11):2034-2044.

45. Guasti L, Squizzato A, Moretto P, Vigetti D, Ageno W, Dentali F, Maresca AM, Campiotti L, Grandi AM, Passi A. In vitro effects of Apixaban on 5 different cancer cell lines. PLoS One 2017;12(10):e0185035.

46. Wong PC, White A, Luettgen J. Inhibitory effect of apixaban compared with rivaroxaban and dabigatran on thrombin generation assay. Hosp Pract (1995) 2013;41:19-25.

47. Sadeghi N, Kahn D, Jeske W, Hoppensteadt D, Fareed J. Tissue factor-mediated activation of the prothrombin complex concentrate (PCC) is differently inhibited by dabigatran, rivaroxaban, and apixaban: potential clinical implication. Clin Appl Thromb Hemost 2013;19:589-599.

48. Adelmann D, Wiegele M, Wohlgemuth RK, Koch S, Frantal S, Quehenberger P, Scharbert G, Kozek-Langenecker S, Schaden E. Measuring the activity of apixaban and rivaroxaban with rotational thrombelastometry. Thromb Res 2014;134:918-923.

49. Wong PC, Crain EJ, Xin B, Wexler RR, Lam PY, Pinto DJ, Luettgen JM, Knabb RM. Apixaban, an oral, direct and highly selective factor Xa inhibitor: in vitro, antithrombotic and antihemostatic studies. J Thromb Haemost. 2008;6(5):820-829.

50. Gardiner C, Kohama K, Patel I, Lane P, Dwyer S, Machin SJ, Mackie IJ. A performance evaluation of a novel human recombinant tissue factor prothrombin time reagent (Revohem(™) PT). Int J Lab Hematol. 2017;39(5):532-538.

51. Pinto DJ, Orwat MJ, Koch S, Rossi KA, Alexander RS, Smallwood A, Wong PC, Rendina AR, Luettgen JM, Knabb RM, He K, Xin B, Wexler RR, Lam PY. Discovery of 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo [3,4-c]pyridine-3-carboxamide (apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. J Med Chem. 2007;50(22):5339-5356.

52. Jourdi G, Siguret V, Martin AC, Golmard JL, Godier A, Samama CM, Gaussem P, Gouin-Thibault I, Le Bonniec B. Association rate constants rationalise the pharmacodynamics of apixaban and rivaroxaban. Thromb Haemost. 2015;114(1):78-86.

53. Wong PC, Luettgen JM, Rendina AR, Kettner CA, Xin B, Knabb RM, Wexler R, Priestley ES. BMS-593214, an active site-directed factor VIIa inhibitor: enzyme kinetics, antithrombotic and antihaemostatic studies. Thromb Haemost. 2010;104(2):261-269.

54. Sadeghi N, Kahn D, Jeske W, Hoppensteadt D, Fareed J. Tissue factor-mediated activation of the prothrombin complex concentrate (PCC) is differently inhibited by dabigatran, rivaroxaban, and apixaban: potential clinical implication. Clin Appl Thromb Hemost. 2013;19(6):589-599.

55. Wong PC, Jiang X. Apixaban, a direct factor Xa inhibitor, inhibits tissue-factor induced human platelet aggregation in vitro: comparison with direct inhibitors of factor VIIa, XIa and thrombin. Thromb Haemost. 2010;104(2):302-310.

56. Nieman MT. Protease-activated receptors in hemostasis. Blood. 2016;128(2):169-177.

57. Rothmeier AS, Ruf W. Protease-activated receptor 2 signaling in inflammation. Semin Immunopathol. 2012;34(1):133-149.

58. McCoy KL, Traynelis SF, Hepler JR. PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. Mol Pharmacol. 2010;77(6):1005-1015.

59. Alberelli MA, De Candia E. Functional role of protease activated receptors in vascular biology. Vascul Pharmacol. 2014;62(2):72-81.

60. Rousseau A, Van Dreden P, Mbemba E, Elalamy I, Larsen A, Gerotziafas GT. Cancer cells BXPC3 and MCF7 differentially reverse the inhibition of thrombin generation by apixaban, fondaparinux and enoxaparin. Thromb Res 2015;136:1273-1279.

Figure Legends

Figure 1. Analysis of the procoagulant activity of released microvesicles. MDA-MB-231 and AsPC-1 cells were adapted to DMEM serum-free medium, for 2 h and the released microvesicles were collected by ultracentrifugation. The activity of the microvesicles prepared from MDA-MB-231 (A) and AsPC-1 cells (B) was measured by CAT assay, in the presence and absence of an TF-inhibitory antibody (HTF-1; 20 µg/ml) and compared (C). (n = 4; * = $p < 0.05$ vs the respective untreated sample). In addition, the fXa-generation activity of the microvesicles was analysed in the absence of fVIIa (D). (n = 3; * = $p < 0.05$ for microvesicle preparations vs the sample devoid of fVIIa). The activity of the microvesicles from MDA-MB-231 (E) and AsPC-1 cells (F) was also measured by CAT assay in complete and fVII-deficient plasma and compared (G). The presence of fVIIa subunits in the cells was examined by western blot analysis (H). (The micrographs are representative of 4 experiments)

Figure 2. Analysis of the inhibition of fVIIa and fXa activity by DOAC. To determine the optimal concentrations for DOAC, fXa (10 nM) was pre-incubated for 30 min with a range of Apixaban (0-1.8 µM) and Rivaroxaban (0-1.8 µM). Factor Xa activity was then determined using a chromogenic substrate (A). (n = 3; * = p < 0.05 vs fXa alone). To ensure that the released microvesicles were free of any DOAC carry over, the microvesicles were incubated with fXa (10 nM) and fXa-chromogenic substrate (0.2 mM) and any reduction in fXa activity was recorded (B). A synthetic fVIIa-chromogenic substrate (NH2-Asn-Leu-Thr-Arg-pNA; 0.4 mM), or Perfachrome (0.4 mM) was incubated with purified fVIIa (5 nM) or purified fXa (10 nM) at 37°C for 1 h and the absorptions at 410 nm determined (C). (n = 4; * = p < 0.05 vs untreated sample). Samples of purified fVIIa (5 nM) were then pre-incubated Apixaban (1.8 µM), Rivaroxaban (1.8 µM), an inhibitory polyclonal anti-fVIIa antibody or a control isotype, prior to addition of the fVIIa-chromogenic substrate and incubated at 37°C for 1 h and measuring the absorption at 410 nm (D). (n = 4; * = p < 0.05 vs untreated sample, # = p<0.05 vs fVIIa alone). Combinations of TF (1 U/ml), fVIIa (5 nM) and Apixaban (1.8 µM) were incubated with Perachrome substrate for 1 h at 37°C and the absorptions measured at 410 nm (E). (n = 5; * = p < 0.05 vs untreated sample). Samples of purified fVIIa (50 nM) were incubated with a range of concentrations of Perfachrome substrate (0.4-1.6 mM) in the presence or absence of Apixaban (1.8 µM) and the rates of reaction measured (F). (n = 4). By constructing Lineweaver Burk plots, the Ki for the competative inhibition of fVIIa by Apixaban was determined to be 4.4 nM (G). Samples of purified microvesicles (50 nM), purified from the conditioned media of MDA-MB-231 cells were pre-incubated with Apixaban (1.8 µM), Rivaroxaban (1.8 µM) or DMSO vehicle, and then incubated with the VIIa-chromogenic substrate (NH2-Asn-Leu-Thr-Arg-pNA; 0.4 mM) and incubated at 37°C for 1 h. The absorption values at 410 nm were then determined (H). (n = 4; * = p < 0.05 vs untreated sample, # = p<0.05 vs DMSO vehicle).

Figure 3. DOAC suppress the release of $TF^{+}MV$ from cells in response to fXa. Sets of MDA-MB-231 and AsPC-1 cells were adapted to serum-free medium and incubated with fXa for

30 min, in the presence of Apixaban (0-1.8 µM) or Rivaroxaban (0-1.8 µM). The density of microvesicles released from MDA-MB-231 (A) and AsPC-1 cells (B) was determined using the Zymuphen MP assay kit. (n = 3; * = $p < 0.05$ vs fXa alone). Also, the TF antigen content of the microvesicles from MDA-MB-231 (C) and AsPC-1 cells (D) was determined using the Quantikine TF-ELISA assay. (n = 3; * = $p < 0.05$ vs fXa alone). Finally, the TF activity of the microvesicles from MDA-MB-231 (E) and AsPC-1 cells (F) was measured using CAT assay. (n = 3; * = $p < 0.05$ vs fXa alone).

Figure 4. Apixaban but not Rivaroxaban suppresses the release of $TF^{+}MV$ from in resting cells. Sets of MDA-MB-231 and AsPC-1 cells were adapted to serum-free medium and incubated for 2 h in the presence of Apixaban (1.8 µM), Rivaroxaban (1.8 µM) or the DMSO vehicle. The density of microvesicles released from MDA-MB-231 (A) and AsPC-1 cells (B) was determined using the Zymuphen MP assay kit. (n = 4; * = $p < 0.05$ vs DMSO control). The TF antigen content of the microvesicles from MDA-MB-231 (C) and AsPC-1 cells (D) was also determined using the Quantikine TF-ELISA assay. (n = 4; * = $p < 0.05$ vs DMSO control).

Figure 5. Apixaban but not Rivaroxaban suppresses the cell proliferation. Sets of MDA-MB-231 (A), AsPC-1 (B) and HDBEC (C) were plated out in 12-well plates (2×10^4) in complete media, and were treated with Apixaban (0-1.8 µM), Rivaroxaban (0-1.8 µM) or the vehicle DMSO. The cells were supplemented on day 2 and the cells numbers were determined after 4 days. (n = 5; * = $p < 0.05$ vs DMSO control). Sets of MDA-MB-231 cells (5×10^3) were transfected with siRNA to silence the expression of fVII (5 pM), PAR2 (5 pM) or a control siRNA (5 pM) and the cell numbers were determined after 2 days (D).

Figure 6. Apixaban prevents the activation of PAR2 and microvesicle release. Sets of cells were adapted to serum-free medium and pre-incubated with blocking antibodies to PAR2 (SAM11; 20 µg/ml) or PAR1 (ATAP2; 20 µg/ml) in the presence and absence of Apixaban (1.8 µM) and Rivaroxaban (1.8 µM) or DMSO control, for 2 h. The density of microvesicles

released from MDA-MB-231 (A) and AsPC-1 cells (B) was determined using the Zymuphen MP assay kit. (n = 4; * = $p < 0.05$ vs fXa alone). In addition sets of cells were incubated with of Apixaban (1.8 µM) and Rivaroxaban (1.8 µM) or DMSO control and activated using PAR2-AP (20 µM) and the density of microvesicles released from MDA-MB-231 (C) and AsPC-1 cells (D) was determined. (n = 4; * = $p < 0.05$ vs PAR2-AP alone). To examine any non-specific effects of DOAC on the activation of PAR2, sets of MDA-MB-231 cells (2×10^4) were activated using PAR2-AP (20 µM) in the absence and in the presence of Apixaban (1.8 µM) or Rivaroxaban (1.8 µM). Cell numbers were then measured after 2 day (E). Sets of cells were adapted to serum-free medium and pre-incubated with blocking antibodies to PAR2 (SAM11; 20 µg/ml) or PAR1 (ATAP2; 20 µg/ml) in the presence of Apixaban (1.8 µM), Rivaroxaban (1.8 µM) or DMSO control. The density of microvesicles released from resting MDA-MB-231 (F) was determined using the Zymuphen MP assay kit. (n = 4; * = $p < 0.05$ vs DMSO alone). Sets of cells were incubated with Apixaban (1.8 µM) or SAM11 antibody (20 µg/ml) and compared to respective vehicle-treated cells. Total RNA was extracted using the Ribozol solution. The expression of fVII mRNA or IL-8 was measured by GoTaq® 1-Step RT-qPCR System using QuantiTect primers for fVII, IL-8 and β-actin and relative amounts determined using as a reference (G). (n = 4; * = $p < 0.05$ vs untreated sample). Sets of MDA-MB-231 cells (2×10^4) were also pre-incubated with antibody (20 µg/ml) and cell numbers determined cell proliferation compared to the untreated cells (H). (n = 5; * = $p < 0.05$ vs untreated sample).

Figure 7. Apixaban prevents cell activation by inhibiting TF-fVIIa activity. Samples of fXa (10 nM) or fVIIa (5 nM) were incubated with an inhibitory anti-fVIIa polyclonal antibody (20 µg/ml) or an equivalent isotope control antibody, for 1 h. Sets of MDA-MB-231 and AsPC-1 (2×10^5) were adapted to serum-free media and incubated with the untreated enzymes, or the samples pre-incubated with the inhibitory or the isotype antibodies, for 2 h. The density of microvesicles released from MDA-MB-231 (A) and AsPC-1 cells (B) was determined using the Zymuphen MP assay kit. (n = 5; * = $p < 0.05$ vs respective enzyme without any

antibody). The TF antigen content of the microvesicles from MDA-MB-231 (C) and AsPC-1 cells (D) was also determined using the Quantikine TF-ELISA assay. (n = 5; * = $p < 0.05$ vs respective enzyme without any antibody). Sets of cells were supplemented with fVIIa (5 nM) in the presence of Apixaban (1.8 µM), Rivaroxaban (1.8 µM) or the DMSO vehicle. The density of microvesicles released from MDA-MB-231 (E) and AsPC-1 cells (F) was determined. (n = 5; * = $p < 0.05$ vs DMSO control). The TF antigen content of the microvesicles from MDA-MB-231 (G) and AsPC-1 cells (H) was also the Quantikine TF-ELISA assay. (n = 5; * = $p < 0.05$ vs DMSO control).

Figure 8. Approximation of the interaction of Apixaban with fVIIa. The crystal structures of fVIIa (4ylq), fXa (2P16) and Apixaban (GG2) were obtained on Brookhaven format (PDB) and used to estimate the location and efficiency of binding using the Autodock 4v2.6. The Autodock graphical interface AutoDockTools 1.5.6 was used, the polar hydrogens were retained and partial charges added to the proteins using the Gasteiger charges. The search space was limited to an area of 20 × 20 × 20 Å, centred around the hydroxyl group of Ser195 in the enzymatic site of fVIIa and fXa. For each enzyme, 25 × ligand orientations (poses) were examined and ranked according to the scoring-function.