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Full Length Article

Dual antiplatelet and anticoagulant (APAC) heparin proteoglycan mimetic with shear-dependent effects on platelet-collagen binding and thrombin generation

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A B S T R A C T

Heparin proteoglycans (HEP-PGs) carry standard heparin-mediated anticoagulant properties as well as novel antiplatelet functions, a combination that may be significant for targeting multiple pathways in a single therapy. Recent work developing semisynthetic HEP-PG mimetics has shown promising results also in vivo, however flow conditions in vitro that replicate in vivo hemodynamics have not been reported. In this work, we present several assays (platelet calcium mobilization, aggregometry, microfluidic tests at venous and arterial hemodynamics) to characterize specific mechanistic effects of dual antiplatelet and anticoagulant (APAC) constructs as mimetics of HEP-PGs. Three APACs with different conjugation levels of heparin chains (CL10, CL18, HICL) were shown to decrease platelet deposition to collagen surfaces in PPACK-treated whole blood at venous shear rate (200 s^{-1}). FXIIa-inhibited whole blood (CTI: corn trypsin inhibitor, 40 μg/mL) perfused over collagen/tissue factor showed reduced both platelet and fibrin deposition when treated with APACs. IC50 values for platelet and fibrin inhibition were calculated for each molecule at venous shear rate. Increasing the shear rate to arterial flows (1000 s^{-1}) and using APAC as the sole anticoagulant, resulted in a more potent antiplatelet effect of APAC, suggesting an added effect on von Willebrand Factor (vWF) function. Additionally, APAC caused an inhibition of calcium mobilization specific to thrombin and collagen stimulation and a dose-dependent reduction in collagen-mediated platelet aggregation. Understanding the sensitivity of APAC activity to shear rate, platelet signaling and procoagulant pathways is important for applications in which APAC administration may have beneficial therapeutic effects.

1. Introduction

Antithrombotic drugs are typically classified into one of three major categories: antiplatelet, anticoagulant, or fibrinolytic agents [1, 2]. Common antiplatelet therapeutics include aspirin and clopidogrel which both inhibit secondary platelet agonist generation (thromboxane A2 and ADP, respectively) [1–3], as well as inhibitors of the integrin αIIb/β3 [4]. Anticoagulants are responsible for preventing thrombin generation and fibrin polymerization. Warfarin and various heparins have been used as an oral and parental anticoagulant for several decades, but recent advances have focused on specifically targeting coagulation factors, such as thrombin and factor Xa [1]. Finally, fibrinolytic or thrombolytic drugs (most notably tPA: tissue plasminogen activator) promote the generation of plasmin, an enzyme that cleaves fibrin [5].

With increasingly complex cardiovascular disease states comes a need for the administration of multiple antithrombotics with different mechanisms of action. While certain classes of drugs have the potential to function synergistically, there is an associated increased bleeding risk as the number of drugs increases [6, 7]. Therefore, identifying a method for combining the antithrombotic functions of antiplatelet and anticoagulant agents into a single therapy can have a potentially great impact on the field, as uncertainty regarding optimal use remains [7].

Heparin (usually referred to as unfractionated heparin; UFH) is yet another common clinically-used antithrombotic agent which carries anticoagulant behavior through its binding and activation of antithrombin. Antithrombin then works to deactivate circulating thrombin and factor Xa to hinder the coagulation process [8]. Heparin can bind directly to thrombin (K_d = 100 nM) resulting in anticoagulant behavior [9]. Heparin is derived from mast cells which line the vascular walls usually in the same general location as tissue factor (TF). Upon tissue injury, mast cells are activated and release heparin proteoglycans (HEP-PGs) which are much higher in molecular weight than UFH [10, 11]. These structures have been shown to exhibit both anticoagulant

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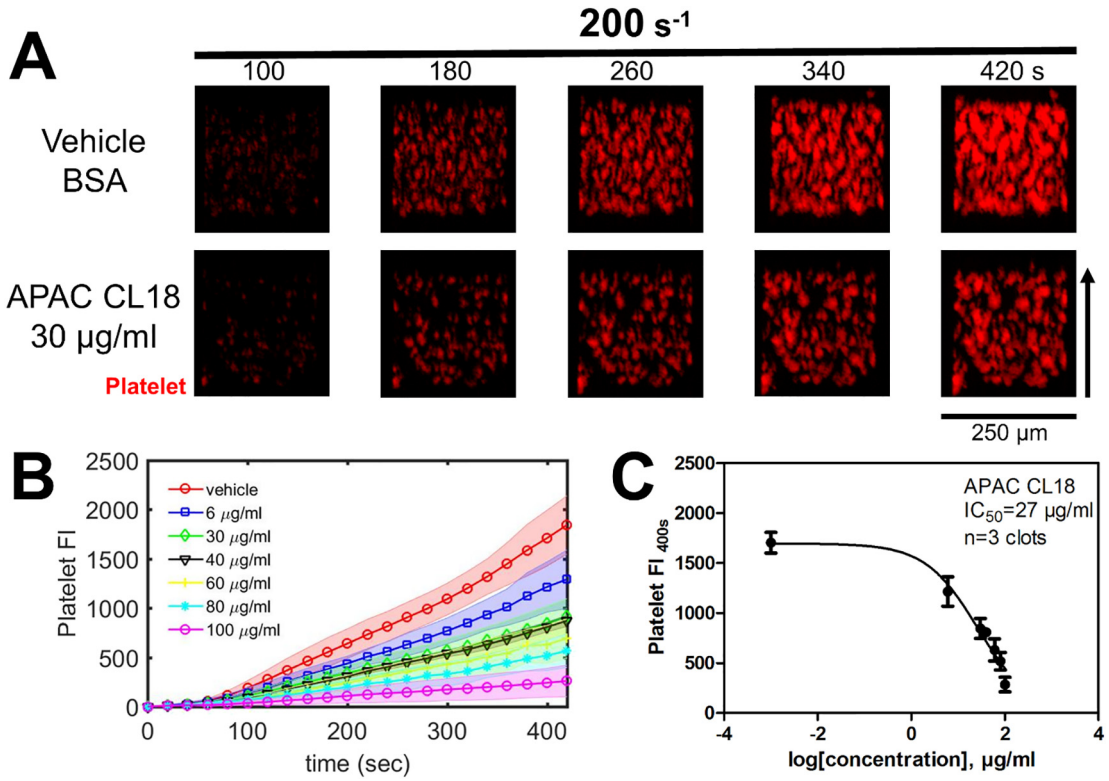

Fig. 1. Platelet deposition is dose-dependently reduced by APAC in the absence of thrombin under 200 s^{-1} over collagen in microfluidic assay. Vehicle (1% BSA) or APAC (CL18, 30 µg/mL) was added to PPACK-treated whole blood and perfused over collagen under venous shear rate. (A) images of platelet deposition, (B) dynamics of platelet deposition and (C) dose-response curve and IC50 (± standard deviation [SD]).

Table 1
IC50 values calculated at venous shear rate for PPACK and CTI-treated whole blood. IC50 values were calculated for each APAC species (CL10, CL18, and HICL) under two different anticoagulated whole blood conditions (PPACK and CTI). Since PPACK inhibits all thrombin activity, APAC-driven inhibition was only observed on platelet deposition. CTI-treated whole blood enabled the calculation of IC50 for both platelet deposition and fibrin polymerization.

	IC_{50} (µg/ml), 200 s^{-1}		
	PPACK	High CTI	
	Platelet	Platelet	Fibrin
APAC CL10	Not determined	25	0.6
APAC CL18	27	71	5.4
APAC HICL	57	90	0.5

features, as does heparin typically [12], as well as specific antiplatelet properties, most notably involving the platelet-collagen interaction and subsequent aggregation and fibrin polymerization [10]. The unconventional ability for a heparin-based entity to impact collagen-dependent platelet activation could be attributed to the fact that type I collagen has binding sites for heparin, in addition to the heparin binding site to von Willebrand Factor (vWF) bridging platelets with collagen, relevant under arterial shear rates [13]. The concept of designing synthetic HEP-PG mimetics, structured with a protein core and conjugated with UFH, has been demonstrated [10, 11, 14, 15].

Despite various results comparing the ability of HEP-PGs and UFH to inhibit collagen-mediated platelet aggregation and serotonin release under flow conditions [10], previous work with dual anticoagulant and antiplatelet (APAC) conjugates has been focused primarily on in vitro platelet aggregometry studies and in vivo vascular models [11, 15]. The importance of understanding the functionality of APACs, as is the case with any novel therapy, in more pathophysiologic scenarios in vitro is crucial. Thus, the focus of this work was to compare the results obtained from various in vitro experimental techniques to gain a broader understanding for the potential therapeutic effect of synthetic HEP-PG mimetics with varying heparin conjugation levels (CL10, CL18, HICL).

2. Materials and methods

2.1. Reagents

Reagents were obtained as follows: Anti-human CD61 (BD Biosciences, San Jose, CA), Alexa Fluor® 647 conjugated human fibrinogen (Life Technologies, Waltham, MA), corn trypsin inhibitor and D-Phe-Pro-Arg-chloromethylketone (CTI and PPACK, respectively; Haematologic Technologies, Essex Junction, VT), Sigmacote® siliconizing reagent (Sigma, St. Louis, MO), Dade® Innovin® PT reagent (Siemens, Malvern, PA), collagen Type I Chrono-Par™ aggregation reagent (Chrono-log, Havertown, PA). Whole blood was drawn via venipuncture from healthy donors following University of Pennsylvania Institutional Review Board approval into a syringe loaded with 100 µM PPACK (to inhibit thrombin activity altogether for the study of platelet deposition on collagen only) or 40 µg/mL CTI (to inhibit contact pathway and measure platelet and fibrin deposition). Prior to each blood draw, donors self-reported to be free of any medications for 7 days and alcohol use for 48 h. Additionally, female donors self-reported to not using oral contraceptives.

Apixaban (SelleckChem, Houston, TX), HEPES (Fisher Scientific, Hampton, NH), Fluo-4 NW calcium dye and probenecid (Invitrogen, Carlsbad, CA), ADP (Sigma-Aldrich, St. Louis, MO), U46619 (Tocris Bioscience, Bristol, UK), convulxin (Cayman Chemical, Ann Arbor, MI), type I fibrillar collagen (Chrono-log, Havertown, PA), thrombin (Haematologic Technologies Inc., Essex Junction, VT), and SFLLRN and AYPGKF amide (Bachem, Torrance, CA), Protein A Sepharose CL-4B (GE Healthcare, US), vWF (Wilfactin®, 100 IU/mL, LFB

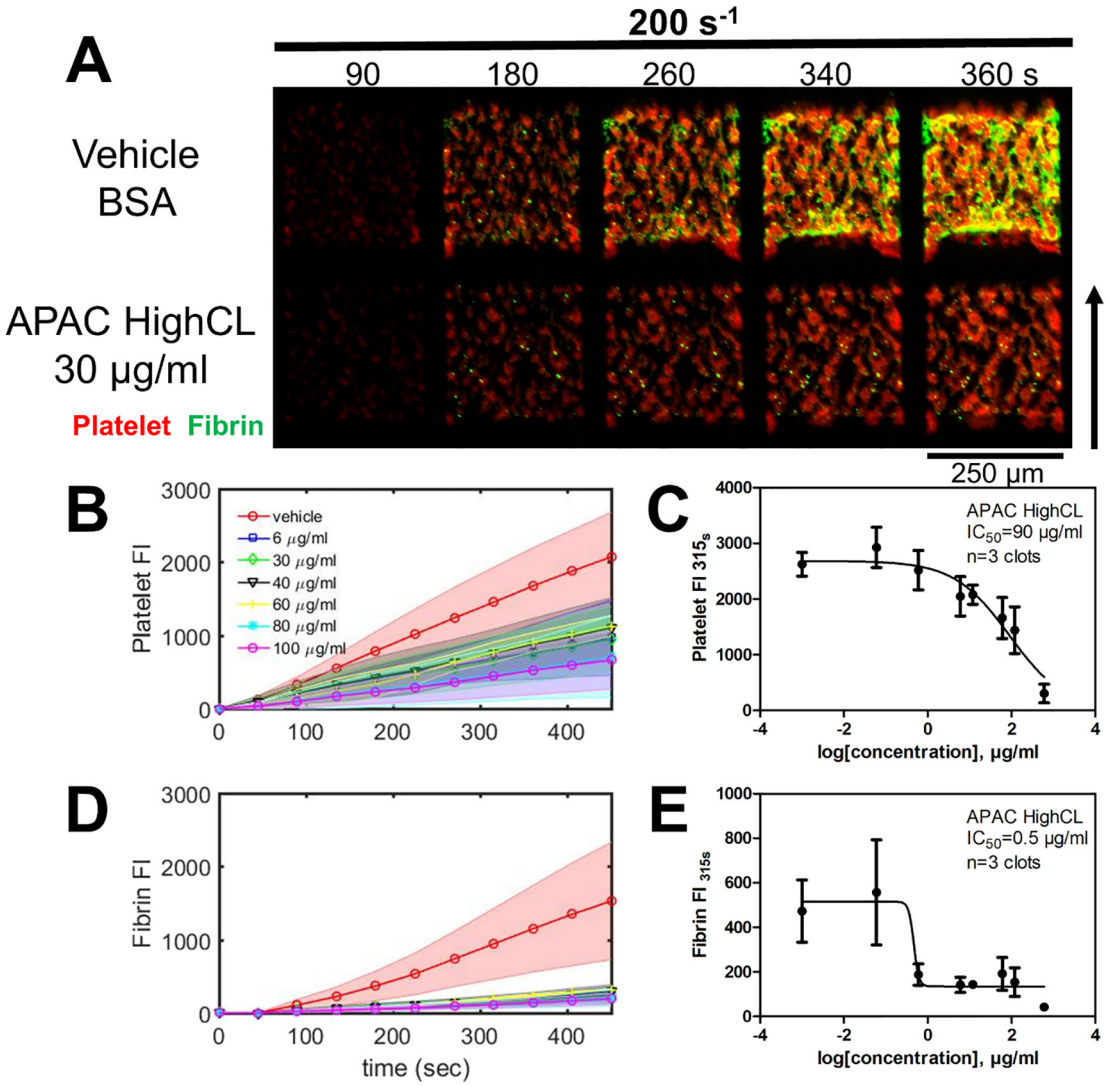

Fig. 2. APAC inhibits platelet deposition dose-dependently with simultaneous anticoagulant efficacy under 200 s^{-1} over collagen/TF in microfluidic assay. Vehicle (1% BSA) or APAC (HICL, 30 µg/mL) was added to CTI-treated whole blood and perfused over collagen/TF under venous shear rate. (A) images of platelet and fibrin deposition, (B) dynamics of platelet deposition, (C) dose-response curve and IC50 for platelet deposition, (D) dynamics of fibrin polymerization, (E) dose-response curve and IC50 for fibrin polymerization (± standard deviation [SD]).

Biomedicaments, Les Ulis, FR), rabbit polyclonal anti-vWF (DAKO, Glostrup, DK), enhanced chemiluminescent (ECL) detection reagents (GE Healthcare, US), goat anti-rabbit HRP, (Jackson Immunoresearch, Westgrove, PA, US), streptavidin-ATTO647 (Immune Biosolutions, Sherbrooke, QE, CA) were stored and used according to each manufacturer's instructions. Three different APAC molecules were synthesized (Aplagon, Helsinki, Finland) as previously described [11, 15]. In brief, dual antiplatelet and anticoagulant (APAC) conjugate comprises of protein core, where UFH chains are bound by covalent di-sulfide bridges provided by a cross-linker molecule to reach various conjugation levels (CL) of heparin.

2.2. Microfluidic assays

Microfluidic experiments were run as previously described [16]. Glass slides were treated with Sigmacote®. A volume of 5 µL of collagen was perfused through a patterning channel device (250 µm wide and 60 µm high) to create a single strip of fibrillar collagen. Lipidated TF was then sorbed to the collagen surface by introduction of 5 µL of Dade Innovin PT reagent (20 µM stock concentration). The Dade Innovin PT reagent was incubated with the collagen for 30 min without flow and then blocked and rinsed with 20 µL of bovine serum albumin (1% BSA in Hepes-buffered saline). An 8-channel microfluidic device was vacuum-mounted perpendicularly to collagen/TF surfaces forming 8 parallel-spaced prothrombotic patches (250 × 250 µm). APAC (CL10 7.12 mg/mL; CL18 11.94 mg/mL or HICL 6.71 mg/mL in phosphate-buffered saline, PBS, 10 mM Na_2HPO_4, 0.137 M NaCl, pH 7.5) was diluted in 1% BSA for appropriate concentrations for the analysis. Vehicle (1% BSA) or APAC-treated blood was perfused across the 8 channels at an initial wall shear rate controlled by a syringe pump (Harvard PHD 2000; Harvard Apparatus, Holliston, MA). Each thrombus was formed under constant flow rate (constant Q). Platelet and/or fibrin deposition were monitored simultaneously by epifluorescence microscopy (IX81; Olympus America Inc., Center Valley, PA). Images were captured with a charged coupled device camera (Hamamatsu, Bridgewater, NJ) and were analyzed with ImageJ software (National Institutes of Health). To avoid side-wall effects, fluorescence values were taken only from the central 75% of the channel. The background-corrected fluorescence values were fitted by use of a log (inhibitor concentration) vs. response routine in GraphPad Prism 5.00 (GraphPad Software) to calculate the half-maximal inhibitory concentration (IC50).

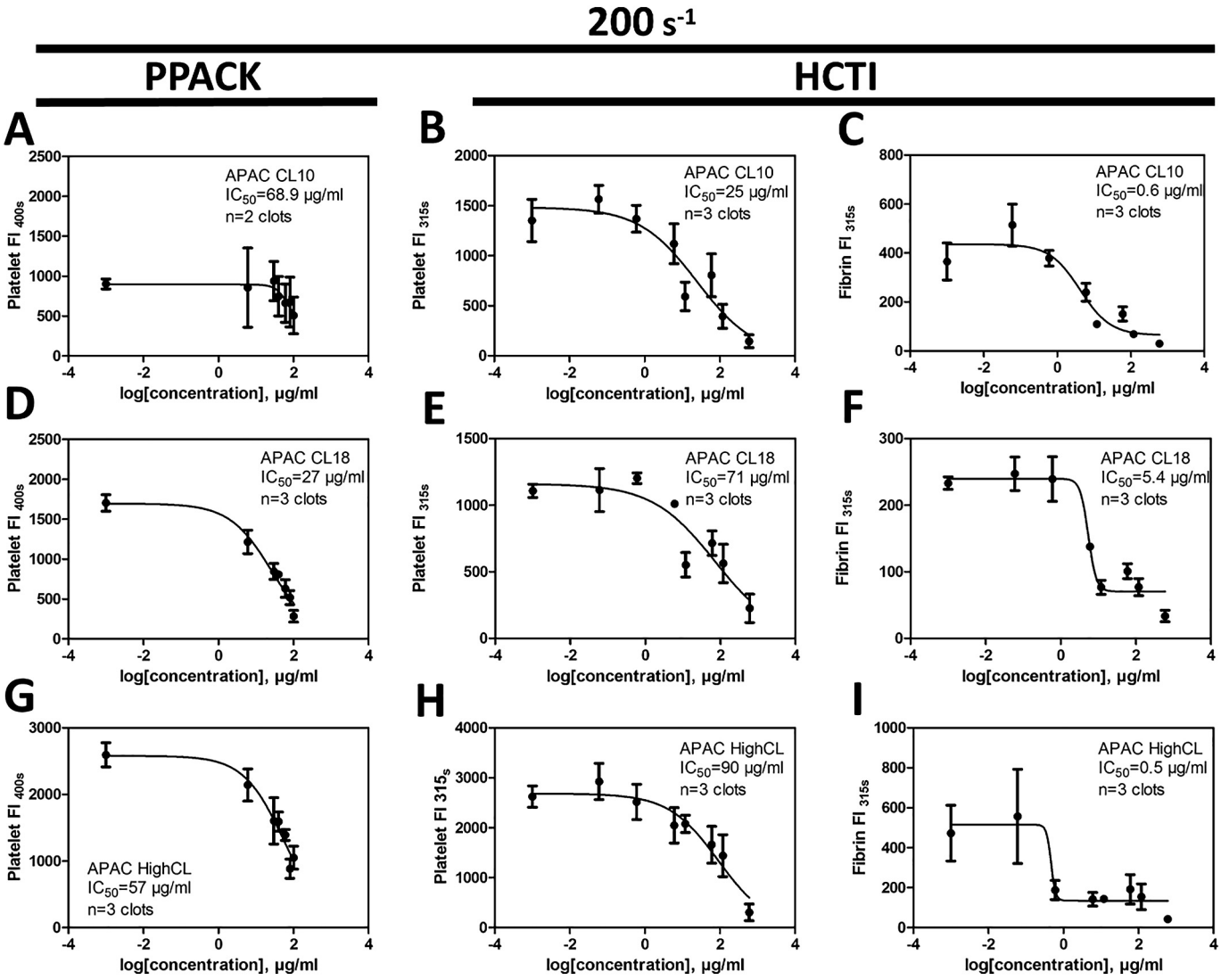

Fig. 3. Dose-response curves for each APAC species at venous shear rate. Inhibition on platelet deposition and fibrin formation under two anticoagulated whole blood conditions (PPACK and CTI) at venous shear rate (200 s^{-1}) of CL10 (A), (B), (C). CL18 (D), (E), (F). HICL (G), (H), (I) (± standard deviation [SD]).

2.3. Immunoprecipitation to detect vWF and APAC binding

Rabbit polyclonal anti-vWF (1:5000) was first attached to Protein A Sepharose CL-4B and used to capture vWF (50 µg of Wilfactin®, final) and vWF-specific APAC-biotin (CL10, 8 µg in PBS, pH 7.5). Between each step Protein A Sepharose was spun down, and washed thrice with PBS. Samples were run under reduced conditions (β-mercaptoethanol) on SDS-PAGE (6%) and Western-blotted on polyvinylidene difluoride membrane. VWF was detected with goat anti-rabbit HRP and APAC-biotin with Streptavidin-ATTO647 (1:1000).

2.4. Platelet calcium assays

Measurements of platelet calcium mobilization were conducted in 384-well plate assay format, as described previously [17]. Briefly, fresh whole blood treated with 250 nM apixaban (a Factor Xa inhibitor used to eliminate endogenous thrombin generation) was centrifuged (120 *g*, 10 min) to isolate platelet-rich plasma (PRP). The PRP was incubated with a vial of Fluo-4 NW calcium dye prepared by reconstitution with sterile 20 mM HEPES-buffered saline (HBS, pH 7.4) and 77 mg/mL probenecid to prevent dye leakage for 30 min. After incubation, two separate 384-well plates were assembled. One plate contained dye-loaded PRP (30 µL/well) and the other plate contained platelet agonists at previously determined EC50 concentrations [17, 18], as well as serial dilutions of three different APAC molecules of varying heparin conjugation levels (CL10, CL18, HICL). The agonist-containing plate was prepared using a JANUS liquid handling system (PerkinElmer, Inc., Waltham, MA) such that all 144 combinations of the six platelet agonists and eight APAC concentrations (0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 µg/mL) were prepared in a time-efficient manner with replicates. The two plates were loaded into a FlexStation 3 microplate reader (Molecular Devices, Inc., Sunnyvale, CA) and agonists (20 µL/well) were dispensed column-wise into PRP. The dynamic fluorescence intensity F(t) was read and normalized by the pre-dispense baseline (F_o) for 4.5 min with readings every 2.5 s (Ex: 485 nm; Em: 525 nm). The final PRP concentration after agonist addition was 12% by volume and previously no evidence of autocrine signaling has been reported in these dilute conditions [17]. Testing of type I fibrillar collagen (20 µg/mL) in the platelet calcium assay was performed as previously described [19], in which manual pipetting and reading using a Fluoroskan Ascent plate reader was required due to variable delivery of collagen by the FlexStation automated pipetting system.

2.5. Platelet aggregometry

Platelet aggregation studies were performed using a Chrono-log

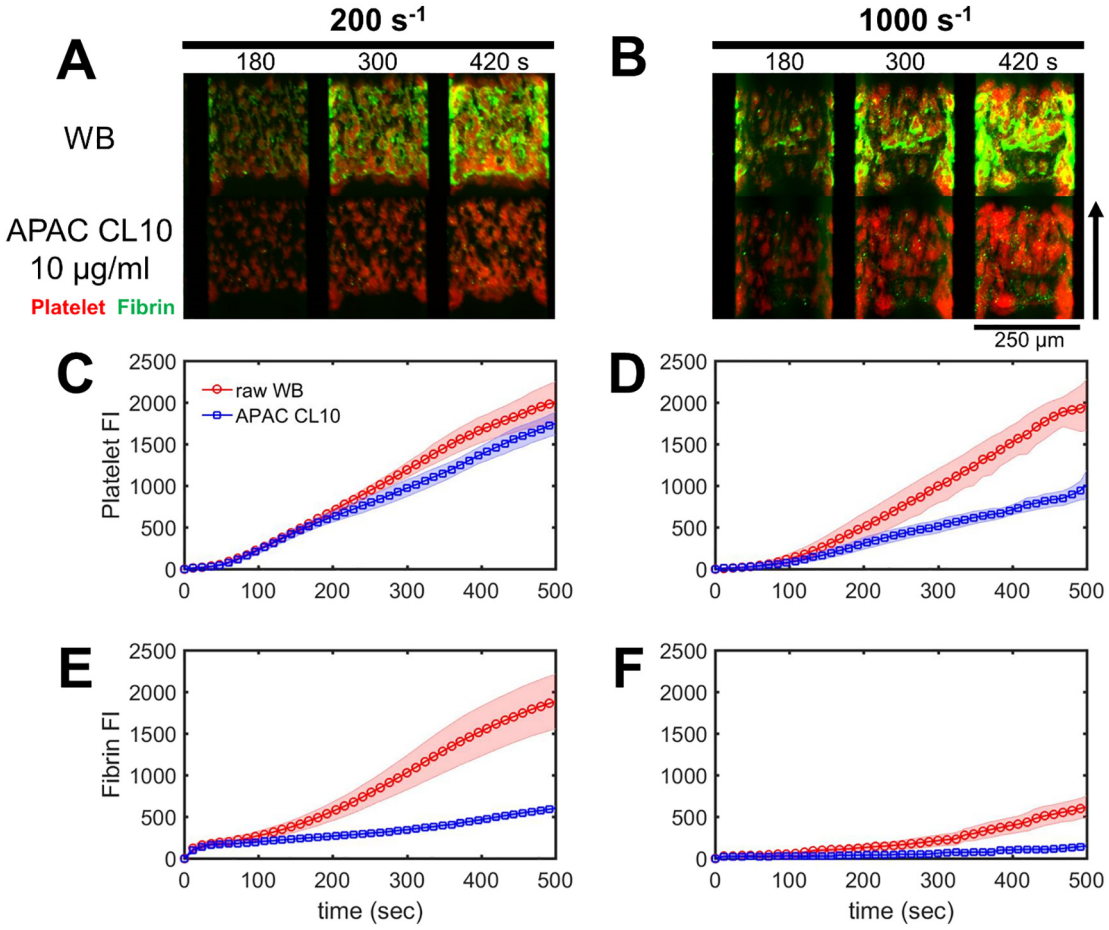

Fig. 4. APAC is more efficient in antiplatelet activity under arterial shear rate compared to venous shear rate. APAC (CL10, 10 μg/mL) was added to non-anticoagulated, freshly-drawn whole blood and perfused over collagen/TF under (A) venous shear rate and (B) arterial shear rate. APAC has stronger effect on inhibition of platelet deposition. (C), (D) dynamics of platelet deposition and (E), (F) dynamics of fibrin generation (± standard deviation [SD]).

Model 700 Whole Blood/Optical Lumi-Aggregation System. Whole blood was drawn via venipuncture into a syringe loaded with 250 nM apixaban and 4% (*w/v*) sodium citrate (Sigma-Aldrich) under the same IRB approval as above. Citrated whole blood is commonly used to chelate calcium ions and prevent platelet activation, but here we use citrate in conjunction with apixaban to further ensure minimal endogenous thrombin generation. Type I fibrillar collagen (1 μL) was added to PRP after 10 min incubation of APAC (or buffer as control) and aggregation was measured for 4 min post-dispense.

3. Results

3.1. APAC has antiplatelet activity in the absence of thrombin under venous shear rate upon collagen

The 8-channel device, developed by Maloney et al. [20], has been used to investigate platelet function and coagulation in whole blood under flow. Briefly, eight inlets of treated or untreated blood were perfused over collagen (with or without TF) while platelet and fibrin deposition can be monitored. The first aim was to determinate the antiplatelet effectiveness alone, without the influence of thrombin and fibrin. PPACK, a direct thrombin inhibitor, was added as an anticoagulant and blood was perfused over collagen at a shear rate of 200 s^{-1}. CL18 (30 μg/mL) inhibited platelet deposition (Fig. 1A) at both early times (100 s) of attachment to collagen and at later times (180 to 420 s) where secondary deposition was occurring, via ADP/thromboxane enhancement [21, 22]. CL18 caused a dose-dependent inhibition (IC50 = 27 μg/mL, based on endpoint fluorescence at 400 s) (Fig. 1B-C). In similar tests with PPACK-treated whole blood, HICL also reduced platelet deposition (IC50 = 57 μg/mL), while CL10 had unclear effect of inhibition on platelet deposition (IC50, not determined) (Table 1, Fig. 3A).

3.2. APAC shows both antiplatelet and anticoagulant activity under venous shear rate upon collagen/TF

High level of CTI (40 μg/mL), a FXIIa inhibitor, was used to avoid clot formation in the reservoir, which allowed the combined study of platelet deposition in the presence of thrombin/fibrin production. Blood was perfused (at the shear rate of 200 s^{-1}) over collagen/TF strip to initiate extrinsic pathway. HICL at 30 μg/mL reduced both platelet and fibrin deposition at venous flow condition with fibrin production strongly antagonized (Fig. 2A). HICL produced a dose-dependent inhibition of both platelet (IC50 = 90 μg/mL) and fibrin deposition (IC50 = 0.5 μg/mL) (Fig. 2B-E, Fig. 3H-I). Similar tests were run for CL10 (Platelet IC50 = 25 μg/mL; Fibrin IC50 = 0.6 μg/mL) and for CL18 (Platelet IC50 = 71 μg/mL; Fibrin IC50 = 5.4 μg/mL) (Table 1, Fig. 3B-C, Fig. 3E-F). For the 3 APAC constructs tested at venous thrombotic conditions, each construct was considerably more potent (> 13 to 180-fold) against thrombin generation/fibrin deposition in comparison to inhibition of platelet deposition.

3.3. APAC in whole blood is more potent as an antiplatelet agent under arterial than venous flow

Microfluidic methods allow the rapid testing of non-anticoagulated,

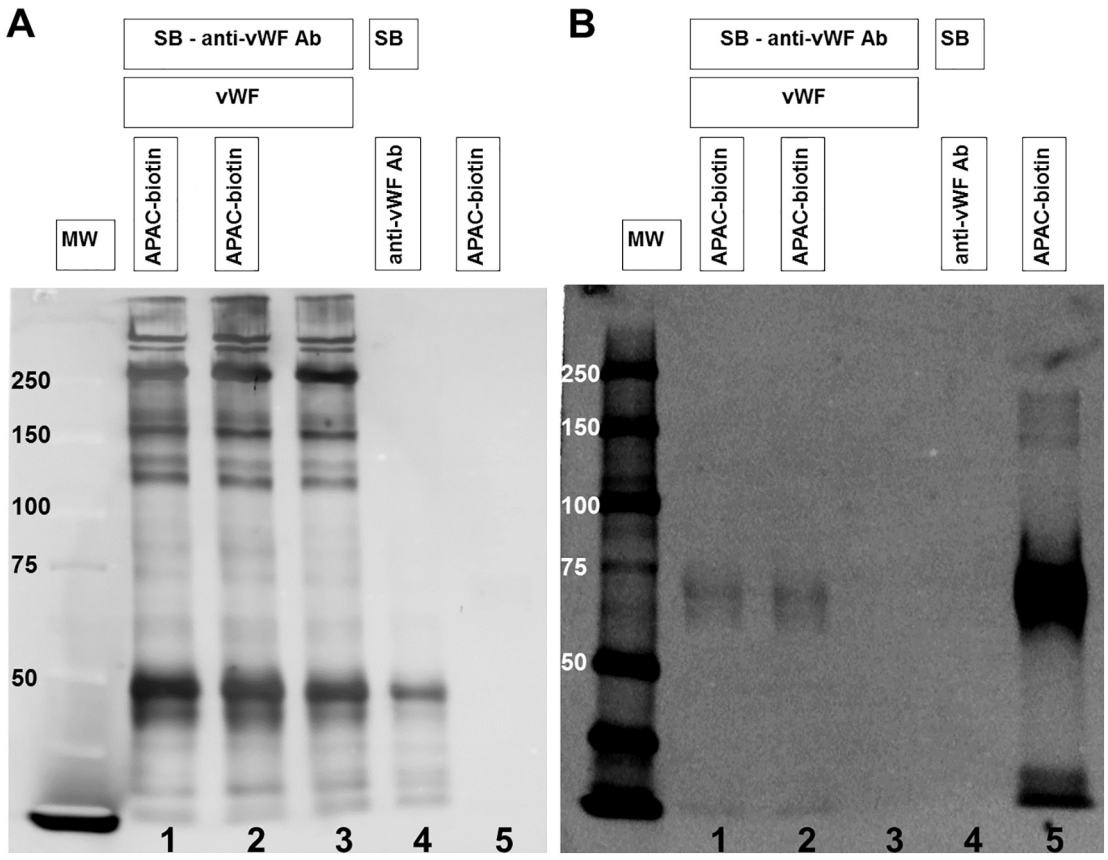

Fig. 5. VWF captures APAC under static conditions, (A) detection of vWF and (B) of APAC. Direct interaction of vWF and APAC was studied with immunoprecipitation. vWF was bound to rabbit polyclonal anti-vWF coated beads and used to capture APAC. In (A), vWF (Wilfactin®) was detected with goat anti-rabbit HRP, and in (B), APAC (CL10)-biotin conjugate was detected with streptavidin-ATTO647. Samples for both (A) and (B) were as follows: 1. and 2. vWF captured APAC (in duplicate), 3. vWF bound to rabbit polyclonal anti-vWF coated beads, 4. rabbit polyclonal anti-vWF coated beads, and 5. APAC-biotin (8 μg). SDS-PAGE (6-%) was run under reducing conditions, resulting in APAC splitting into its constituents, a core protein and UFH. SB = Protein A Sepharose CL-4B beads. vWF = von Willebrand factor.

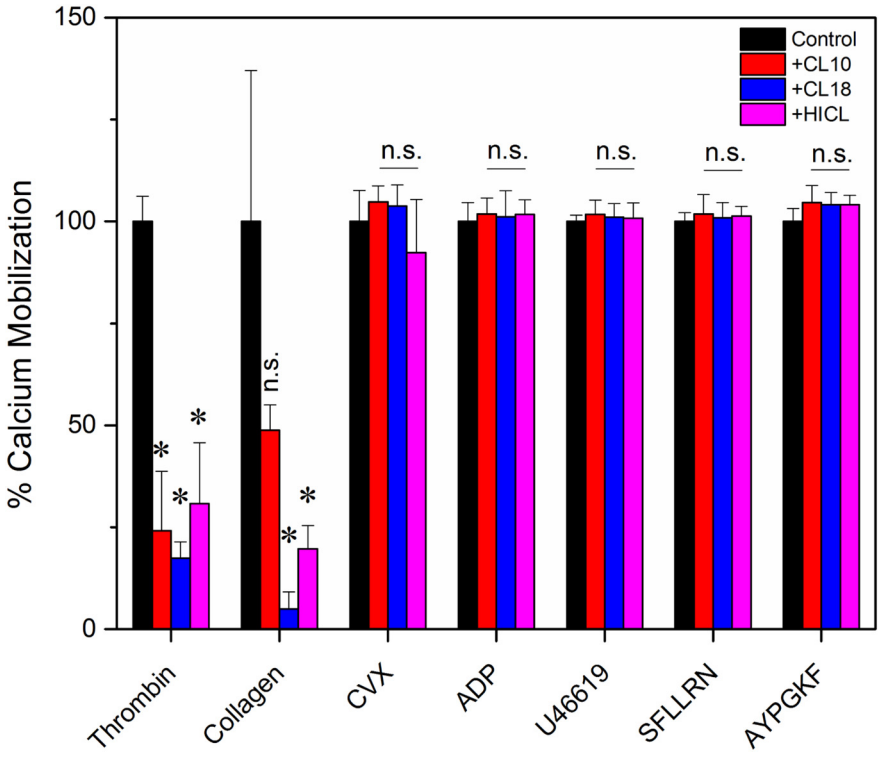

Fig. 6. Thrombin- and collagen-dependent calcium mobilization is specifically reduced in the presence of APAC. Previously determined EC50 concentrations [17, 18] of several platelet agonists were added to dilute apixaban-treated PRP to stimulate platelet activation. A control condition (absence of APAC) was used as the 100% calcium mobilization baseline to which the effect of three APACs (12.5 μg/mL) was compared. Antiplatelet effects were seen by reduced activation levels in cases where thrombin and fibrillar collagen were used as the stimulus. However, all other platelet agonists were not significantly hindered by APAC. Final agonist concentrations: thrombin, 20 nM; collagen, 20 μg/mL; CVX, 2 nM; ADP, 1 μM; U46619, 1 μM; SFLLRN, 10 μM; AYPGKF, 300 μM. (n = 3 donors, $^{*}p < 0.05$, mean ± SD). PRP = platelet-rich plasma.

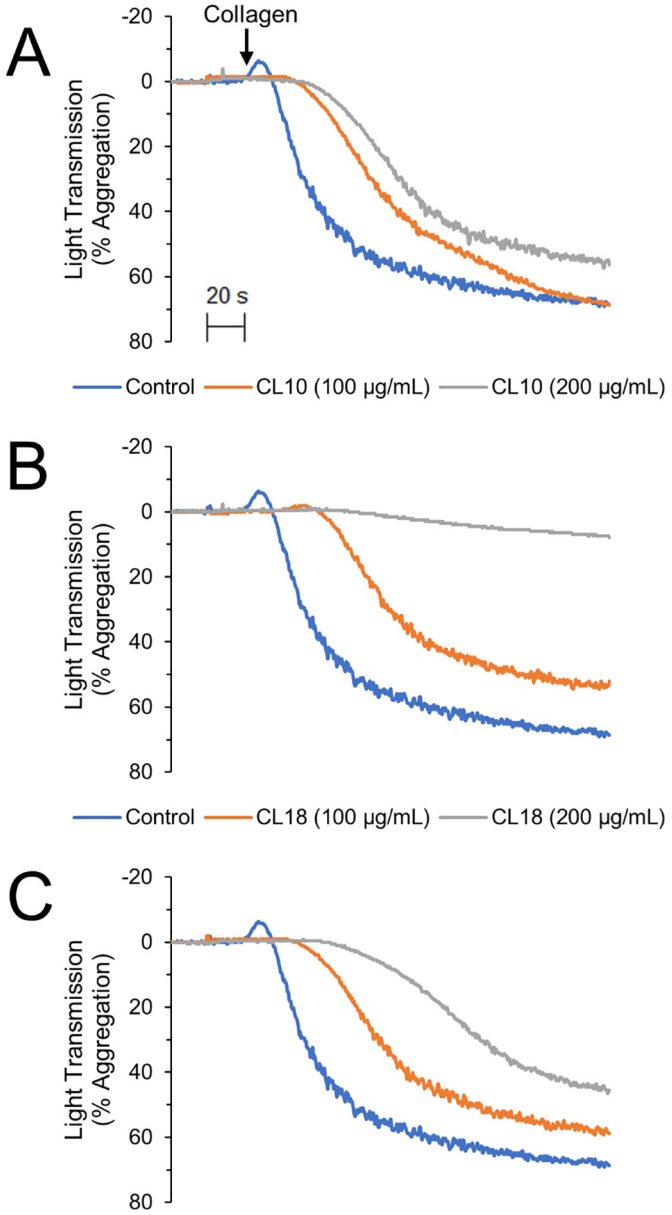

Fig. 7. Platelet aggregation in PRP is inhibited by APAC. A dose-dependent reduction in collagen-mediated aggregation of apixaban/sodium citrate-treated PRP was observed for each of three APACs (A. CL10; B. CL18; C. HICL). A high dose of CL18 was shown to have the greatest effect as aggregation was almost completely abolished, consistent with the calcium mobilization findings reported in the previous section. PRP = platelet-rich plasma.

freshly-drawn whole blood, enabling the determination of clotting in the presence of FXII [16]. Blood samples need to be carefully handled and minimally perturbed. The concentration of APAC was chosen to be 10 μg/mL to match the IC50 order in the previous experiment. Whole blood treated with CL10 (10 μg/mL) was immediately perfused over a collagen/TF surface at either venous (200 s^{-1}) or arterial (1000 s^{-1}) perfusion. At this low concentration under venous perfusion, there was little antagonism of platelet deposition, but strong antagonism of fibrin deposition (as shown in Table 1). In contrast, under the arterial perfusion condition, CL10 displayed considerable potency against both platelet and fibrin deposition (Fig. 4), suggesting that CL10 may antagonize vWF pathways required for platelet deposition at arterial flow conditions. As expected, there was considerably less fibrin generated under control conditions (no CL10) at arterial flow compared to venous flow (Fig. 4E-F). Finally, APAC and vWF interaction was confirmed by immunoprecipitation, where APAC (CL10, biotinylated) was captured by vWF under static conditions (Fig. 5A and B).

3.4. APAC specifically inhibits calcium mobilization due to thrombin and fibrillar collagen but has no effect on other platelet agonists

Intracellular calcium mobilization of dilute apixaban-treated PRP in response to several platelet agonists was measured in the presence and absence of three different APAC molecules. Platelet agonists included thrombin (20 nM), convulxin (a potent platelet glycoprotein (GPVI) activator derived from snake venom; 2 nM), ADP (1 μM), U46619 (a stable thromboxane A2 analog; 1 μM), fibrillar collagen (20 μg/mL), and two PAR-specific ligands SFLLRN (PAR-1 agonist; 10 μM) and AYPGKF (PAR-4 agonist; 300 μM). All APAC concentrations (1.56–100 μg/mL) reduced the platelet calcium response to thrombin and collagen at least 50% compared with the negative control condition. Representative results for 12.5 μg/mL of APAC are shown in **Supplementary Fig. S1**.

For each condition, the area under the curve of the resultant calcium fluorescence trace was calculated and converted to a percentage of the control response (Fig. 6). The effect of heparin conjugation level was also identified to impact the extent of inhibition. Incubation of PRP with CL10 resulted in a 76% decrease in thrombin-induced platelet calcium signal and 51% reduction in the collagen response. CL18 showed the highest attenuation of calcium mobilization, yielding an 83% and 95% reduction in response to thrombin and collagen, respectively. However, increasing the conjugation level above 18 (HICL) still exhibited a statistically significant inhibition, but slightly less than that seen with CL18 and closer to that of CL10. For each of the other five agonists tested, virtually no inhibitory effect was observed, as is evident by ~100% calcium mobilization (Fig. 6). Heparin inhibition of thrombin-mediated platelet calcium mobilization was fully consistent with accelerated thrombin complexation with antithrombin via the heparin functionality of the constructs, while maintaining PAR-1/4 functionality, as evidenced by the unchanged responses to SFLLRN and AYPGKF stimuli. In addition to this anticoagulant feature, previous work has shown the APAC conjugates to also display antiplatelet effects [11], however the effects on intracellular calcium mobilization have not been reported. The antiplatelet effects appear to be specific to collagen and thrombin, since all other platelet signaling pathways and receptor agonists (CVX, ADP, U46619, SFLLRN, and AYPGKF for GPVI, P2Y1/12, TP, PAR1, and PAR4, respectively) were active in the presence of APAC. While collagen can bind heparin, convulxin has no known heparin-binding domain, thus likely explaining the striking difference in APAC activity against collagen stimulation of the platelets that remain fully responsive to the GPVI activator, convulxin.

3.5. APAC reduces collagen-induced platelet aggregation

In a similar test as previously reported [11], aggregometry was used to investigate the antiplatelet features of the heparin proteoglycan mimetic APAC. Each individual APAC (100 or 200 μg/mL final concentration) or buffer was added to PRP, and type I fibrillar collagen (2 μg/mL) was used as the stimulus to measure platelet aggregation in citrated PRP with apixaban addition. The negative control condition (buffer treatment) produced 70% aggregation over the course of the experiment (4 min). For each APAC species (CL10, CL18, and HICL), aggregation was impaired in a dose-dependent manner (Fig. 7A-C). In all cases, the maximal aggregation was lowered, the lag time prior to aggregation was prolonged, and the slope and area under the curve were reduced. The intermediate-chain conjugated species (CL18) was again observed to have the most significant effect, as platelet aggregation was completely abolished at high concentrations (200 μg/mL) (Fig. 7B). Other platelet agonists were screened in aggregometry and the same observations were made in that APAC conjugates only affect thrombin- and collagen-mediated platelet aggregation

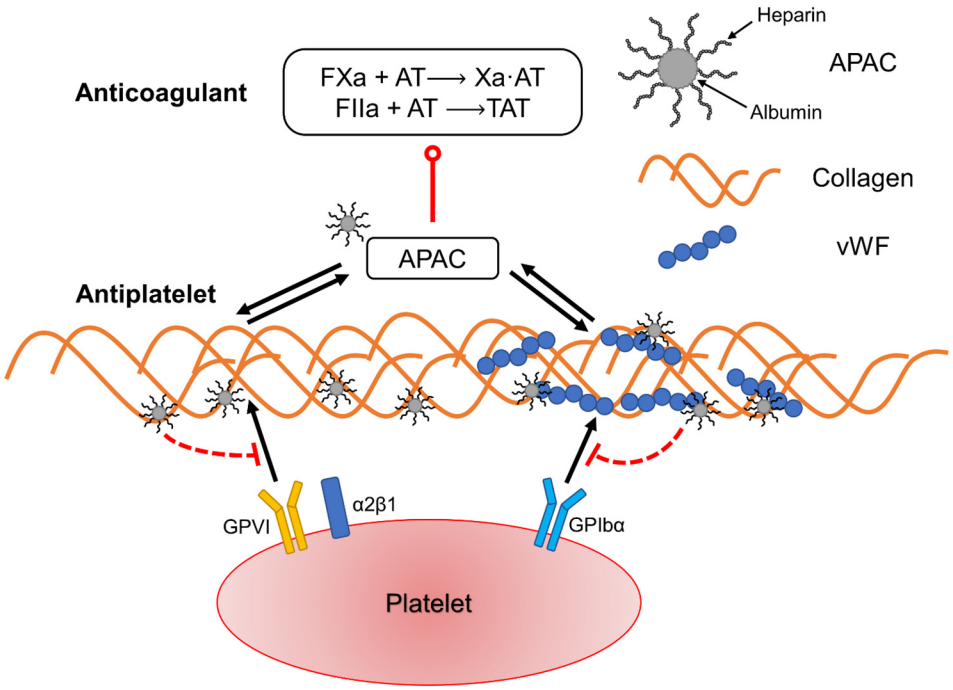

Fig. 8. Schematic of APAC functionality as both antiplatelet and anticoagulant agent. APAC has anticoagulant effect by accelerating the inactivation of thrombin and factor Xa through an antithrombin-dependent mechanism. Its antiplatelet activity results from inhibition on platelet-collagen interactions. APAC strongly inhibited platelet deposition under arterial shear rate by attenuating GPIbα-vWF mediated activation. vWF = von Willebrand factor.

(**Supplemental Fig. S2**), confirming the calcium mobilization results.

4. Discussion

Concern of bleeding risks associated with combinations of two or more blood modulating drugs have sparked interest in developing cardiovascular therapies with dual antiplatelet and anticoagulant (APAC) activity. Using naturally-produced HEP-PGs as a framework for synthetic alternatives, protein functionalized with conjugated UFH chains offers a promising route [11, 15]. With our 8-channel device, we demonstrated APAC antiplatelet activity with PPACK-treated blood perfused upon collagen. Secondly, we analyzed the ability of APAC to interfere with the thrombus growth when CTI-treated blood was perfused upon a collagen/TF surface. We also provide evidence that APACs can directly interact with collagen to reduce platelet deposition under flow and to decrease collagen-induced calcium mobilization. Additionally, the increased inhibitory activity against platelets under arterial flow conditions suggests that APAC when studied in the absence of any other anticoagulant may also reduce vWF binding to collagen or modulate the VWF-GPIbα interaction. APAC interaction with vWF was supported by the immunoprecipitation studies where vWF captured APAC (Fig. 5). A schematic summarizing the major results from Figs. 1–3 is shown in Fig. 8.

To investigate the observed antiplatelet effects of APAC more specifically, we utilized intracellular calcium mobilization as a metric to test the influence of APAC on various platelet signaling pathways. The activation of several platelet receptors leads to distinct signaling cascades that converge on mobilization of calcium ions, so we can infer the level of agonist-induced activation through this reading. A screen of seven common platelet agonists in the presence and absence of three APAC conjugates revealed inhibitory action only towards thrombin and fibrillar collagen (Fig. 5, **Supplementary Fig. S1**). All other agonists (ADP, U46619, convulxin, SFLLRN, and AYPGKF) were sufficiently active with respect to the control buffer condition. Collagen-induced aggregation was also impaired by each APAC molecule (Fig. 7). These results suggest that APAC has specific antiplatelet targets without being a universal inhibitor of platelet activation, leaving the other activation routes intact.

Other than its traditional anticoagulant mechanism, heparin has been implicated to exhibit other antithrombotic effects such as inhibiting endoperoxide metabolites that lead to thromboxane A2 production, suggesting aspirin-like functions [23]. Also, collagen has previously been reported to have unique heparin-binding sites separate from those involved in heparin-triggered thrombin inactivation [13, 24, 25]. Though the functional significance of heparin-collagen binding is still unclear, it may explain the observed inhibitory phenomenon of heparin proteoglycans and synthetic APAC conjugates on collagen-induced platelet aggregation, especially under blood flow.

Collagen activates platelets primarily through GPVI, a uniquely non-G protein-coupled receptor. GPVI belongs to a class of proteins known as immunoreceptor tyrosine-based activation motifs (ITAM) [26]. The converse to ITAM receptors are immunoreceptor tyrosine-based inhibition motifs (ITIM), which inhibit ITAM signaling in order to suppress platelet activation, a pathway that functions along with the prostacyclin-activated IP receptor [27, 28]. Upon first consideration, it would appear, that the APAC conjugates would qualify as ITIM-activating molecules, but the calcium mobilization results showing full GPVI activity via convulxin disproves this hypothesis and points more towards the collagen-heparin interaction. Though certain explanations may be incorrect, and collagen and thrombin appear to be the sole targets, more work should be performed to further refine the specific antiplatelet and platelet anticoagulant mechanisms of APAC.

Currently available treatment by antithrombotics is available only systemically. In our previous, publications we have shown that during local application at the vascular injury site, either in vivo (arterial model of crush injury in baboon) or on the collagen-coated surface ex vivo (collagen coated chamber - shunt model in baboons) APAC inhibits platelet thrombosis and reduces platelet accumulation under arterial shear forces [11]. In addition, we have previous data to show by PET scan that APAC ([^{64}Cu]-APAC) binds at the injury site with extended retention time in comparison with UFH ([^{64}Cu]-UFH) [15]. Our recent data (K. Barreiro et al., submitted) also show targeting and localization of APAC at the injury site in porcine models of balloon denuded iliac and carotid arteries and at the arteriovenous fistula of femoral artery and vein. Based on the results of our current manuscript showing reduced platelet deposition on the highly thrombogenic collagen/tissue factor surface and the inhibition of collagen- and thrombin-induced platelet activation and aggregation support the suggestion to combine

antiplatelet and anticoagulant action in a single antithrombotic, locally acting modality. These actions do not exclude the simultaneous systemic use of e.g. acetylsalicylic acid. Importantly, polyvalent APAC constructs may offer PK/PD advantages relative equivalent free drug.

Contributions

J.C., C.C.V., and S.L.D. designed and ran flow experiment; A.J. and R.L. designed and synthesized APAC reagents and were responsible for the immunoprecipitation. All authors contributed to the manuscript.

Conflict-of-interest disclosure

AJ is employed by Clinical Research Institute Helsinki University Central Hospital (HUCH) Ltd., Aplagon purchases services, including those of AJ, from HUCH Institute. RL is employed by HUCH and has permission to act as CSO of Aplagon. RL has a minority shareholding (< 10%) in Aplagon.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2018.07.026.

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