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Full Length Article

Intravenously administered APAC, a dual AntiPlatelet AntiCoagulant, targets arterial injury site to inhibit platelet thrombus formation and tissue factor activity in mice

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ABSTRACT

Introduction: Arterial thrombosis is the main underlying mechanism of acute atherothrombosis. Combined antiplatelet and anticoagulant regimens prevent thrombosis but increase bleeding rates. Mast cell-derived heparin proteoglycans have local antithrombotic properties, and their semisynthetic dual AntiPlatelet and AntiCoagulant (APAC) mimetic may provide a new efficacious and safe tool for arterial thrombosis. We investigated the *in vivo* impact of intravenous APAC (0.3–0.5 mg/kg; doses chosen according to pharmacokinetic studies) in two mouse models of arterial thrombosis and the *in vitro* actions in mouse platelets and plasma.

Materials and methods: Platelet function and coagulation were studied with light transmission aggregometry and clotting times. Carotid arterial thrombosis was induced either by photochemical injury or surgically exposing vascular collagen after infusion of APAC, UFH or vehicle. Time to occlusion, targeting of APAC to the vascular injury site and platelet deposition on these sites were assessed by intra-vital imaging. Tissue factor activity (TF) of the carotid artery and in plasma was captured.

Results: APAC inhibited platelet responsiveness to agonist stimulation (collagen and ADP) and prolonged APTT and thrombin time. After photochemical carotid injury, APAC-treatment prolonged times to occlusion in comparison with UFH or vehicle, and decreased TF both in carotid lysates and plasma. Upon binding from circulation to vascular collagen-exposing injury sites, APAC reduced the *in situ* platelet deposition.

Abbreviations: ACS, acute coronary syndrome; ADP, adenosine diphosphate; ANOVA, analysis of variance; APAC, AntiPlatelet and AntiCoagulant; APTT, activated partial thromboplastin time; CLEC, C-type lectin-like receptor 2; CVD, cardiovascular disease; FIIa, activated coagulation factor II; FVIIa, activated coagulation factor VII; FX, coagulation factor X; FXa, activated coagulation factor X; HEP-PG, heparin proteoglycan; i.v., intravenous; P, probability value; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; SD, standard deviation; SEM, standard error of the mean; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TT, thrombin time; UFH, unfractionated heparin; VWF, von Willebrand factor.

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Conclusions: Intravenous APAC targets arterial injury sites to exert local dual antiplatelet and anticoagulant actions and attenuates thrombosis upon carotid injuries in mice. Systemic APAC provides local efficacy, highlighting APAC as a novel antithrombotic to reduce cardiovascular complications.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of mortality worldwide. With an estimated 17.3 million annual deaths, it accounts for 32 % of all fatalities – more than double the number attributed to cancer [1] and raises along the proportion of elderly people [2,3]. The essence of cardiovascular complications, *i.e.*, myocardial infarction, ischemic stroke, and peripheral arterial occlusion, is arterial thrombosis [4], where platelets play a crucial role. Platelets undergo rapid activation after adhering to sites of endothelial erosion or plaque rupture, and initiate thrombus formation.

Accordingly, platelet antagonists are the first line therapy for patients suffering from acute coronary syndrome (ACS), peripheral arterial occlusive disease, and ischemic stroke [5,6]. Together with tissue factor (TF) and thrombo-inflammation, platelets reinforce arterial thrombosis and clot stability [7–9]. Anti-TF treatment inhibits arterial thrombosis in animal models [10] and elevated circulating TF levels are observed in patients suffering from ACS [11], diabetes [12], dyslipidemia [13], and hypertension [14], supporting the clinical role of coagulation.

The combined role of platelets and coagulation in the setting of CVD is supported by the COMPASS *(*Cardiovascular Outcomes for People Using Anticoagulation Strategies) trial, where the combination of very low-dose rivaroxaban and aspirin improved outcomes in patients with stable atherosclerosis compared to aspirin alone [4]. While this regimen improved outcomes, it also resulted in higher bleeding rates [15,16]. Therefore, the discovery of novel strategies with both antiplatelet and anticoagulant efficacy but without excess bleeding remains an unmet medical need [17].

Mast cell-derived heparin proteoglycans (HEP-PG) carry localized antithrombotic properties in vascular tissue [18]. The HEP-PGs from porcine intestinal mucosa or bovine lung provide the clinical anticoagulant heparin, which is isolated from the protein backbone [17]. The original macromolecular HEP-PGs inhibit collagen-induced platelet activation, especially under von Willebrand factor (VWF)-dependent high shear rates, unlike the medically used heparins [19–21].

We have previously studied the antithrombotic properties of the semisynthetic heparin bioconjugate APAC, a mimic of HEP-PGs, comprising of 5–9 unfractionated heparin (UFH; ~17 kDa) chains covalently bound to a human serum albumin (66 kDa) core [22]. APAC displays both platelet-inhibitory and anticoagulant effects in human blood *in vitro*, unlike UFH [22–25]. Specifically, APAC inhibits only collagen- and thrombin-induced platelet aggregation, and calcium mobilization in human platelets, leaving the other pathways intact [23,25]. In rodents, intravenous (i.v.) APAC distributes to kidneys, liver, and spleen, with similar clearance rates to UFH [22,24]. APAC's anticoagulant action is assessed by thrombin time (TT) and activated partial thromboplastin time (APTT) [22,24,26]. Interestingly, tail-bleeding times in rats were shorter with APAC than UFH, suggesting a favorable risk-benefit profile [22].

Locally applied APAC at the site of stenosed vascular injury or a collagen-coated graft, inhibited thrombus propagation *via* reduced local platelet deposition in two baboon models *in situ* [22]. To porcine angioplasty or arteriovenous fistula, the *in situ* -administered APAC co-localized with VWF and laminin, unlike intact endothelium [27]. I.v. APAC protected kidneys from ischemia-reperfusion injury in rats [24]. These antithrombotic effects recapitalized in human blood, where APAC alone as an anticoagulant (without citrate) halved the VWF-dependent platelet deposition on the collagen-TF surface under high shear-rate flow conditions [25]. Our overall studies support APAC as a local therapeutic for vascular injuries, such as arterial grafting or stenting.

In this study, we explored for the first time the local antithrombotic potential of i.v.- administered APAC at two dose levels based on the pharmacokinetic and toxicology program [26]. We compared the vascular targeting and subsequent antithrombotic impact of circulating APAC with UFH or vehicle (phosphate-buffered saline, PBS, or saline) in two mouse models with either endothelial-specific photochemical injury [28–30] or surgically exposed adventitial collagen surface in the carotid arteries [31].

2. Materials and methods

2.1. Mice

Study design and experimental protocols were approved by the respective institutional animal ethics committees (ZH219/18 and UNC APAC #19-104), and the care complied with the European and American convention on animal care. Experiments of arterial thrombosis were performed in 12–week old male C57BL/6 or /6 J wildtype mice (Jackson Laboratories, Bar Harbour, Maine, USA). All animals were maintained at a 12-h light dark cycle with ad libidum access to food and water. Two *in vivo* carotid injury thrombosis models were used: laser-induced photochemical injury [29–31], and adventitial collagen exposure by insertion of a small epigastric artery within a carotid artery [28].

2.2. Concentrations of APAC

APAC (Aplagon Ltd., Helsinki, Finland) concentration was determined with a colorimetric Blyscan assay (Biocolor Ltd., Carrickfergus, Northern Ireland, UK) on the heparin-equivalent UFH- (Leo Pharma, Ballerup, Denmark) reference curve. Two clinically relevant doses were selected based on the pharmacokinetic and toxicology program and our previous experience [26].

2.3. Platelet aggregation

Platelet aggregometry studies were performed as previously [32]. Briefly, whole blood was collected (3.8 % citrate anticoagulant) from mice, and either washed platelets or platelet-rich plasma (PRP) were isolated. Washed platelets were incubated with APAC at 5 μg/ml, and PRP at 2.5, 5, and 10 μg/ml, or with UFH, at the respective concentrations, or with PBS (pH 7.5), for 15 min prior to initiation of aggregation with collagen (2 μg/ml; Chrono-Log, Havertown, PA, USA) or ADP (2 μM; Chrono-log, Havertown, PA, USA). Platelet aggregation was studied (Chrono-Log response to collagen, as assessed) under constant stirring (600 rpm) at 37 °C. The results are expressed as maximal aggregation, lag time, and slope (inclination).

2.4. Anticoagulant activity

Since the low blood volume in mice restricted the sample collection in the *in vivo* phase, the anticoagulant activity of spiked (0.25–1.5 μg/ml) APAC and UFH was studied in (3.8 %-citrated) plasma (mouse C57BL6 plasma, Innovative Research, Novi, MI, USA) with thrombin time (TT) (STA Thrombin 2, Stago, Asnières sur Seine, France) and activated partial thromboplastin time (APTT) (SynthAFax, HemosIL, Instrumentation Laboratory, Bedford, MA, USA) in Start Max analyzer (Stago). We refer to our earlier i.v. anticoagulant actions in our toxicology program [26].

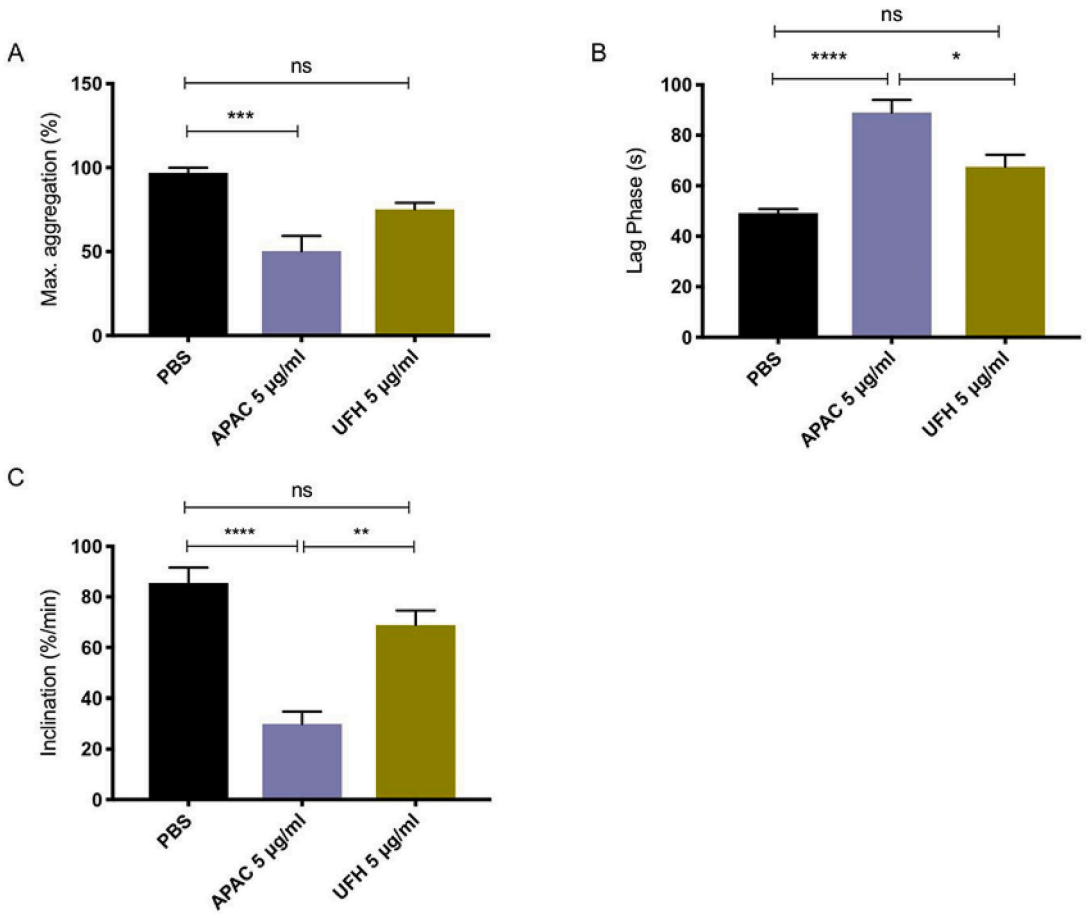

Fig. 1. APAC, unlike UFH, inhibited aggregation of washed platelets in response to collagen *in vitro*. APAC (at 5 μg/ml) *in vitro* inhibited aggregation of washed murine platelets in response to collagen, as assessed by maximal (Max.) aggregation (A), lag time (B), and slope of aggregation (C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = nonsignificant, $n = 6$–7, mean ± SEM.

2.5. Photochemically-induced carotid thrombosis in vivo

To assess the antithrombotic potential of i.v. administered APAC, mice were tail vein-injected with either APAC or UFH (anti-FIIa activity ~200 IU/mg) at equivalent doses of 0.3 mg/kg (60 IU/kg) or with an equivalent volume (100 μL) of PBS. Injections were administered 15 min prior to the onset of the laser injury.

In vivo carotid thrombosis was induced as previously [30]. Briefly, animals were anaesthetized using 87 mg/kg sodium pentobarbital (Butler, Columbus, OH, USA). The right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Transonic Systems, Ithaca, NY, USA) was connected to a flowmeter (Model T106, Transonic Systems, Ithaca, NY, USA) to follow carotid blood flow and heart rates. To induce photochemical endothelial injury, Rose Bengal (63 mg/kg body weight) was injected into the tail vein, and the right common carotid artery was exposed to a laser light beam (1.5 mW, 540 nm, Mellesgriot Inc., Carlsbad, CA, USA) at distance of 6 cm for 60 min. Blood flow was monitored from the laser onset for 120 min or until occlusion (flow ≤ 0.1 ml for 1 min) occurred. After thrombosis had developed, or at 120 min, the animals were euthanized by cervical dislocation to allow the harvesting of blood and carotid arteries. Possible bleeding symptoms were evaluated during and after surgery.

2.6. Collagen-induced carotid artery thrombosis in vivo

To assess the antithrombotic potential of i.v. administered APAC at a 0.5 mg/kg dose, APAC was studied with the collagen-induced carotid artery model [31] and compared with PBS. APAC was fluorescently labeled with CruzFluor647 succinimidyl ester (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a molar ratio of 1:8 in 0.9 M Na_2HPO_4, pH 9 and purified with a PD-10 column (Cytiva, Marlborough, MA, USA) in PBS, pH 7.4, according to manufactures instructions. Integrity of the labeled APAC was confirmed by polyacrylamide gel electrophoresis and platelet aggregation studies. Since the number of free amines targeted by the fluorescent label are limited in UFH, APAC was compared with saline only.

Animals were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were pre-injected with rhodamine 6G (0.5 mg/kg, i.v. through jugular vein) to label mainly platelets, and either with APAC-CruzFluor647 at 0.5 mg/kg i.v. or saline control (50 μl volume for both). The superficial inferior epigastric artery was isolated and dissected from the medial surface of the right leg. A nylon 11–0 suture was placed into one end of the artery and tied in a single-throw knot. The right carotid artery was dissected with background material inserted underneath. It was clamped at proximal and distal extremes. The prepared epigastric artery was brought into the site, and the needle of the suture was inserted into and out of a ventral site on the clamped region of the common carotid artery, over a ~ 0.5 mm distance. By pulling on the needle, the suture advanced through this luminal portion, and subsequently drew the epigastric artery into and out of the site. The passage was stopped when a portion of this small artery was completely inside the carotid artery adjacent to the inner surface of the arterial wall. The occluding clamps were then removed, and intravital fluorescence time-lapse videography of the site was started 1 min after clamp release using 100× magnification for 60 min. Mice were also followed for any

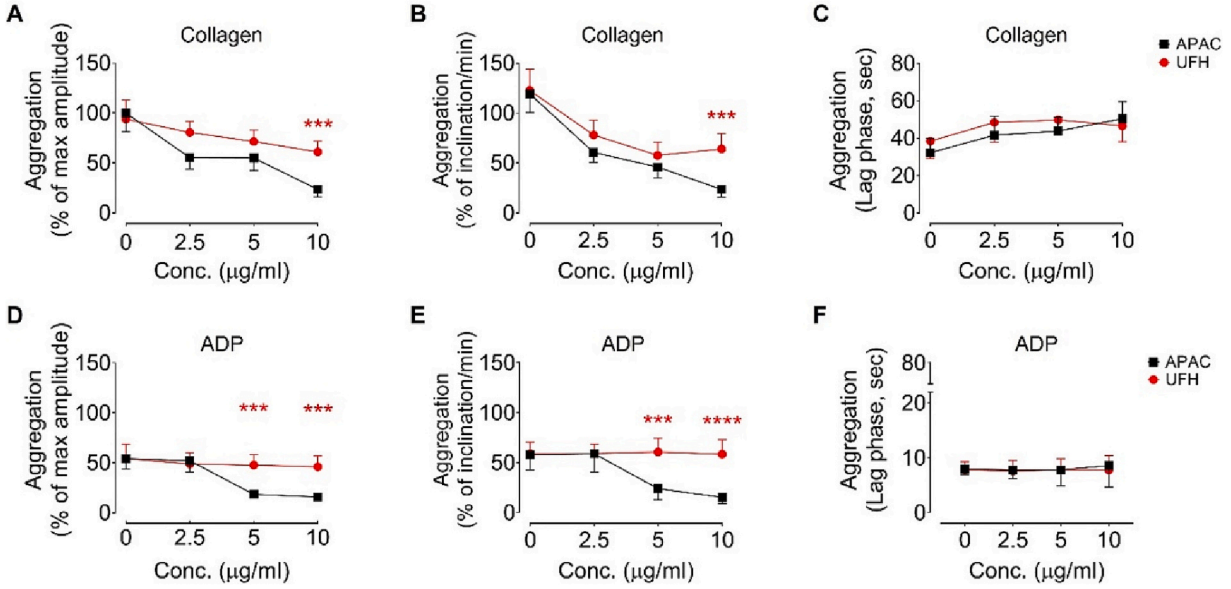

Fig. 2. APAC reduced collagen- and ADP-induced platelet aggregation in PRP in comparison to UFH *in vitro*. Conc. is concentration.
Collagen- and ADP-induced platelet aggregation in murine PRP was reduced by pre-treatment (15 min) with specified concentrations of APAC when compared with UFH. The data are presented as maximal aggregation (A, D), slope of aggregation (B, E), and lag time (C, F). ***P < 0.001, ****P < 0.0001, n = 6, mean ± SEM.

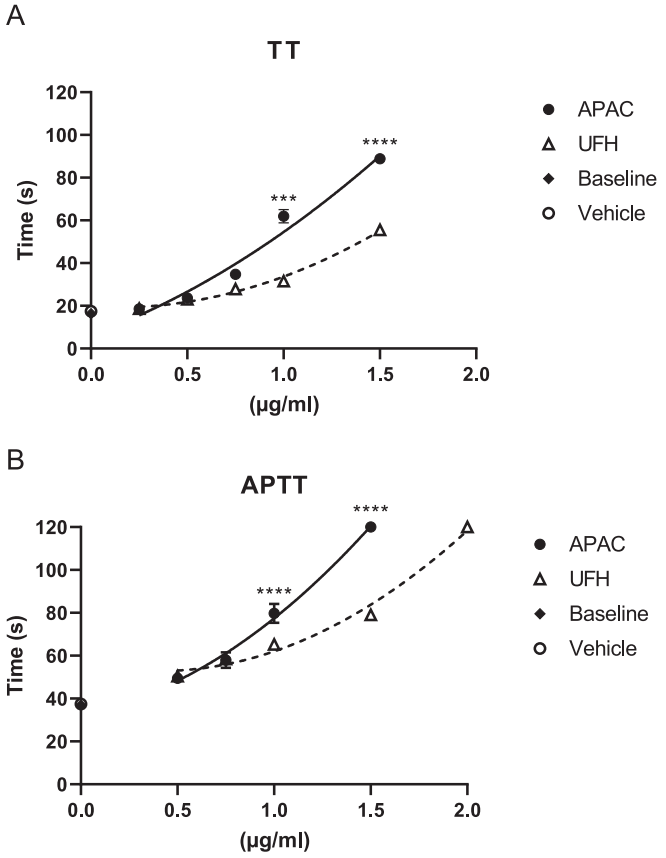

Fig. 3. APAC prolonged TT and APTT *in vitro*.
Concentration-dependent prolongation of thrombin time (TT) (A) and activated partial thromboplastin time (APTT) (B) in APAC- or UFH-spiked citrated mouse plasma. APAC and UFH at concentrations exceeding 0.5 μg/ml prolonged coagulation times compared with baseline. APAC was more potent than UFH at concentrations above 0.75 μg/ml (TT) and above 1.0 μg/ml (APTT). ***$P < 0.001$, ****$P < 0.0001$, $n = 3$, mean ± SD.

bleeding signs. Image analysis for APAC deposition and platelet accumulation was performed with background subtraction for relative intensity, normalizing data for the amounts of injected fluorophores and body weight.

2.7. Tissue factor (TF) assay

TF levels were determined as previously [28] in both plasma and left carotid arteries from APAC-treated and control mice by colorimetric assay, according to the manufacturer (Actichrome®TF, Sekisui Diagnostics, Stamford, CT, USA). Blood was collected *via* intracardiac puncture and immediately mixed with EDTA (1.8 mg/ml of blood) before centrifugation for 15 min at 3000 G. EDTA-plasma was collected and snap-frozen in liquid nitrogen. Carotid arteries were lysed (50 mM Tris–HCl, 100 mM NaCl, 0.1 % Triton X-100, pH 7.4) and total protein concentration was determined by the Bradford protein assay according to the manufacturer's recommendations (VWR Life Science AMRESCO, Solon, OH, USA).

EDTA-plasma and carotid lysates were mixed with coagulation factor (F) VIIa and FX (Actichrome®TF)), converting FX to FXa; FXa subsequently cleaves the chromogenic substrate SPECTROZYME FXa to measure TF activity. Optical density of cleaved SPECTROZYME FXa was determined at 490 nm by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), and subtracted from absorbance at 405 nm. Finally, functional TF (pM) content was calculated, according to a standard curve. For carotid lysates, TF concentration, detected by the colorimetric assay, was normalized to the total protein content of the sample, and expressed as pM/g of total protein.

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). All statistical analyses were performed using GraphPad Prism 7–9 software (GraphPad Software, Inc., La Jolla, CA, USA). Results were confirmed to follow a normal distribution with the Kolmogorov-Smirnov test. The data that passed the normality assumption were analyzed with two-tailed unpaired Student's *t*-test. The data that failed the normality were analyzed with the nonparametric

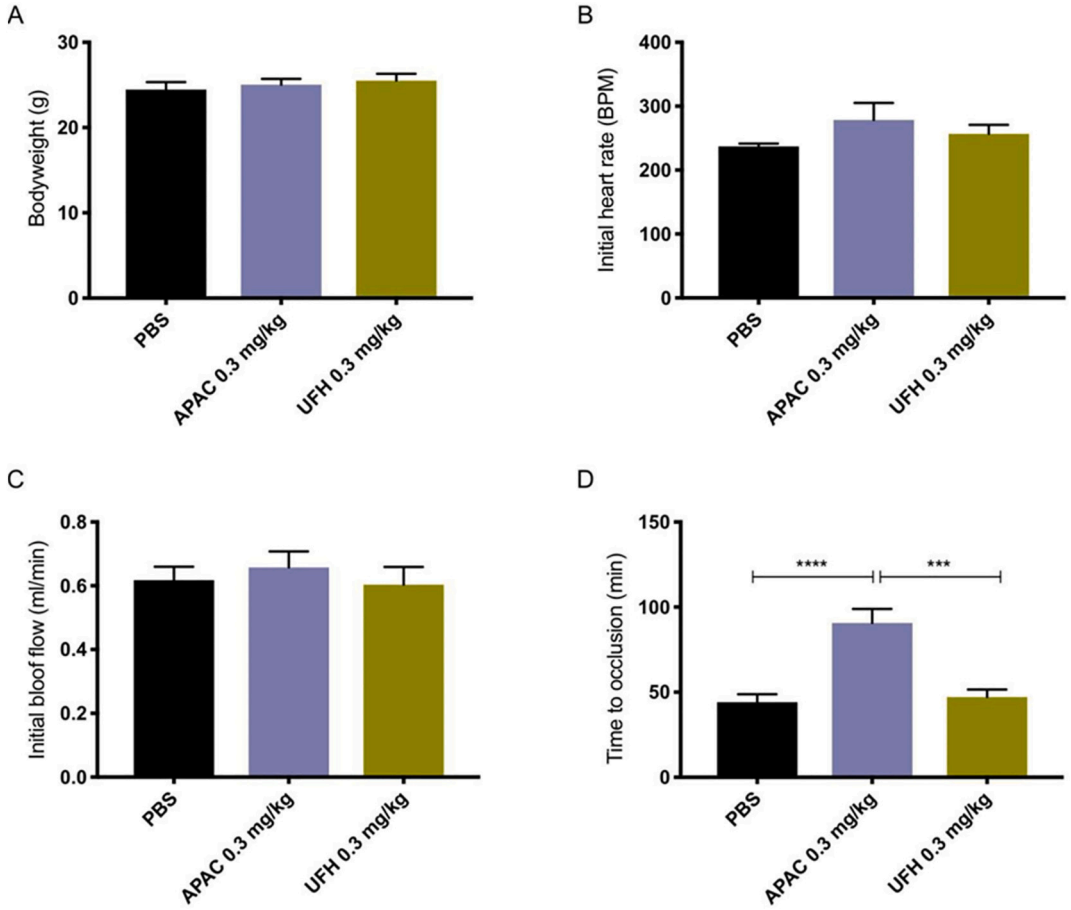

Fig. 4. APAC, unlike UFH, decelerated thrombus formation in photochemically induced carotid artery thrombosis.
APAC- or UFH-treatment did not change body weight (A), initial heart rate (B) or initial blood flow (C). APAC significantly prolonged the time to thrombotic occlusion (D), both compared with PBS and UFH. ***$P < 0.001$, ****$P < 0.0001$, n = 8, mean ± SEM.

Mann–Whitney *U* test. For repeated measurements, one- or two-way ANOVA with Sidak *post hoc* test was applied. A probability value (P) below 0.05 was considered significant.

3. Results

3.1. APAC inhibited platelet aggregation in vitro

To assess the platelet-inhibitory effects of APAC *in vitro*, murine washed platelets and PRP were incubated (15 min) with specific doses of APAC or UFH or with PBS prior to stimulation with collagen or ADP (Figs. 1 and 2).

3.2. Washed platelets in response to collagen

APAC (5 μg/ml) remarkably inhibited maximal aggregation of washed platelets in response to collagen, when compared with PBS (by 48 % Fig. 1A), prolonged lag phase (by 1.8-fold, Fig. 1B), and decreased slope of aggregation (by 65 % Fig. 1C) (Supplementary Fig. 1 for the aggregation curves). APAC, compared to UFH, prolonged the lag phase (by 1.3-fold, Fig. 1B) and decreased the slope of aggregation (by 56 %, Fig. 1C). UFH did not inhibit platelet aggregation in any of the assessed variables.

3.3. PRP in response to collagen and ADP

Moreover, for collagen, according to our dose-response studies APAC at 2.5–5 μg/ml, compared with PBS, reduced maximal aggregation in PRP by 45 %, and APAC at 10 μg/ml by 76 % (Fig. 2A, Supplementary Fig. 2). APAC at 2.5 μg/ml, declined the slope of aggregation (% of inclination/min) for collagen by 49 %, and at 5 μg/ml by 62 %, and at 10 μg/ml by 80 % (Fig. 2B). APAC at 10 μg/ml prolonged lag phase for collagen by 1.6-fold (Fig. 2C).

When compared with UFH, APAC-treatment (10 μg/ml) reduced collagen-induced aggregation by 2.5-times more potently in PRP (Fig. 2A-C).

ADP-induced aggregation in PRP was reduced with APAC at 5–10 μg/ml by 58–73 % (Fig. 2D-F) in comparison to PBS. ADP-induced aggregation was not influenced by UFH under the respective concentrations. The lag phases were similar during APAC- and UFH-treatment whether ADP or collagen were the agonists (Fig. 2C and F).

3.4. APAC prolonged thrombin time (TT) and activated partial thromboplastin time (APTT) in vitro

APAC and UFH concentration-dependently prolonged TT in mouse plasma (17.4 ± 0.6 s, $n = 3$) (Fig. 3A). At 0.5 μg/ml, both APAC and UFH prolonged TT ~1.3-fold the baseline value, while at 0.75–1.5 μg/ml, APAC was 1.2- to 2-fold more potent than UFH (Fig. 3A).

APTT prolonged similarly with APAC- and UFH-treatment at 0.5 and 0.75 μg/ml (from 37.5 ± 0.9 s, by 1.3- to 1.5-fold), respectively (Fig. 3B). At higher concentrations, above 1.0 μg/ml, APAC again was 20–50 % more potent than UFH (Fig. 3B); and APAC reached the upper limit (120 s) of APTT at 1.5 μg/ml, whereas UFH did so at 2 μg/ml.

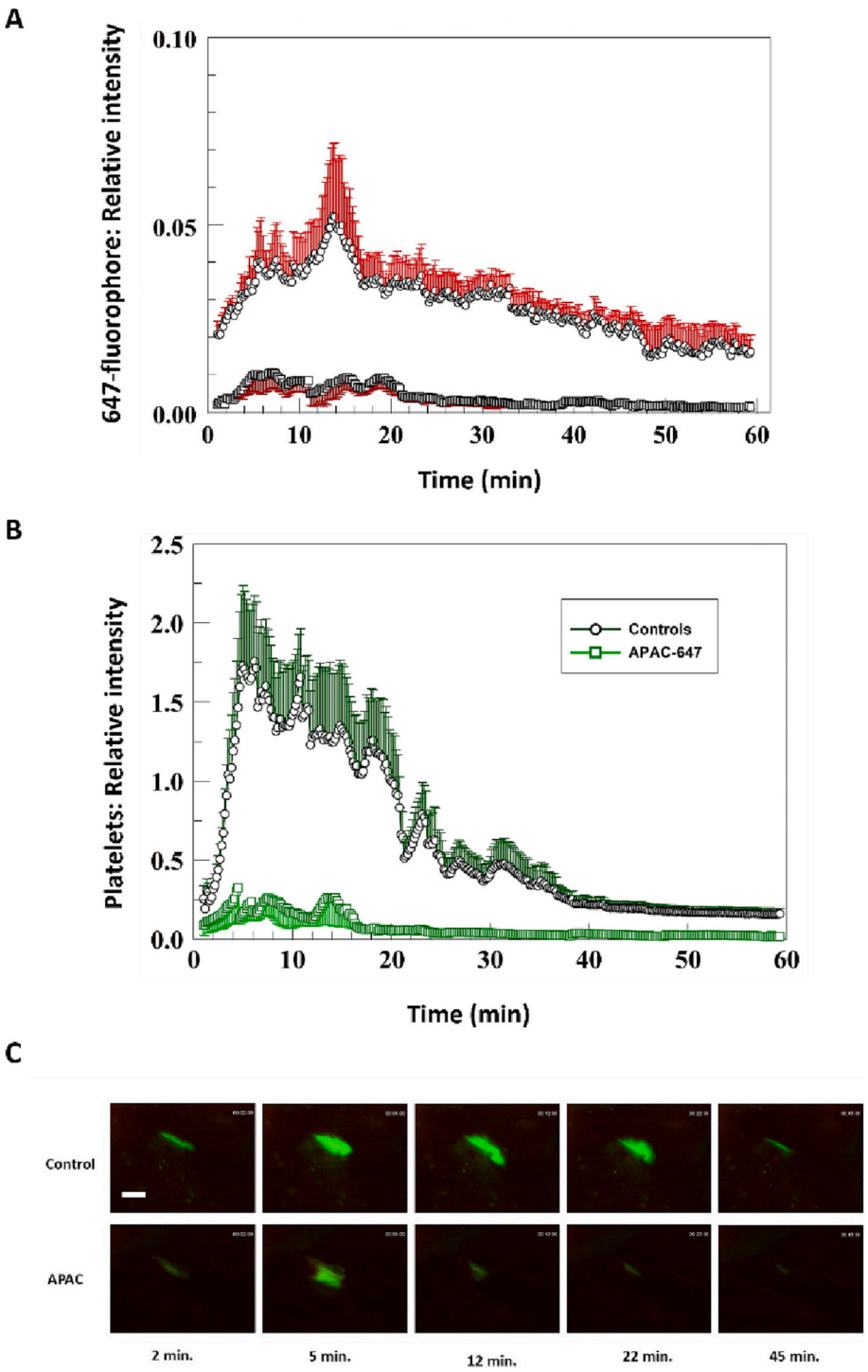

Fig. 5. APAC (0.5 mg/kg) reduced platelet deposition upon targeting to the vascular wall in a collagen-induced thrombosis model.
APAC (circle) binding to the thrombogenic collagen surface *versus* the saline background (square) in the collagen-induced thrombosis model (A) ($P < 0.05$, n = 3, mean of every 10 s ± SEM). Subsequent platelet accumulation with APAC (labeled with Cruz647 fluorophore) (square) or saline control (circle) ($P < 0.05$, n = 6, mean of every 10 s ± SEM) (B). Representative images of time-dependent platelet (green) and 647-fluorescing (red) accumulation at 2, 5, 12, 22 and 45 min after APAC- or saline-treatment in the collagen-induced thrombosis model (flow in the carotid artery is from upper left to lower right, the scale of the white bar is 500 μm) (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. APAC, unlike UFH, prolonged time to arterial thrombotic occlusion in vivo

To investigate the role of systemic APAC on arterial thrombosis, mice received i.v. 0.3 mg/kg of APAC, UFH or volume-matched PBS, 15 min before induction of an endothelial-specific vascular injury by reactive oxygen species in a photoreactive dye (Rose Bengal) and laser. Body weight, heart rate and carotid blood flow did not differ among the groups (Fig. 4A-C). APAC prolonged the time to thrombotic occlusion by 2-fold, both compared with PBS and UFH (mean ± SEM, APAC 91 ± 23 min, $n = 8$ *versus* PBS 44 ± 13 min, n = 8; $P < 0.0001$; APAC *versus* UFH 47 ± 12 min, n = 8; $P = 0.0001$, Fig. 4D). The thrombotic occlusion time was similar with UFH and PBS. No signs of bleeding were detected.

3.6. APAC decreased platelet deposition when binding to the thrombogenic vascular surface in vivo

To assess APAC binding and platelet accumulation to the thrombogenic surface *in vivo*, animals were treated with i.v. infusion of either 0.5 mg/kg of APAC or saline, and then subjected to collagen-induced thrombosis. APAC binding at the thrombogenic surface was confirmed by Cruz647-fluorophore accumulation ($n = 3$) (Fig. 5A). The saline treated controls accumulated more platelets at all time points after 3 min ($P < 0.05$; $n = 6$ per group) than the APAC-treated mice, indicating a

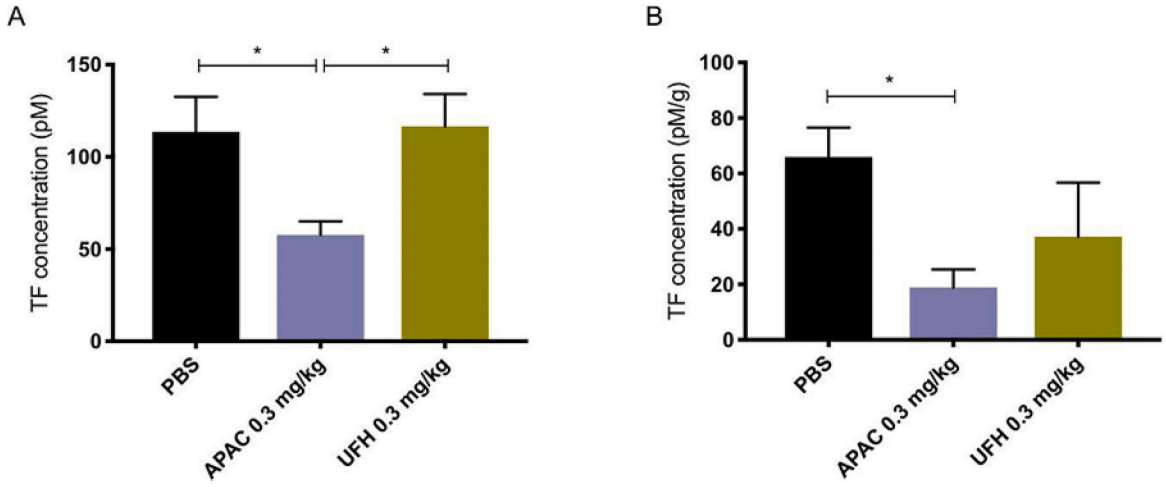

Fig. 6. APAC, unlike UFH, reduced circulating and vascular tissue factor activity.
Upon thrombosis, APAC decreased tissue factor (TF) activity in plasma compared with both PBS and UFH (A), and the content of vascular TF when compared to PBS (B). *$P < 0.05$, n = 5–8, mean ± SEM.

strong inhibitory effect by the systemically administered APAC (Fig. 5B). The relative effect on platelet deposition at the injury site by rhodamine 6G accumulation is shown in Fig. 5C. No signs of bleeding were detected.

3.7. APAC, unlike UFH, decreased TF levels in plasma and in carotid lysates during arterial thrombosis

Due to the key role of TF in arterial thrombosis, TF activity was studied in plasma and carotid lysates in the laser-induced injury model. Plasma TF activity decreased in APAC-treated animals when compared with both PBS- and UFH-treated mice (mean ± SEM, APAC 58 ± 21 pM, $n = 8$ *vs.* PBS 114 ± 50 pM, $n = 7$; $P = 0.038$; APAC *vs.* UFH 117 ± 46 pM, n = 7; $P = 0.028$; Fig. 6A). UFH had no effect on TF activity when compared with PBS.

Vascular TF activity in laser-injured carotid arteries was also reduced by APAC treatment when compared with PBS (APAC 18.8 ± 6.5 pM/g, n = 8 *vs.* PBS 65.9 ± 10.6 pM/g, $n = 5$; $P = 0.043$; Fig. 6B), whereas UFH was less effective (UFH 37.2 ± 19.6 pM/g, n = 5, *vs.* PBS 65.9 ± 10.6 pM/g; P = ns).

4. Discussion

Here we, for the first time, demonstrate that APAC from the systemic circulation is clearly able to target arterial injury sites with dual antiplatelet and anticoagulant action *in vivo*. Our previous work has addressed the *in situ* administration and effects of APAC in crushed arterial injury and arteriovenous fistula [22,27]. Upon vascular targeting, the local thrombotic occlusion is delayed in association with laser-injury or precluded at the sites of surgical, collagen-exposing carotid injury in mouse models. More specifically, i.v. administered APAC decreases local platelet deposition, and reduces circulating and vascular TF activity after photochemically induced endothelial injury. No signs of bleeding were observed during or after the interventions. These findings underscore the dual antiplatelet and anticoagulant effects of APAC and highlight the homing of the antithrombotic effect to the critical sites of arterial damage without hemostatic problems. Likewise, APAC's dual antiplatelet and anticoagulant properties were confirmed in mouse plasma *in vitro*.

Platelets respond rapidly to endothelial injury and vascular collagen exposure [33]. VWF is involved to bridge the platelet adhesion also to collagen. We were careful to address the APAC response in PRP as well as in washed platelets because we have shown earlier that differential mechanisms apply under these conditions, with a direct interaction of platelets with collagen *via* GPIa/IIa and an indirect one with GPIIb/IIIa [34]. Interestingly, APAC was an effective inhibitor under both conditions, pointing at its multimodal effects. Upon stimulation, extracellular matrix not only propagates platelet activation, but also promotes coagulation and subsequent fibrin formation. Antiplatelet agents are the current standard of care for secondary prevention of CVD. To manage acute thrombotic events, they are co-administered with anticoagulants, since the current platelet antagonists alone have limited effects on the platelet procoagulant actions.

We confirmed in mouse plasma and platelets, that beyond being an anticoagulant, APAC also inhibits platelet reactivity to collagen and ADP *in vitro*. We extended these findings to carotid arteries *in vivo* by demonstrating that i.v. administered APAC both delayed occlusion at the sites of photochemically-induced injury and reduced platelet deposition at the collagen-exposing sites. In deviation from our previous human *in vitro* studies, where only collagen and thrombin-induced aggregation were readily inhibited by APAC [22,23,25], in murine platelets, APAC also inhibited ADP. The species differences have been observed earlier [35]. This antiplatelet efficacy of APAC was evidenced without observing bleeding signs, and the hemostatic potential accorded with our earlier published experimental and pharmacokinetic data [26]. In baboons we have shown that when compared with UFH, APAC reduces not only platelet deposition but also, fibrin formation by 50 % [22]. Administration of local APAC, without any other antithrombotics present, inhibited arterial occlusion at the site of 30–90 % stenosed and crush-injured femoral artery (platelet-dependent Folts model). Also, in another baboon model APAC treatment of a collagen-coated graft (shear rate of 265 s^{-1}) inhibited distal thrombus growth and fibrin formation [22]. These *in vivo* finding are supported in human whole blood anticoagulated merely with APAC and explored under blood flow conditions modifying shear rates *in vitro* [25]. In these perfusion studies over surfaces coated with collagen and TF, under the low shear rate, platelet accumulation was not much affected, whereas fibrin formation was reduced. However, under the known VWF-dependent conditions of high shear rate the dual antithrombotic action of APAC was clear: platelet deposition was reduced by 50 % and subsequent fibrin formation even more [25].

Apart from platelet activation, plasma protein- and fibrin-mediated coagulation stabilizes the arterial thrombus. Upon disruption of endothelial integrity, TF activates the coagulation cascade [36,37]. Accordingly, TF is constitutively expressed by cells of the adventitia and media [38] and circulating TF sustains thrombin generation under some pathological conditions [36,39]. Interestingly, HEP-PGs, the model for

APAC, reside abundantly in the adventitial layer, where they may regulate local coagulation activity [40]. Mainly, TF pathway inhibitor (TFPI) regulates TF activity, so only minor amounts of active TF are present in circulating blood [41]. In states of chronic inflammation [42,43], coronary artery disease or atherosclerosis [44,45], circulating levels of TF rise. Activated platelets enhance monocytic TF expression *via* platelet-derived 12-HETE and secrete heparinases which enhance TF-mediated FXa [46–48]. Platelet phosphatidylserine activates the latent monocytic TF by exposing the critical binding sites [49]. Also, protein disulfide isomerase is expressed by activated platelets and is required for thrombus formation in mice [50].

In the current study, we found decreased TF concentration in plasma and injured vascular tissue in APAC-treated animals. In the light of the above and our unpublished data, the platelet-inhibitory effect of APAC may diminish platelet-dependent monocyte activation, a concept to be further studied. Indeed, we have found that APAC binds to adventitial collagen, where it may provide localized inhibition of thrombo-inflammation [51]. Additionally, APAC may hinder endothelial TF to co-localize with VWF, and thereby blocking interaction with activated platelets. In the current study, platelet deposition significantly decreased when APAC bound to the arterial injury site. Moreover, also members of intrinsic pathway, and platelet-derived platelet-factor 4 (PF 4) can be targets of the highly negatively charged APAC.

Relevant to the antithrombotic potential of APAC against platelet-collagen interactions, in previous broad signaling studies in mice, APAC, but not heparin, inhibited platelet degranulation and fibrinogen binding in response to C-type lectin-like receptor 2 (CLEC-2) stimulation [23]. This inhibition by APAC on degranulation was absent in platelets from G6b-B-deficient mice and depended on APAC suppressing CLEC-2-mediated platelet activation *via* G6b-B recruitment of the downstream phosphates.

5. Conclusions

In summary, our study confirms the dual antiplatelet and anticoagulant effect of systemically administered APAC and its targeting from the circulation to the vascular injury site for local antithrombotic action. In addition to concentration-dependent reduction of platelet aggregation and local platelet deposition to the vascular injury, the decreased circulating and vascular TF mechanistically further explains the anticoagulant effects of APAC. APAC therefore uniquely targets the sites of vascular injury and aligns with the physiological role of HEP-PGs in hemostasis [19]. Our aim is to target peripheral arterial disease with APAC based on its localization and VWF recognition [6,27,52].This targeted intervention is reflected by the delayed thrombotic vessel occlusion and reduced platelet deposition under the systemic APAC treatment in our models, importantly without any signs of bleeding. Our findings support the efficacy of APAC after systemic administration and expand on possible therapeutic indications for this novel antithrombotic beyond its local application [53].

CRediT authorship contribution statement

J. H. Beer, R. P. Lassila, G. G. Camici, N. R. Bonetti, B. C. Cooley, A. S. Jouppila and T. F. Lüscher designed the studies. N. R. Bonetti, B. C. Cooley, A. Jouppila, L. Pasterk, L. L. Liberale, and S. Gobbato performed the analyses. All authors contributed to the interpretation of data, writing of the manuscript, and approved the manuscript.

Declaration of competing interest

The corresponding and shared last author of the manuscript, Riitta Lassila has worked with this concept for 15 years and is the CSO of the Aplagon OY. A. Jouppila and B. C. Cooley received research funding from Aplagon Ltd. L. Liberale, and G. G. Camici are coinventors on the International Patent WO/2020/226993 filed in April 2020. The patent relates to the use of antibodies which specifically bind IL-1α to reduce various sequelae of ischemia-reperfusion injury to the central nervous system. G. G. Camici is scientific consultant to Sovida. N. R. Bonetti; S.S. Saeedi Saravi; L. Pasterk; S. Gobbato; T. F. Lüscher and J. H. Beer have no conflicts of interest to declare.

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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.2023.04.010.

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