Blocking the EP3 receptor for PGE2 with DG-041 decreases thrombosis without impairing haemostatic competence

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Aims
Haemostasis interrupts bleeding from disrupted blood vessels by activating platelet aggregation and coagulation. A similar mechanism termed thrombosis generates obstructive thrombi inside diseased arteries. As a consequence of this similarity, current anti-thrombotic agents increase the risk of bleeding. Atherosclerotic plaques produce significant amounts of prostaglandin E2 (PGE2), which activates its receptor EP3 on platelets and aggravates atherothrombosis. We investigated whether blocking EP3 could dissociate atherothrombosis from haemostasis.

Methods and results
Inhibiting in vivo the receptor EP3 for PGE2 with the blocking agent DG-041 reduced murine thrombosis triggered by local delivery of arachidonic acid or ferric chloride on healthy arteries. Importantly, it also reduced thrombosis triggered by scratching murine atherosclerotic plaques. PGE2 was not produced at the bleeding site after tail clipping. Consistently, blocking EP3 did not alter murine tail, liver, or cerebral haemostasis. Furthermore, blocking EP3 reduced murine pulmonary embolism and intensified platelet inhibition by clopidogrel leaving tail bleeding times unchanged. Human atherosclerotic plaques produced PGE2, which facilitated platelet aggregation in human blood and rescued the function of P2Y12-blocked platelets. Finally, in healthy patients, DG-041 reduced platelet aggregation, but did not significantly alter the cutaneous bleeding time at doses up to eight times the dose that inhibited the facilitating effect of PGE2 on platelets.

Conclusion
In mice, blocking EP3 inhibited atherothrombosis without affecting haemostasis and intensified efficiency of conventional anti-platelet treatment without aggravating the bleeding risk. In patients, blocking EP3 should improve the prevention of cardiovascular diseases, which is currently limited by the risk of bleeding.

Keywords
Atherothrombosis • Prostaglandins • Haemostasis • Anti-platelet agents

1. Introduction
Atherothrombosis, the leading cause of death worldwide, is partly prevented by current anti-platelet agents.1 More potent drugs2,3 or drug combinations increase the risk of bleeding,4–6 and bleeding itself increases the risk of re-infarction and death.7 Thus, there is a need for drugs able to intensify the anti-platelet effect without altering haemostasis, although the molecular mechanisms at play in haemostasis and thrombosis are highly intertwined. Some pathways involved in coagulation or platelet aggregation have been shown to predominate in thrombosis over haemostasis with the promise to reduce thrombotic events without increasing the bleeding risk.1 Another strategy is to seek pro-thrombogenic factors that are produced in much higher amounts by atherosclerotic plaques than by healthy vascular wall.
Drugs targeting such factors could reduce atherothrombosis with a minor impact on haemostasis.

Atherosclerotic plaques are inflammatory lesions in which the arachidonic acid (AA) pathway is activated, producing for instance prostaglandin E2 (PGE2), which activates its EP2, EP3, and EP4 specific receptors on platelets, whereas EP2 and EP3 activate the adenylate cyclase. Both higher affinity of PGE2 for EP3 and murine in vivo models of thrombosis showed that the EP3-induced inhibition of adenylate cyclase predominates over EP2 and EP4 activations. So, PGE2 globally decreases the intraplatelet production of cyclic AMP (cAMP), which itself inhibits calcium mobilization triggered by conventional platelet activators, such as adenosine diphosphate (ADP), collagen, thrombin, or thromboxane A2 (TXA2). Hence, PGE2 increases platelet response, i.e. sensitizes platelets to its activators while alone it does not induce platelet aggregation. Specific inactivation of EP3 synergizes with activation of EP2 and EP4 receptors by PGE2 to increase the amount of intraplatelet cAMP that inhibits the platelet response. Consistent with this, we previously reported that in vivo murine atherothrombosis was drastically reduced by the lack of EP3 on platelets.

We also previously observed that tail transection of EP3-deficient mice did not increase bleeding in mice. Since human plaques produce PGE2, these data suggest that targeting the PGE2/EP3 pathway might prevent atherothrombosis without altering haemostasis. However, the lack of impact on murine haemostasis has been challenged, the impact of PGE2 on human platelets has been questioned and demonstration that targeting the PGE2/EP3 pathway dissociates thrombosis from haemostasis is lacking.

To test the hypothesis that atherothrombosis can be controlled without inducing bleeding in mice, we used a selective EP3 blocker, DG-041, known to inhibit PGE2-induced potentiation of aggregation of rat and human platelets.

Using several models, the pharmacological EP3 blockade reduced in vivo thrombosis, but did not alter haemostasis in several vascular beds. We showed that PGE2 re-sensitized platelets in which the ADP pathway was blocked by clopidogrel, and that associating DG-041 to clopidogrel allowed the anti-thrombotic effect to be intensified without aggravating the clopidogrel-induced bleeding. Furthermore, we have shown here that PGE2 was produced by human plaques, and that PGE2 facilitated platelet aggregation in human whole blood. Finally, DG-041 inhibited human platelet aggregation, but not human bleeding time. These data show that blocking the PGE2/EP3 pathway can reduce thrombosis without altering haemostasis.

2. Methods

2.1 Mice

Mouse procedures were approved by ethical committees (Strasbourg), conforming to the directive 2010/63/EU. Anaesthesia and euthanasia were performed with isoflurane. All experiments were performed by investigators blinded to treatments and/or genotypes (full methods in Supplementary material online).

2.2 Platelet aggregation tests

About 250 μL of platelet-rich plasma (PRP, 300 000 platelets/μL) isolated from citrated blood were used for aggregation tests: when indicated, platelets were incubated with DG-041 at 25 °C for 10 min.

2.3 Quantification of murine thrombosis by intravital macroscopy

Left carotid arteries of mice injected with calcein (300 ng/mL)-loaded platelets were exposed to either 4 μL of AA (100 mg/mL, 1 min) or ferric chloride 4% (1 μL, 5 min). Fluorescent thrombosis was video-recorded through a MacroFluo (Leica).

2.4 Mouse model of atherothrombosis

Atherosclerotic plaques were scratched in blood flow using a tiny needle introduced through a branch of the external carotid artery (Figure 1).

2.5 Measurement of PGE2 at the mouse site of bleeding

After amputating 5 mm of murine tail tips, another 3 mm wide piece was cut off for PGE2 detection immediately vs. after bleeding cessation. PGE2 was detected by Enzyme ImmunoAssay (EIA).

2.6 Quantification of murine blood loss

After amputating 7 mm of murine tail tips, surging blood was collected in distilled water (30 mL) up to bleeding cessation. Blood volume was determined from the OD540 plotted against a calibration curve, corrected by blood haemoglobin.

2.7 Liver bleeding

In mice gavaged with vehicle, DG-041 (60 mg/kg) or clopidogrel (10 mg/kg), the median abdominal line was cut 1 h (water, DG-041) or 6 h (clopidogrel) after the gavage. A calibrated piece (3.90 ± 0.15 mg, n = 46) was chopped off from the inferior edge of the right lobe of the liver and the cavity closed. Red blood cells (RBCs) were counted in the 4 mL peritoneal lavage 20-min later.

2.8 Cerebral bleeding

A small borehole was drilled at 2 mm laterally to the bregma. A 25-G needle was lowered down to a 4 mm mark, rotated 360°, and removed. After euthanasia and transcardial perfusion 30-min later, the brain was extracted and cut (1 mm thickness). Haematoma was measured using Image J after deconvolution (fast red-fast blue-DAB; threshold: 160).

2.9 Determinations of murine bleeding time

To test DG-041 increasing doses, a transverse incision was made over the right lateral tail vein. Before AA-induced thrombosis, mice receiving clopidogrel or DG-041 had their 5 mm tail tips transected. In all these cases, the tail was immersed into phosphate buffered saline (37 °C). Bleeding times were measured from incision or transection to first cessation of bleeding.

2.10 Model of mouse pulmonary thromboembolism

Thirty minutes after gavaging mice with DG-041, 5 mL/kg of a solution combining a TXA2 mimetic, U46619 (0.6 μmol/L), and the selective EP3 agonist sulprostone (0.05 mg/mL) was injected via a lateral tail vein to induce intravenous thrombosis through the PGE2/EP3 pathway. Survival was noted 60 min later.

2.11 Measurement of PGE2 in human atherosclerotic plaques

Human carotid endarterectomy samples and non-atherosclerotic endarteries were obtained from patients undergoing carotid surgery or coronary bypass. These tissues, considered as surgical waste, were incubated for 24 h in a volume of Roswell Park Memorial Institute medium adjusted to the wet weight (6 mL/g of tissue) before performing EIA.
2.12 Blood sample collection from human subjects
Informed consent was obtained from healthy volunteers (20–40 years old) in accordance with the Helsinki protocol from local bioethical committees (Strasbourg: CPP#09/504 or the National Bioethics Committee of Iceland). Fresh blood (venipuncture) was collected in citrated tubes from subjects not taking non-steroidal anti-inflammatory drugs.

2.13 Phosphorylated VAsodilator-Stimulated Phosphoprotein assay
Lysed platelets (radioimmunoprecipitation assay buffer) were used to measure protein concentration or phosphorylated VAsodilator-Stimulated Phosphoprotein (pVASP) by EIA. The EIA plates were coated with a monoclonal anti-VASP antibody (Immunoglobe); the primary antibody directed against pVASP Ser157 (Santa Cruz) was revealed by a Horse Radish Peroxidase-α rabbit secondary antibody (DakoCytomation).

2.14 DG-041-CV-007 human clinical trial
This single-blind, randomized, placebo-controlled study (Institutional Review Board at the clinical site) studied three doses of DG-041 (100, 400, and 800 mg twice a day for 7 days). Ten healthy volunteers (18–50 years old) were enrolled into each of the three cohorts. In each cohort, eight subjects were randomized to DG-041 and two to placebo. Bleeding times and platelet aggregation were measured at entry (pre-dose), and 4 h after the morning dose on the seventh day of treatment.

2.15 Human bleeding times
Under blood pressure maintained at 40 mmHg (local cuff), small punctures (1 mm wide and 3 mm deep) using Surgicutt™ (ITC) were performed into the upper forearm of healthy individuals. Blood was blotted by the filter paper below the wound at 30-s intervals.

2.16 Statistics
Student’s t-tests were bilateral and non-paired, except where otherwise indicated. The Mann–Whitney test was used when variances differed. The Kruskal–Wallis test followed by Dunn’s post-test was used instead of one-way analysis of variance when the distribution was not Gaussian.

Means are expressed ± SEM. A P-value of <0.05 denoted significance. Tests were performed using the GraphPad Prism, Version 5.0, for Windows and SAS/STAT software, Version 9.

3. Results
3.1 Blocking the EP3 receptor with DG-041 reduced murine thrombosis
Gavaging wild-type mice with DG-041 (60 mg/kg) dramatically inhibited the facilitating effect induced by PGE2 (10.0 ± 4.8%, n = 7 vs. 60.4 ± 6.0% of maximal aggregation, n = 7 control mice), as efficiently as Ep3−/− platelets (see Supplementary material online, Figure S1).

Figure 1 In vivo blockage of EP3 with DG-041 reduced murine atherothrombosis. (A) Diagram picturing the scratching procedure used to injure the surface of plaques in carotid arteries. (B) a–b: Carotid plaque in a vehicle-gavaged Apoe−− mouse before (a) and after (b) the scratching. Under fluorescence, vascular walls are orange-reddish, plaques are yellow, and thrombosis is green. (c–d) Carotid plaque in a DG-041-gavaged mouse before and after scratching. (C) Quantitative analysis of atherothrombosis in vehicle (n = 12) vs. DG-041 (n = 13) groups.
Furthermore, DG-041 inhibited thrombosis in vivo induced by AA or ferric chloride superfused on carotid arteries (see Supplementary material online, Figure S2). Moreover, we scratched the luminal surface of atherosclerotic plaques in Apeo⁻/⁻ carotid arteries to provoke atherothrombosis. DG-041 strikingly reduced the fluorescent atherothrombosis (0.02 ± 0.01 × 10⁵ pixels/min, n = 12) observed in control mice (1.80 ± 0.60 × 10⁵ pixels/min, n = 13, Figure 1).

Thus, the selective blockade of EP₃ inhibited murine thrombosis and atherothrombosis.

3.2 The PGE₂/EP₃ pathway was not involved in murine haemostasis

To examine the role of the PGE₂/EP₃ pathway in haemostasis, we first examined whether PGE₂ is produced in response to injury. After the amputation of tail tips (5 mm), PGE₂ amounts detected in the next 3 mm distal part of the tails were similar in samples taken upon beginning (555 ± 72 pg/mg, n = 5) vs. cessation (580 ± 88 pg/mg, n = 5, Figure 2A) of bleeding. This lack of difference suggests that the cessation...
of bleeding did not require local production of PGE2. This basal amount of PGE2 detected in the tail tissue does not interfere with the platelet function, since amputation of the tail tips did change the volume of blood loss neither in Ep3−/− mice (see Supplementary material online, Figure S2) nor in Apeo−/− mice of which platelets in carotid arteries were scratched. In the latters, the volumes of lost blood were not significantly different in mice treated with vehicle (128 ± 30 µL, n = 10), DG-041 (85 ± 27 µL, n = 10), or in sham-operated and age-matched WT mice (163 ± 33 µL, n = 10, Figure 2B). We concluded that the PGE2/EP3 pathway was not significantly involved in tail haemostasis.

Since haemostasis is tissue-specific,76,27 we further explored the impact of EP3 blockade with DG-041 on liver and brain bleeding. After a calibrated piece of liver was chopped off from the inferior edge, the number of RBC in the peritoneal lavage did not change in mice gavaged with DG-041 (60 mg/kg) when compared with mice receiving water (1.04 ± 0.39 × 10^6 RBC/µL, n = 14 vs. 0.95 ± 0.18 × 10^6 RBC/µL, n = 15). Conversely, mice gavaged with 10 mg/kg clopidogrel lost significantly more blood (5.22 ± 0.97 × 10^5 RBC/µL, n = 17, Figure 2C). To investigate the effect of EP3 on brain haemostasis, we used a model of needle-induced intracerebral haemorrhage.28 The extent of the cerebral haematoma was significantly increased only in mice receiving 10 mg/kg clopidogrel (22086 ± 5475 red pixels, n = 10), not in DG-041- (60 mg/kg) or water-gavaged mice (6864 ± 1247 red pixels, n = 13 vs. 4967 ± 668 red pixels, n = 10, Figure 2D).

Thus, blocking the EP3 function did not alter tail, liver, or brain haemostasis.

### 3.3 High doses of DG-041 protected against pulmonary embolism, but maintained haemostasis

Next, we used a model of pulmonary thromboembolism to test whether haemostasis remains competent, although doses of DG-041 are increased. Almost all mice (86%) co-infused with sub-stimulating amounts of U46619 and sulprostone, an EP3 agonist, died. This percentage dropped to 20% (n = 15, Figure 3A—bottom) in the group of mice pre-treated with DG-041 at a dose of 10 mg/kg or greater. Conversely, bleeding induced by a calibrated transverse incision of the tail was unaltered by the EP3 antagonist (88 ± 7 s, n = 27 receiving 100 mg/kg vs. 69 ± 6 s, n = 26 controls, Figure 3A—top). This experiment which involved a strong EP3 stimulation clearly shows that increasing the doses of DG-041 decreased thrombosis, while haemostasis remained unchanged.

### 3.4 Activating the PGE2/EP3 pathway restored the sensitivity of murine P2Y12-blocked platelets

Since EP3 activation decreases the cAMP platelet content and hence increases platelet sensitivity, we examined whether PGE2 can re-sensitize platelets subjected to clopidogrel, which blocks the ADP receptor P2Y12 to prevent thrombosis.29 Platelets from mice gavaged with clopigogrel (5 mg/kg) and exposed to ADP (1 µmol/L) aggregated only in the presence of 10^−6 M PGE2 (3.81 ± 0.69%, n = 10 vs. 0.40 ± 0.14%, n = 10; Figure 3B). Thus, PGE2 partially restored the sensitivity of clopigogrel-treated-treated platelets to ADP. Therefore, blocking the PGE2/EP3 pathway should increase the anti-thrombotic effect induced by conventional platelet activators.

### 3.5 Blocking EP3 intensified platelet inhibition without exacerbating bleeding

We next used the model of AA-induced thrombosis to test whether DG-041 can intensify platelet inhibition induced by clopidogrel. Clopidogrel at 2.5 mg/kg reduced thrombosis, but also significantly increased the bleeding time from 120 ± 10 s (controls, n = 9) to 301 ± 58 s (n = 13, Figure 3C—top). Adding DG-041 to clopidogrel further decreased thrombotic scores from 10.72 ± 2.46 × 10^5 (n = 14) to 3.12 ± 1.13 × 10^5 pixels/min (n = 13, Figure 3B—bottom), while the bleeding time did not significantly increase (279 ± 52 s, n = 15). To obtain a similar anti-thrombotic effect with clopidogrel alone, we had to increase the dose up to 10 mg/kg, which clearly exacerbated bleeding (463 ± 51 s, n = 13). Thus, blocking EP3 intensified the platelet inhibition without worsening the bleeding induced by clopidogrel. This opens the possibility to dissociate thrombosis from haemostasis in vivo, at least in mice.

### 3.6 Human atherosclerotic plaques produced PGE2

To examine whether this possibility to control thrombosis independently from haemostasis is clinically relevant, we first checked whether human atherosclerotic plaques produce PGE2. Carotid plaques or fragments of non-atherosclerotic endarteries collected from patients were incubated in adequate culture medium. The 24-h cumulative amounts of PGE2 produced by stenotic (279 ± 48 pg/mg, n = 10) or non-stenotic (144 ± 23 pg/mg, n = 10) fragments of plaques were significantly higher than those yielded by fragments of non-atherosclerotic arteries (6.38 ± 2.12 pg/mg, n = 11, Figure 4A). Thus, human plaques produce PGE2.

### 3.7 The PGE2/EP3 pathway facilitated human platelet aggregation in whole blood

Since EP3 gene sequences30 and platelet expression10,11 are very close in mice and humans, platelet reactivity might be similar in both species. Indeed, PGE2 facilitates in vitro human platelet aggregation in PRP.31,32 To check whether PGE2 also facilitates platelet aggregation in whole blood, blood samples from nine healthy volunteers were subjected to the lowest dose of U46619 triggering a sub-maximal platelet aggregation (166 ± 15 nmoI/L). The adjuvention of PGE2 (10−7 mol/L) to this U46619 dose significantly increased the platelet aggregation (Figure 4B), showing that PGE2 also facilitated platelet aggregation in human whole blood.

### 3.8 Activating the PGE2/EP3 pathway restored the sensitivity of human P2Y12-blocked platelets

Next, we tested whether PGE2 can restore the function of human platelets inhibited by standard medications. We obtained platelets from patients with peripheral arterial disease and medicated with aspirin (80 mg/day, n = 6, ages 61–74) or clopidogrel (75 mg/day, n = 5, ages 48–91), and from non-medicated control subjects (n = 5, ages 66–71). VASP, a cytoskeleton protein, is phosphorylated by cAMP/cGMP kinases; pVASP is thus a marker of platelet cAMP levels.33,34 Thereby, the dephosphorylation of pVASP indicates a cAMP decrease resulting from platelet increased sensitivity or from platelet activation. As expected, adding ADP to ex vivo platelets dephosphorylated pVASP in platelets from control- or aspirin-treated patients, but not in those from clopidogrel-treated patients (93.6 ± 18.5 vs. 32.1 ± 4.8% in
controls, Figure 4C). In contrast, the EP₃ agonist sulprostone dephosphorylated pVASP in all platelet samples, including the group protected by clopidogrel (31.1 ± 4.9 vs. 29.9 ± 6.6% in controls). Thus, these human platelets subjected to standard oral anti-platelet treatments were re-sensitized by EP₃ activation. These results suggest that PGE₂ produced locally by the plaque can increase the sensitivity of platelets to their activators, even in patients receiving anti-platelet therapy.

3.9 Blocking EP₃ on human platelets inhibited the facilitating effect of PGE₂ and preserved haemostasis

DG-041 potently inhibits EP₃ on human platelets (see Supplementary material online, Figure S4). In healthy volunteers ingesting DG-041 for 7 days, the twice-daily 100 mg dose abrogated the ex vivo platelet aggregation induced by sub-threshold collagen in the presence of 10⁻⁶ M PGE₂/EP₃ dissociates thrombosis from haemostasis

Figure 3 Blocking EP₃ dissociated thrombosis from haemostasis in mice. (A) Increasing doses of DG-041 (top, n = 27, 10, 16, and 26, respectively) did not alter tail bleeding times while (below) death induced in the same mice by intravenous co-injection of U46619 (0.6 µmol/L) and the EP₃ agonist sulprostone was prevented by DG-041. *p < 0.05; **p < 0.01; ***p < 0.001. (B) Example of traces (left) and quantitative analysis (right) of platelet aggregation showing that platelets isolated from mice gavaged with clopidogrel were insensitive to 1 µmol/L of ADP (upper trace) when compared with water-gavaged mice (lower trace). However, PGE₂ restored partial aggregation of these P₂Y₁₂-blocked platelets in response to the same amount of ADP (intermediate trace). (C) Bleeding times (top, tail transection) in mice receiving clopidogrel alone or combined with DG-041 (n = 9, 13, 13, and 15, respectively) and (below) effect of the same treatments on carotid thrombosis induced by topical delivery of AA (100 mg/mL, n = 12, 14, 13, and 13, respectively). DG-041 potentiated the anti-thrombotic effect of P₂Y₁₂ blockade without further increasing the bleeding times prolonged by clopidogrel.
Figure 4  PGE₂ was produced by human plaques and altered human platelet function in whole blood. (A) PGE₂ produced by stenotic (‘culprit’: C, $n = 10$) or less stenotic (‘non-culprit’: NC, $n = 10$) human plaque fragments vs. atherosclerosis-free arterial samples ($n = 11$) incubated in culture media for 24 h. (B) Top left, a typical trace of platelet aggregation in whole human blood in response to sub-maximal activation induced by U46619 (200 nmol/L) after adding PGE₂ ($10^{-7}$ mol/L) vs. saline. Below is another example in which the facilitating effect of PGE₂ was minor at 5 min, but more important at 8 min. Right: data from the nine volunteers are reported in ohms (top) measured at 5 min or at maximal aggregation, and below as AUC at 5 or 8 min. Each point is the mean of three replicates. (C) Dephosphorylation of pVASP (i.e. low pVASP ratio) by ADP or sulprostone in patients with peripheral artery occlusive disease (PAOD) medicated with clopidogrel ($n = 5$) or aspirin ($n = 5$) when compared with disease-free platelet donors (controls, $n = 6$). Blocking P₂Y₁₂ with clopidogrel prevented pVASP dephosphorylation by ADP, not by the EP₃ agonist sulprostone. DG-041 prevented pVASP dephosphorylation by sulprostone in all groups; *$P < 0.05$, ***$P < 0.005$, #$P < 0.001$. 

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The effect of EP3 blockade on the scratching model confirms that the PGE2 amount produced by plaque inflammation is sufficient to sensitise platelets. Moreover, it was not known whether it could re-sensitise platelets that have been blocked by conventional anti-platelet drugs. From the total amount of PGE2 detected in Apoe−/− aorta, the average number of plaques (10) in our 50- to 60-week-old mice and murine plaque volumes (0.1–0.001 µL as measured by micro-CT), we calculated that the PGE2 concentration in murine plaques varies from 1 to >400 µmol/L. Here, 1 µmol/L of PGE2 was sufficient to re-sensitize murine clopidogrel-blocked platelets, consistent with the decrease of pVASP observed in human clopidogrel-blocked platelets in response to sulprostone. Thus, plaque-produced PGE2 can reduce the efficacy of conventional anti-platelet treatments, and platelets from clopidogrel-treated patients which appear insensitive to ADP in ex vivo clinical aggregation tests may actually become reactive on contact with ruptured atherosclerotic plaques, which release PGE2. These sensitizing and re-sensitizing effects of PGE2 pinpoint the EP3 receptor as an attractive target to control thrombosis.

Two previous studies showed opposed results on bleeding times in Ep3−/− mice. Here, blocking EP3 did not significantly alter bleeding times after tail transection or vein incision, neither did it alter blood loss after tail transection. Two other approaches (peritoneal RBC counting and brain slice imaging) led to the same conclusion. Based on these five different approaches in the three organs, we concluded that the PGE2/EP3 pathway does not interfere with murine haemostasis. This is in agreement with our failure to detect any significant production of PGE2 at the injury site; it is likely that inflammation is too slow a process to play any significant role in urgent haemostasis.

Thus, targeting EP3 should dissociate thrombosis from haemostasis in humans, provided that the mechanisms at play are similar.

The PGE2 content of human lipid-rich plaques excised from atherosclerotic arteries was previously found too low to alter blood platelet function. We similarly obtained negative results when we measured PGE2 in isolated plaques. Conversely, we detected PGE2 after mashing aortas immediately after harvest or while unfreezing, which suggests that excising plaques from surrounding tissues degrade PGE2. Here, human plaques quickly immersed in culture media produced PGE2, demonstrating that they can produce it. The same authors reported that PGE2 did not alter human platelet aggregation in blood, as shown by the 5-min area under the curve (AUC). We reproduced similar data (P = 0.063); however, maximal aggregations at 5- and 8-min (ohms) and the 8-min AUC were significantly increased. This late response in some individuals may be due to initial higher cAMP platelet contents. Thus, PGE2 is produced by human plaques, facilitates platelet aggregation in whole blood, and therefore can impact atherothrombosis.

Consistent with the lack of any impact on murine haemostasis and in agreement with another recent study, DG-041 up to 1600 mg/day (eight times the dose that inhibits the facilitating effect of PGE2 on platelets) did not alter the human bleeding time. Hence, blocking EP3 opens the possibility to reduce thrombosis without altering haemostasis in humans.

Apart from aspirin, the efficacy of which is limited by bleeding, NSAIDs, or COX-2 blockers increased cardiovascular events in clinical trials, although they inhibit PGE2 production. Actually, these drugs inhibit the entire prostaglandin pathway, unfavourably impacting the balance PGI2/TXA2 and increasing blood pressure. Conversely, blocking specifically the PGE2/EP3 pathway should preserve other prostaglandin functions and avoid vasoconstriction. Blocking the production of PGE2 will also inhibit its multiple physiological functions. Hence, blocking...
specifically the EP3 receptor is an attractive strategy. The EP3 receptor has been detected at significant levels in the kidneys, pancreas, adipocytes, and uterus, but EP3-deficient mice did not display renal, metabolic, or fertility disorders. In human volunteers (Clinical Study Reports for trials DG-041-CV-007, DG-041-CV-008), neither renal function nor glucose levels were altered by DG-041 at doses up to 1600 mg daily for 28 days.

Compared with other strategies developed to preserve haemostatic function, targeting the PGE2/EP3 pathway allows for the restriction of the anti-thrombotic effect to the plaque site, since plaques produce PGE2 as opposed to the non-inflamed bleeding site. Since PGE2 also reduces the protection conferred by clopidogrel, blocking the EP3 receptor increases platelet inhibition at the ruptured site, where anti-thrombosis is crucial.

To conclude, PGE2 is produced by human atherosclerotic plaques and facilitated platelet function in human blood. Blocking EP3 in mice or human volunteers inhibited platelet function while preserving haemostatic function. Thus, haemostasis and thrombosis differ by the selective involvement of the PGE2/EP3 pathway, and therefore targeting the EP3 receptor should increase the efficiency of conventional anti-platelet drugs without increasing the bleeding risk in myocardial infarction or strokes.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interests:** J.E.F. was a consultant at deCODE Genetics for 1 year (2008). K.S. is deCODE Genetics’ CEO and M.E.G. is a former employee of deCODE Genetics.

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