Despite their limitations, unfractionated heparin (UFH) and bivalirudin remain standard-of-care parenteral anticoagulants for percutaneous coronary intervention (PCI). We discovered novel direct thrombin inhibitors (DTIs) from tick salivary transcriptomes and optimised their pharmacologic activity. The most potent, ultravariegin, inhibits thrombin with a $K_i$ of 4.0 pM, 445-fold better than bivalirudin. Unexpectedly, despite their greater antithrombotic effect, variegin/ultravariegin demonstrated less bleeding, achieving a 3-to-7-fold wider therapeutic index in rodent thrombosis and bleeding models. When used in combination with aspirin and ticagrelor in a porcine model, variegin/ultravariegin reduced stent thrombosis compared with antiplatelet therapy alone but achieved a 5-to-7-fold lower bleeding time than UFH/bivalirudin. Moreover, two antibodies screened from a naïve human antibody library effectively reversed the anticoagulant activity of ultravariegin, demonstrating proof-of-principle for antidote reversal. Variegin and ultravariegin are promising translational candidates for next-generation DTIs that may reduce peri-PCI bleeding in the presence of antiplatelet therapy.
DURING Percutaneous coronary intervention (PCI), balloon angioplasty is frequently followed by stent implantation. The procedure causes extensive endothelial disruption and injury, leading to an intense burst of thrombin generation. As such, patients are routinely pre-treated with dual antiplatelet therapy (DAPT) and administered an injectable anticoagulant, commonly unfractionated heparin (UFH), during the PCI procedure. The limitations of UFH include major bleeding, heparin-induced thrombocytopenia (HIT) and the need for coagulation monitoring due to its unpredictable pharmacokinetics. However, UFH remains one of the most widely used parenteral anticoagulants due to low cost (~$4–$10 USD per PCI) and the wealth of clinical experience accumulated in more than eight decades of use. To overcome some of UFH’s disadvantages, an injectable direct thrombin inhibitor (DTI), bivalirudin, was developed as an alternative to UFH in PCI. Although bivalirudin is more expensive (~$400 to $600 per PCI without post-procedural infusion), initial randomised trials showed that bivalirudin was associated with similar antithrombotic efficacy but less bleeding when compared with a combination of UFH and a platelet glycoprotein IIb/IIIa inhibitor. The protocol-mandated use of glycoprotein IIb/IIIa inhibitors in the UFH arm of these trials could have contributed to the higher bleeding rates among patients randomised to UFH. More recent trials with balanced use of glycoprotein IIb/IIIa inhibitors in both bivalirudin and UFH arms have shown less favourable results for bivalirudin. Therefore, in the 2018 European Society of Cardiology/European Association for Cardio-Thoracic Surgery guidelines on myocardial revascularization, routine use of UFH received a higher recommendation (class I) than bivalirudin (class IIb) for peri-PCI anticoagulation. Bivalirudin has been more widely adopted in the United States than in the rest of the world. One study estimated that 47.1% of PCI cases between July 2009 and December 2014 in the US used bivalirudin (52.9% UFH) but also noted a decline in bivalirudin use after 2013. With increasing use of potent platelet P2Y12 antagonists such as ticagrelor, prasugrel and clopidogrel, there remains an even greater unmet need for safer peri-PCI anticoagulants to adequately improve the efficacy-safety balance of antithrombotic therapy during PCI. Combination antiplatelet and anticoagulant therapy has become more common especially among patients with atrial fibrillation or venous thromboembolism undergoing PCI.

Haematophagous animals such as leeches, mosquitoes, ticks, tsetse flies and others are rich sources of antithrombotic agents. Molecules such as hirudin from the medicinal leech, anophelin from mosquitoes, tsetse and tsetse flies are potent and selective thrombin inhibitors that are highly amenable to customisation as synthetic inhibitors with improved properties. We have previously characterised a new class of DTIs, variegin and other variegin-like peptides, from the salivary gland of the tropical bont tick, *Amblyomma variegatum*. Variegin is a 32-residue peptide, binds to both thrombin’s active site and exosite-I. Variegin inhibits thrombin more potently than bivalirudin by sixfold and demonstrates superior selectivity for thrombin. Variegin has a half-life of 25 min and must therefore be administered as a continuous infusion during PCI as the majority of these procedures last ~30 min to an hour. Variegin has a half-life of 52.3 ± 4.4 min and may potentially be given as a single bolus for peri-PCI anticoagulation.

In this study, we hypothesised that a high-affinity, high-specificity bivalent DTI with non-covalent binding to thrombin like variegin would sufficiently prevent thrombus formation during PCI when administered in low doses in combination with aspirin and ticagrelor (DAPT); this low-dose approach to peri-PCI anticoagulation would then preserve the ability to regenerate thrombin when bleeding occurred. Through iterative design and optimisation, we develop a picomolar affinity DTI, named ultrivariegin. We perform global coagulation experiments in plasma to better understand the antithrombotic efficacy versus preservation of haemostatic capacity by taking into consideration the interaction of DTIs with platelets and DAPT. In dose-ranging studies using rodent models of carotid artery thrombosis and tail bleeding, we compare the therapeutic indices of variegin and ultrivariegin with UFH and bivalirudin. Then, in an ex vivo porcine model of coronary stent thrombosis and venous bleeding, we test the efficacy and safety of these anticoagulants in the absence and presence of DAPT. Finally, we identify specific antidotes for ultrivariegin through a screen against a naïve human antibody library and test them against ultrivariegin in vitro and in vivo.

### Results

#### Design of ultrivariegin, a picomolar thrombin inhibitor, from the tropical bont tick *A. variegatum*

Variegin-like thrombin inhibitors in *Amblyomminae* are synthesised as larger precursor proteins containing multiple repeats that are post-translationally processed into shorter peptides. Potential thrombin inhibitor precursors in *A. variegatum* were identified from its salivary gland transcriptome (Fig. 1). Proteomic data and sequence alignment suggest that these peptides have serine residue at the amino terminus. However, the post-translational cleavage site remains unidentified. Considering the variability in sequences, there is uncertainty about the identity of carboxyl-terminal amino acids of these inhibitors (Fig. 1). We synthesised three peptides representing repeats from one of the precursors (GenBank accession number DAAA34688.1 repeat 1, 1B and 1C) with different possible C-termini (Supplementary Figs. 1–14 and Supplementary Table 1). These peptides potently inhibited thrombin’s amidolytic activity on chromogenic substrate S2238 with significant inhibition at equimolar concentrations of inhibitor and thrombin with linear inhibition progress curves (Fig. 2a). This is consistent with the previously reported fast−and−tight-binding behaviour of variegin-like peptides. Truncation of amino acids from the C-terminus resulted in progressively stronger inhibition as indicated by the corresponding inhibitory constant (K_i) values (Table 1). The K_i for DAAA34688.1 repeat 1C is 6.5 ± 1.3 pM, which is 42-fold and 96-fold stronger than synthetic variegin and avathrin, respectively (Table 1). We hypothesised that mutating the non-conserved Thr22 in DAAA34688.1 repeat 1C to Glu would enrich negative charges needed for binding to thrombin exosite-I. Consistent with this design, the K_i improved to 4.0 ± 0.5 pM (Table 1 and Fig. 2b). We named this mutant ultrivariegin, which has a K_i for thrombin that is 445-fold greater than bivalirudin (Table 1). The apparent K_i (K’_i) of ultrivariegin increased linearly with increasing concentration of thrombin chromogenic substrate, indicating that ultrivariegin was a competitive inhibitor of thrombin with respect to thrombin’s active site (Fig. 2c).

#### Novel sequences located at the C-terminal of ultrivariegin impart high affinity towards thrombin

As reported earlier, the N-terminal segment of variegin (residues 1–7) interacts with thrombin through long-range electrostatic steering. Residues 8–14 of variegin target thrombin’s active site and the C-terminal segment binds to thrombin’s exosite-I. Thrombin-inhibiting peptides found in *A. variegatum* (Fig. 1) show the highest sequence variability within the C-terminal region. In UV003, UV004 and UV005, we substituted ultrivariegin residues in segments to the sequence of variegin to investigate which local segment of ultrivariegin is most likely responsible for improved
activity over variegin. Substitutions in the C-terminal segment, predicted to target thrombin’s exosite-I, is most intolerable. $K_i$ of ultrivariegin increased fourfold to 16 pM in UV005 (Table 1). In contrast, replacement of the N-terminal segment (UV003) and thrombin’s active site binding segment (UV004) in ultrivariegin with corresponding sequences of variegin resulted in minimum changes in $K_i$. The sensitivity of ultrivariegin activity to changes in C-terminal residues was also demonstrated by UV012, whereby the A27E mutation increased the $K_i$ fivefold. Overall, ultrivariegin was the most potent peptide (Table 1 and Fig. 2b).

We have previously reported that thrombin cleaves variegin between the Lys10-His11 scissile bond but the cleavage product C-terminal to the scissile bond (sequence: MHKTAPPFDFAETDDES) is a non-competitive inhibitor of thrombin’s active site with a $K_i$ of ~14.1 nM. We synthesised the equivalent cleavage product of ultrivariegin UV011 and showed that it inhibits thrombin (Table 1 and Fig. 2d). Assuming the same non-competitive mode of inhibition, the $K_i$ of UV011 is $1.66 \pm 0.76$ nM. Bivalirudin is also cleaved by thrombin upon binding and we synthesised the equivalent cleavage product of bivalirudin (BV001) (Table 1). Surprisingly, BV001 was not only unable to inhibit thrombin but instead activate thrombin’s active site by up to 20% when tested at concentrations higher than 1 µM (Fig. 2d).

Ultrivariegin was then screened for selectivity against 11 serine proteases involved in blood coagulation and fibrinolysis, as well as trypsin and chymotrypsin. At 0.1 nM, ultrivariegin inhibited thrombin at around 27%, whereas even at 100 µM, ultrivariegin did not inhibit any other serine protein by more than 20%, indicating at least 1,000,000-fold selectivity in preference for thrombin over other serine proteases (Fig. 2e). Thus, ultrivariegin appeared to be a viable lead molecule with a substantially optimised $K_i$ at 4 pM and enhanced selectivity for thrombin.

Haemostatic capacity of blood is largely preserved with variegin or ultrivariegin in combination with DAPT. We then compared variegin and ultrivariegin with UFH and bivalirudin. The activated partial thromboplastin time (aPTT) was dose-dependently prolonged by all four compounds (Fig. 3a). Molar estimates of the concentrations of UFH used were derived to allow for comparisons with the other three compounds on the same axis. Among the three DTIs, ultrivariegin appeared to be most potent, followed by variegin and bivalirudin, consistent with their respective affinity towards thrombin (Table 1). We then performed clot waveform analysis (CWA), in which successive derivatives of the clotting curves indicate activity of individual coagulation enzymes or complexes. The first derivative (min1) represents thrombin activity (i.e., thrombin burst) and bleeding
risk (low absolute value is associated with greater bleeding risk)\textsuperscript{36,37}, the second derivative (min2) represents the activity of the prothrombinase complex and the third derivative (min3) represents activity of the tenase complex\textsuperscript{38}. All three derivatives dose-dependently decreased with each of the four anticoagulants tested. However, the rates declined very sharply above 25 nM of UFH while a decline only occurred when variegin, ultravariegin and bivalirudin were above 1 µM (Fig. 3b).

In the thrombin-generation test (TGT), lag-time (LT) and time-to-peak (TTpeak) for thrombin generation generally increased with increasing concentrations of all four anticoagulants in platelet-poor plasma (PPP) or platelet-rich plasma.
Fig. 2 Inhibition of thrombin by DTIs. a Amidolytic activity of thrombin (0.8 nM) on chromogenic substrate (S2238, 100 μM) in the presence of various concentrations of inhibitors were monitored as an increase in absorbance over time. A representative progression curve of thrombin inhibition by DAA34688.1 repeat 1 is depicted. All experiments were repeated independently as indicated below with similar results. The linear progression curves are characteristic of fast-binding inhibitors. b Residual thrombin amidolytic activity in the presence of various concentrations of various peptides were fitted to a kinetic equation describing tight-binding inhibitors to estimate apparent Ki (K′i). c Ultravariegin showed a linear increase of K′i with increasing concentrations of substrate [100 µM (n = 7); 150 µM (n = 6); 200 µM (n = 6); 300 µM (n = 5); 400 µM (n = 6)], indicating competitive inhibition. The K′i were calculated to be 4.0 ± 0.5 pM. d Despite cleavage by thrombin, the cleaved peptide C-terminus to scissile bond for ultrivariegin (UV011, coloured salmon) retained strong inhibition against thrombin amidolytic activity with IC50 = 1.66 ± 0.76 nM (n = 3). In contrast, the cleaved peptide C-terminus to scissile bond for bivalirudin (BV001, coloured olive) does not inhibit thrombin but instead paradoxically activates thrombin amidolytic activity by around 20% at high concentrations (1–100 µM, n = 4). e Inhibition of the amidolytic activity of various serine proteases by ultrivariegin (n = 3), note the difference in peptide concentrations tested against thrombin compared to other serine proteases. All data are mean ± standard deviation (SD), n is number of independent experiments.

Table 1 Inhibitory constant (K′i) of thrombin-inhibiting peptides.

| Peptide  | Sequence                  | K′i (pM)       |
|----------|---------------------------|----------------|
| Bivalirudin | FPRPGGG-NGDEFEIPEEYL    | 1780 ± 152     |
| BV001     | PGGGG-NGDEFEIPEEYL       | No inhibition; activation at >1 µM |
| Variegin  | SDQGDDAEPKHMTPAPPDFEAIPEEYLDDES | 277 ± 56       |
| Avathrin  | SGHMQTVPKISQQLGDDFEIIPGDIEIE | 624 ± 86   |
| DAA34688.1 repeat 1 | SDAVARA1PKMYSTAPPDPETIPDIAEDEREMKAR | 43.2 ± 8.0 |
| DAA34688.1 repeat 1B | SDAVARA1PKMYSTAPPDPETIPDIAEER | 20.5 ± 4.7 |
| DAA34688.1 repeat 1C | SDAVARA1PKMYSTAPPDPETIPDIAEIE | 6.5 ± 1.3 |
| Ultrivariegin | SDAVARA1PKMYSTAPPDPEDIPDAEIE | 4.0 ± 0.5 |
| UV003     | SDQGDAEIPKHMTPAPPDPEDIPDAIEE | 4.2 ± 1.0 |
| UV004     | SDAVARAEPKHMTPAPPDPEDIPDAIEE | 4.6 ± 0.4 |
| UV005     | SDAVARA1PKMYSTAPPDPEDIPDAEIE | 16.0 ± 0.3 |
| UV012     | SDAVARA1PKMSSHAPPDPEDIPDAIEE | 23.0 ± 8.1 |
| UV013     | SDAVARA1PKMSSHAPPDPEDIPDAEIEE | 4.5 ± 1.6 |
| UV011     | MYSTAPPDPEDIPDAIEIEE | 1660 ± 759 |

All K′i values were determined through inhibition of thrombin amidolytic activity on chromogenic substrate S2238. Values shown are mean ± standard deviation (SD), n = 3 for all peptides except ultrivariegin, in which K′i were determined as described in Fig. 2c. In bivalirudin sequence, F represent D-Ph.

(PRP), with or without DAPT (Supplementary Fig. 15). However, key differences were observed in the rate of increase in LT and TTpeak with increasing concentrations of anticoagulants. The dose–response changes of variegin and ultrivariegin were gradual compared with UFH and bivalirudin (Fig. 3c and Supplementary Fig. 16). Across all conditions, gradients of LT for variegin and ultrivariegin were 1.66 ± 0.3 fold lower than for UFH and bivalirudin; gradients of TTpeak for variegin and ultrivariegin were 2.3- to 13.7-fold lower than for UFH and bivalirudin (Supplementary Table 2). Relative to UFH and bivalirudin, increases in variegin and ultrivariegin concentration result in a more moderate inhibition of thrombin by gradually delaying the thrombin burst.

Endogenous thrombin potential (ETP) for variegin, ultrivariegin and bivalirudin were largely maintained around the baseline, indicating the capacity to generate thrombin was not fully abrogated by the three DTIs (Supplementary Fig. 16). The peak thrombin and velocity index (VI) were increased for variegin across a 100-fold concentration range (0.01–1 μM) in PPP and PRP without DAPT, but this increase was attenuated in PRP with DAPT (Fig. 3d, Supplementary Fig. 16 and Supplementary Table 2). For ultrivariegin, the thrombin peak and VI showed the most gradual change across a 1000-fold concentration range (0.001–1 μM) in PPP and PRP (Fig. 3d, Supplementary Fig. 16 and Supplementary Table 2). In contrast, ETP, peak thrombin and VI were severely impaired in plasma spiked with UFH, indicating inhibition of thrombin production that did not recover within the timeframe of the experiment (Supplementary Fig. 16). The dose–response curves for UFH were steep, with near-complete inhibition achieved within the small (eightfold) concentration range tested (0.025–0.2 μM) (Fig. 3d, Supplementary Fig. 16 and Supplementary Table 2). ETP, peak thrombin and VI increased within the small dose range (fourfold) of bivalirudin tested (1.5–6 μM) under all conditions, suggesting a potential risk for excessive thrombin generation on the rebound (Fig. 3d, Supplementary Fig. 16 and Supplementary Table 2). Taken together, these results suggest that thrombin-generation capacity for haemostasis was best preserved when ultrivariegin, and to a lesser extent, variegin, was used in combination with DAPT.

Variegin and ultrivariegin showed a wider therapeutic index than UFH and bivalirudin in a rodent model. In a rat model of FeCl3-induced carotid artery thrombosis, the time taken for complete carotid artery occlusion increased dose-dependently with all four anticoagulants. A single 5 mg/kg IV bolus injection of variegin resulted in an occlusion time close to 60 min (maximum observed duration) (Fig. 4a). Consistent with its lower K′i value for thrombin inhibition, a lower dose of ultrivariegin at 2 mg/kg fully prevented carotid artery occlusion (Fig. 4b). This maximum level of antithrombotic efficacy was similarly observed with clinically approved doses of UFH and bivalirudin. For UFH, this therapeutic dose was a bolus IV injection of 432 U/kg which translates to a human equivalent dose of 70 U/kg (Fig. 4c). For bivalirudin, it was 10.8 mg/kg/h of IV infusion, which translates to a human equivalent dose of 1.75 mg/kg/h (Fig. 4d).

In the tail bleeding model, bleeding time increased dose-dependently for all four compounds. The occlusion time and bleeding time curves for variegin and ultrivariegin were well-separated, suggesting a wider therapeutic index (TI) than either
UFH or bivalirudin. With the estimated therapeutic dose of variegin (5 mg/kg) and ultravariegin (2 mg/kg), the bleeding time was only at around half of the maximum bleeding time (~30 min). In contrast, the therapeutic dose of UFH and bivalirudin resulted in maximal bleeding (Fig. 4c, d). The dose–response curves for antithrombotic efficacy (occlusion time) and safety (bleeding time) largely overlapped for UFH, indicating a very low therapeutic index, while the same set of curves for bivalirudin showed minor separation. We then estimated the dose of each compound that resulted in a 50% maximum response time (RT50; 30 min) for occlusion time and bleeding time experiments (Fig. 4). The TIs for each compound were calculated as the dose at RT50 for bleeding time divided by occlusion time. The TIs for UFH and bivalirudin were 1.0 and 1.3, respectively, while the TIs for variegin and ultravariegin were 3.8 and 6.7, respectively, around three- to sevenfold better than UFH and bivalirudin (Supplementary Table 3). The wider TIs observed with variegin and ultravariegin are consistent with the preceding in vitro human blood experiments in which inhibition of coagulation is effective but gradual, and haemostatic capacity is preserved.

We further validated the results of the tail bleeding model with a different rodent bleeding model, the saphenous vein bleeding model39,40. We tested variegin, ultravariegin, UFH and bivalirudin at their respective doses that resulted in a 50% maximum response time of occlusion in the carotid artery thrombosis model (RT50; 30 min). In the saphenous vein bleeding model, a higher number of haemostatic events indicate better preservation of haemostatic capacity 39,40. We showed that the number of haemostatic events ranked as follow: ultravariegin > variegin > bivalirudin > UFH (Fig. 4e). The average time per bleeding event had an identical rank order as tail bleeding time experiments: UFH > bivalirudin > variegin > ultravariegin (Fig. 4f). The bleeding time from both models showed strong, positive correlation ($r = 0.978$) (Fig. 4f, insert), indicating consistent results from both bleeding models.

Variegin or ultravariegin is more effective in preventing stent thrombosis than UFH and bivalirudin. We then compared the efficacy of the four anticoagulants in an ex vivo porcine model of stent thrombosis in the absence and presence of DAPT (Supplementary Fig. 17). This model investigates thrombosis when blood is exposed to thrombogenic tissue under flow conditions, and has been commonly employed for antithrombotic drug testing41–45. Simultaneously, we compared the safety of these compounds by performing bleeding time experiments on a
Fig. 4 Efficacy-safety profiles of anticoagulants in rat models. Efficacy and safety were determined using FeCl₃-induced carotid artery thrombosis and tail incision bleeding models, respectively. The duration of observations (y axis) for both models were standardised at 60 min and titled as Response Time. This facilitates the visualisation of the separation between efficacy (occlusion, red) and safety (bleeding, blue) at any given dose of anticoagulants. Time taken for the carotid artery to be completely occluded (red) was plotted against increasing doses of (a) variegin at 1.0 (n = 6), 1.4 (n = 8), 2.0 (n = 8), 3.0 (n = 6), 5.0 (n = 7) mg/kg; (b) ultravariegin at 0.075 (n = 5), 0.2 (n = 7), 0.75 (n = 5), 2.0 (n = 3) mg/kg; (c) UFH at 100 (n = 5), 200 (n = 5), 300 (n = 6), 432 (n = 8) U/kg; and (d) bivalirudin at 1.0 (n = 6), 2.0 (n = 7), 5.0 (n = 8), 10.8 (n = 5) mg/kg/h. Time for occlusion in rats injected with saline (red dash line) was 8.82 ± 3.57 min (n = 6). Time taken for bleeding to stop (blue), was plotted against increasing doses of (a) variegin at 1.0 (n = 6), 2.0 (n = 7), 5.0 (n = 8), 10 (n = 7) mg/kg; (b) ultravariegin at 0.2 (n = 5), 0.75 (n = 6), 2.0 (n = 5), 7.5 (n = 5) mg/kg; (c) UFH at 100 (n = 5), 140 (n = 6), 200 (n = 8), 300 (n = 7), 432 (n = 5) U/kg; and (d) bivalirudin at 1.0 (n = 6), 2.0 (n = 7), 5.0 (n = 7), 10.8 (n = 6) mg/kg/h. Time for bleeding in rats injected with saline (blue dash line) was 4.25 ± 1.67 min (n = 5). Doses of respective anticoagulants to elicit 50% of response in the models (RT₅₀, dotted lines) were estimated and used to calculate the therapeutic index (TI) as listed in Supplementary Table 3. (e) The number of haemostatic events (i.e., clot formation) and (f) average time per bleeding event (i.e., bleeding time) within 30 min in the saphenous vein bleeding model were used to validate results from tail incision bleeding model at doses in which efficacy for respective treatments are at 50% (ie RT₅₀ for occlusion): saline control (n = 5), 1.4 mg/kg variegin (n = 6), 0.3 mg/kg ultravariegin (n = 5), 225 U/kg UFH (n = 6), 3.1 mg/kg/h bivalirudin (n = 5). Figure insert shows correlation coefficient r = 0.978 (p = 0.004) between bleeding time obtained from tail incision model (y axis) and saphenous vein bleeding model (x axis). Data shown are mean ± SD.
superficial ear vein of the same animals. Without anticoagulation (saline), the stents were completely occluded with thrombus within 60 min of blood flow through the extracorporeal circuit (Fig. 5a, b). With 1 mg/kg of variegin, the therapeutic dose translated based on rodent experiments, thrombus formation was reduced by ~83% (Fig. 5a, b). Near-complete thrombus reduction was achieved with pigs receiving 0.25 mg/kg of ultravariegin (Fig. 5a, b). The administration of therapeutic doses of UFH and bivalirudin reduced thrombus formation by ~38% and 61%, respectively (Fig. 5a, b). Compared with saline, all anticoagulants increased bleeding time by 1.9- to 2.4-fold although only the difference between variegin and saline achieved statistical significance (4.2 min vs 10.3 min, multiplicity-adjusted P value = 0.0376) (Fig. 5c).

Low doses of variegin or ultravariegin in combination with DAPT are highly efficacious in preventing stent thrombus formation without increasing bleeding time. When DAPT (300 mg aspirin and 180 mg ticagrelor loading dose) was orally administered 16 h prior to the experiments, in the absence of anticoagulant, thrombus formation on both the aortic strip and stents was reduced significantly compared with saline (10.8 g vs 83.8 mg, two-tailed Student’s t test P ≤ 0.0001), implying that DAPT was partially effective in preventing PCI-related stent thrombosis (Figs. 5a, b and 6a, b). Given the high affinity of variegin and ultravariegin for thrombin, we hypothesised that their combination with DAPT should provide enough antithrombotic efficacy at a much reduced dose of either variegin or ultravariegin, thereby avoiding excessive inhibition of haemostasis. We, therefore, reduced
Fig. 6 Efficacy-safety profiles of anticoagulants (with DAPT) in porcine models. Efficacy and safety were determined using ex vivo stent thrombosis and superficial ear vein bleeding models, respectively. All pigs were administered 300 mg aspirin and 180 mg ticagrelor orally (DAPT regimen) 16 h prior to the experiments. 

a) Representative photographs showing thrombi formed on coronary stents and endothelium-denuded pig aorta strips in pigs administered with DAPT only ($n=13$), DAPT with 0.1 mg/kg variegin (i.v. bolus, $n=5$), DAPT with 0.025 mg/kg ultravariegin (i.v. bolus, $n=3$), DAPT with 100 u/kg UFH (i.v. bolus, $n=4$), and DAPT with 0.75 mg/kg (i.v. bolus) plus 1.75 mg/kg/h (i.v. continuous infusion) bivalirudin ($n=4$). Photograph from a pig receiving saline without DAPT, as depicted in Fig. 5a, is reproduced here for comparison. Experiments were repeated the indicated number of animals independently with similar results.

b) Total weight of thrombi (one-way ANOVA $P=0.0007$).

c) Bleeding time (one-way ANOVA $P<0.0001$). Multiplicity-adjusted $P$ values for post hoc Tukey's multiple comparisons between respective anticoagulants with DAPT are indicated immediately above each treatment. Multiple comparison among anticoagulants were indicated on top of the plot. Data shown are mean ± SD.
Reversal agents for ultravariegin were identified from a naive human antibody library. The availability of a reversal agent would provide an additional safeguard against bleeding complications from anticoagulation. We, therefore, explored the potential of generating ‘active and specific’ reversal agents for ultravariegin. Screening against a naive human antibody library, seven antibodies were found to bind to biotinylated ultravariegin. These clones were expressed and purified as IgG antibodies. These antibodies dose-dependently reversed ultravariegin’s inhibition of thrombin’s amidolytic activity (Fig. 7a). Almost complete reversal of 0.5 nM ultravariegin occurred with two antibodies (Ab1282 and Ab1283). The binding affinity between biotinylated ultravariegin and Ab1282 or Ab1283, measured by biolayer interferometry (BLI), was 1.25 and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultravariegin and Ab1282 or Ab1283, measured by binding affinity to ultravariegin was identified and 1.40 nM (mean of two biotinylated ultrivar...
Fig. 7 Reversal agents for ultravariegin. a Antibodies discovered by screening against a human naive antibody phage-display library dose-dependently reversed ultravariegin’s (0.5 nM) inhibition of thrombin’s (0.8 nM) amidolytic activity (n = 2 independent experiments for each antibodies, except Ab1277, for which n = 3). Representative BLI-binding sensorgrams between biotinylated ultravariegin and Ab1282 or Ab1283 fitted to a 1:1 kinetic model. Concentrations of Ab1282 and Ab1283 used were 20, 6.7, 2.2, 0.74, 0.25, 0.082 and 0.027 nM in a series. Steady-state analyses were performed to obtain dissociation constant $K_D$ at equilibrium (n = 2 independent BLI experiments). d Tail bleeding time from rats injected with 0.75 mg/kg ultravariegin at t = 0 min, and subsequently saline (red, n = 5) or 10 mg Ab1283 (beige, n = 4) at t = 4 min. $P = 0.0078$ from two-tailed Student’s $t$ test between the two groups. The incision for tail bleed starts at t = 5 min. Data shown are mean ± SD.
model, UFH plasma levels corresponded to ~0.6 µM based on clotting time assays (Supplementary Fig. 20 and 21). The plasma concentration of bivalirudin was reported to be around 2.7 µM for PCI51. For 0.1 mg/kg variegin used in combination with DAPT, the average plasma concentration was ~30 nM53. Since ultravariegin was given at a fourfold lower dose compared with variegin, the plasma concentration of ultravariegin was estimated to be 7.5 nM. At low doses, variegin and ultravariegin appeared more potent than UFH and bivalirudin (Fig. 3a) without impacting the rate of synthesis of various enzymes/complexes important to haemostasis, thus causing less bleeding (Fig. 3b). In contrast, 0.6 µM UFH and 2.7 µM bivalirudin resulted in severe impairment of coagulation (e.g., min1 was less than half of the control); a decrease in the absolute value for min1 is clinically associated with an increase bleeding risk (Fig. 3b)54. The TGT also showed that the anticoagulant intensity of UFH and bivalirudin increased sharply over a very narrow concentration range (Fig. 3, Supplementary Fig. 16 and Supplementary Table 2), suggesting a tendency to excessively suppress haemostasis with just small increases in their plasma concentration. By comparison, variegin or ultravariegin produced a smaller, more moderated increase in anticoagulation response over a wider concentration range. In in vitro assays, cleavage of ultravariegin results in two species of thrombin inhibitors: ultravariegin with higher affinity (Kf = 4.0 pM) and UV011 with 415-fold weaker affinity (Kf = 1660 pM). Hypothetically, UV011 may ‘compete’ with ultravariegin for thrombin binding and hence an overall moderated increase in anticoagulation response was observed. In contrast, bivalirudin’s cleavage product does not bind to thrombin, and any increase or decrease in the concentration of bivalirudin is sharply reflected in its anticoagulation intensity (Fig. 3, Supplementary Fig. 16 and Supplementary Table 2). Furthermore, in our rodent models, variegin/ultravariegin also achieved a three- to sevenfold wider therapeutic index compared with UFH/ bivalirudin.

Our study has limitations. First, the experiments using human blood were performed in vitro. Although we used thrombin-generation parameters that are widely validated to predict bleeding in clinical settings32, the in vitro findings may not fully reflect thrombin regeneration capacity in vivo. Second, we used established small and large animal models to test the efficacy and safety of the four compounds but the results will need subsequent verification in human trials. Pre-clinical animal bleeding models may not always predict clinical bleeding risks in human trials because of a variety of reasons53,54. Here, we used three different bleeding models (tail, saphenous vein and superficial ear vein) to increase confidence in the results. Third, we did not perform dose optimisation of UFH and bivalirudin when used together with DAPT, since we aimed to validate variegin/ultravariegin in comparisons with clinically approved doses of UFH and bivalirudin. It remains possible that dose optimisation will result in a more balanced efficacy-to-bleeding profile of UFH or bivalirudin. Fourth, although the two antibodies were able to fully reverse the thrombin inhibition effect of ultravariegin in vitro, the in vivo studies showed that large doses of Ab1283 may be required for complete reversal, hence the molecule may require further optimisation as an antidote.

In conclusion, we discovered a unique class of bivalent, non-covalent DTIs from tick saliva transcriptomes and showed that optimisation of drug affinity-dose relationships can vastly improve the efficacy-safety balance of DTIs in ACS and PCI. Compared with UFH and bivalirudin, this new class of DTIs achieved greater efficacy at preventing ex vivo stent thrombosis but caused far less bleeding. We also demonstrated proof-of-concept in antidote development by identifying two antibody antidotes that effectively reversed the effect of ultravariegin in vitro. These results require corroboration in clinical trials to determine if the salutary effects of this relatively simple affinity-dose optimisation approach is translatable to humans.

Methods

Study approvals. All animal experiments were approved and conducted in accordance with the guidelines of the National University of Singapore (NUS) Institutional Animal Care and Use Committee (R16-008 and R15-0165). All human studies were approved by the NUS Institutional Review Board (B-15-099) and in compliance with the declaration of Helsinki. All participants gave written informed consent prior to study participation.

Peptide synthesis and purification. Peptides were synthesised using an automated microwave peptide synthesiser (CEM, NC, USA). The C-terminal amino acid was loaded to CI-MPA ProTide resin (CEM, NC, USA). Subsequent coupling of amino acids (0.2 M) were performed using 0.5 M N,N’-disopropylecarbodiimide as activator and 0.1 M N,N’-disopropylethylamine in 2 M Oxyam as activator base. Fmoc deprotection was achieved with 10% w/v piperazine in ethanol-N-methyl-2-pyrrolidone (1:9). Cleavage of the synthesised peptides were performed at room temperature for 3 h in the following cocktails: trifluoroacetic acid (TFA)/trisopropylsiliane/water/dioxia-1,8-octane-dihiol (92:5.2:5.2:5.2:5). Peptides were precipitated using cold diethylether. Purification of peptides was performed on Jupiter® 4 µm Protein 90 Å (250 × 21.2 mm) reversed-phase column (Phenomenex, CA, USA) and by electrospray ionisation mass spectrometry (ESI-MS) using an LCQ Fleet Ion Trap MS and Thermo Xcalibur 2.2 software (Thermo Fisher Scientific, CA, USA) (Supplementary Figs. 1-14 and Supplementary Table 1). Mass spectra were deconvoluted by ProMass for Xcalibur 3.0 software. Concentrations of peptide solutions were estimated using UV absorbance at 280 nm and the extinction coefficient was calculated from the peptide sequence. For the two peptides without Tyr (avathrin and UV004), we measured absorbance at 205 nm and estimated the concentrations based on standard curves constructed using peptides of identical length and similar sequences with known concentrations (e.g., ultravariegin and UV003). Ultravariegin and a peptide with scrambled ultravariegin sequence were synthesised with an additional Cys at the N-terminal for conjugation to biotin using EZ-link724iodoacetyl-LC-biotin (Thermo Fisher Scientific, MA, USA) using standard protocols recommended by the manufacturer.

Inhibition of thrombin amidolytic activity. Assays for the inhibition of thrombin (Haematologics technologies, VT, USA) amidolytic activity on chromogenic substrate S2238 (Chromogenix, NY, USA) by peptides were used to estimate half-maximal inhibitory concentration (IC50) and inhibitory constant (Kf). All assays were performed in 50 mM Tris buffer (pH 7.4), 100 mM NaCl and 1 mg/ml BSA at room temperature using 0.83 nM thrombin, varying concentrations of peptides and 100 µM S2238. The rates of formation of product p-nitroaniline were determined at 405 nm for 10 min with an Infinite Pro M200 microplate reader using Tecan Magellan 7.0 software (Tecan, Mannedorf, Switzerland). Dose–response curves were fitted using Prism 6.0 software (GraphPad, CA, USA) to calculate IC50 values with a logistic sigmoidal equation:

\[
y = A_0 + (A_1 - A_0)/(1 + (x/x_0)^h)
\]

where \(y\) is the percentage of inhibition, \(A_0\) and \(A_1\) are the right and left horizontal asymptote, respectively, \(x\) is \(\log_{10}\) of the inhibitor concentration, \(x_0\) is the point of inflection and \(h\) is the slope of the curve.

When an enzyme was inhibited by an equimolar concentration of inhibitor, the binding of the inhibitor to enzyme caused a significant depletion in the concentration of free inhibitors. To determine the apparent inhibitory constant, \(K_f\), the following tight-binding equation was considered:

\[
v_r = (v_c/2E_0)[(K_f^2 + I_c - E_0^2)^{1/2} - (K_f^2 + I_c - E_0^2)]
\]

where \(v_r\) is the steady-state velocity in the presence of inhibitor, \(v_c\) is the velocity observed in the absence of inhibitor, \(E_0\) is the total enzyme concentration, \(I_c\) is the total inhibitor concentration and \(K_f^2\) is the apparent inhibitory constant.

For competitive inhibition, the inhibitory constant, \(K_f\), is related to \(K_c\) by this equation:

\[
K_f = K_c(1 + S/K_c)
\]

where \(K_f\) increases linearly with \(S\), \(K_c\) is the inhibitory constant, \(S\) is the concentration of substrate and \(K_m\) is the Michaelis–Menten constant for S2238. For non-competitive inhibition, the inhibitory constant, \(K_c\), is equal to \(IC_{50}\).

Serine protease specificity. The selectivity profile of ultravariegin was examined against 13 serine proteases: fibrinolytic serine proteases (plasmin, TPA and urokinase), anticoagulant serine protease-activated protein C (APC), procoagulant serine proteases (FXIIa, FXIa, FXa, FXIIa, kalikrein and thrombin) and classical serine proteases (chymotrypsin and trypsin). Effects of ultravariegin on these serine proteases were determined by inhibition of their amidolytic activities
 Assaday using chromogenic substrates specific for the respective enzymes. The final concentrations of proteases and substrates used are given in parentheses in mM and μM, respectively, unless mentioned otherwise: a-thrombin/S2238 (10.8/100), trypsin/S2222 (0.87/100), FXa/Spectrozyme α (333/0.4), FXa/S2656 (0.012/1000), FXa/S2765 (0.24/650), chymotrypsin/S2586 (1.2/650), tPA/S2288 (36.9/1000), VIIa/S2288 (460/1200), plasmin/S2251 (3.61/1200), APC/S2266 (2.74/600), kallikrein/S2302 (0.93/1100), urokinase/S2442 (32 uM/650), IXa/S2302 (20/1000). The activity of thrombin was tested at lower concentrations of ultraviegin (10 nM, 1 nM and 0.1 nM) compared with other proteases (100 μM, 10 μM and 1 μM).

**Estimation for the molar concentration of UFH.** Three vials of 5000 unit/ml UFH from Leo Pharma (Ballerup, Denmark) were individually lyophilised to approximate the average dry weight of UFH after subtracting stated additives. The average dry weight of UFH obtained after subtraction of the weight of excipients, was 136.3 mg, which is equal to 183.5 unit/mg UFH, consistent with reported values in the literature. Assuming the average molecular weight of 10900 g/mol as reported, the equivalent molar concentrations of UFH were converted from U/ml in order to compare UFH with variegin, ultraviegin and bivalirudin, where data were plotted on the same axis.

**Plasma preparations.** Blood was collected by venipuncture from three healthy male donors into 3.2% trisodium citrate tube. Platelet-poor plasma (PPP) was obtained by centrifugation at 2000 × g for 10 min. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood within an hour of sample collection at 180 × g for 10 min. The platelet count of PRP was adjusted to 150,000 platelets/μl with autologous PPP.

**Activated partial thromboplastin time (APTT) and clot waveform analysis (CWA).** APTT was performed on PPP using the Sysmex CS-5100TM (Kobe, Japan) using Actin FSL APTT reagent (Siemens Healthcare Diagnostics, IL, USA). APTT was spiked with variegin, ultraviegin, UFH, or bivalirudin for APTT according to the standard conditions recommended by the manufacturer. The final concentrations of aspirin and ticagrelor were 333 μg/ml and 3.33 μg/ml, respectively. Changes in light transmission were recorded to determine the clot waveform. Clotting times were as reported and the clot waveform was differentiated to derive the first, second and third derivatives (min1, min2 and min3, respectively) using CWA analysis software IPU version 18.

**Thrombin-generation test (TGT).** Thrombin generation was determined using the calibrated Automated Thrombogram (Diagnostica Stago, Asnieres-sur-Seine, France) method in accordance with the manufacturer’s instructions. In all, 980 μl of PPP or PRP were spiked with 10 μl of variegin, ultraviegin, UFH, or bivalirudin, and 10 μl of either DAPT (final concentrations 333 μg/ml aspirin and 3.33 μg/ml ticagrelor) or vehicle (5% dimethyl sulfoxide) accordingly. Preliminary dose titrations of aspirin and ticagrelor for inhibition of platelet aggregation in whole blood were performed using the Multiple Analyzer (Roche Diagnostics, Basel, USA). The lowest concentrations of aspirin and ticagrelor to produce more than 90% inhibition of platelet aggregation were selected for TGT. Thrombin generation of PRP and PPP was triggered using 1 μM and 5 μM tissue factor, respectively. Thrombin-generation curves were calculated using the ThrombinoScope 5.0 software (Thrombinoscope, Limburg, Netherlands).

**Change in various thrombin-generation parameters with increasing doses differed among the four anticoagulants.** The dose–response was plotted with double logarithmic axes to demonstrate linear relationships in anticoagulant intensity (represented by various TGT parameters) with changes in dose. Data were fitted by regression in Prism 6.0 according to the following equation:

\[ Y = 10^a \cdot \log(X) + C \]  
(4)

where Y is either LT, TTP, peak, peak, TEP or VI, X is the concentrations of anticoagulants, m is the slope and C is the Y-intercept. The slopes calculated from curve fittings are recorded in Supplementary Table 2.

**Rats.** Male Sprague-Dawley rats (InVivos, Singapore) were housed in microisolator cages and were kept on a 12-h light/dark cycle with constant temperature and humidity. Rats were anaesthetised with a mixture of ketamine:xylazine (75:10 mg/kg body weight) and maintained with inhaled 1% isofluorane in oxygen throughout. Male Sprague-Dawley rats (InVivos, Singapore) were housed in microisolator cages and were kept on a 12-h light/dark cycle with constant temperature and humidity. Rats were anaesthetised with a mixture of ketamine:xylazine (75:10 mg/kg body weight) and maintained with inhaled 1% isofluorane in oxygen throughout the experiment. Catheters (23 G) were inserted into the left femoral vein and artery, for drug injection and blood collection, respectively.

**Rat carotid artery thrombosis model.** Rat carotid artery thrombosis model was performed as follows: five minutes after an i.v. bolus injection (saline, variegin, ultraviegin, or UFH) or 15 min after initiation of continuous i.v. infusion (bivalirudin), a spring-loaded blade device (Surgicut Adult bleeding time device, ITC, USA) was applied longitudinally on the ventral surface of the tail to produce an incision (1 mm depth × 5 mm length) at 9–9.5 cm from the tip of the tail. The filter paper was used to blot blood from the side of the wound (without touching the wound) every 15 s. Bleeding time was defined as the time after incision until the cessation of bleeding on eight consecutive blots. The experiment was terminated at 60 min after tail incision. Bleeding time was recorded as 60 min if bleeding did not start within this time.

This model was also used for testing the in vivo reversal activity of Ab1283. Four minutes after rats were injected with an i.v. bolus of 0.75 mg/kg ultraviegin, an i.v. bolus of 10 mg of Ab1283 or saline was injected. One minute later, an incision was made on the tail and bleeding time was measured as described above.

**Rat saphenous vein bleeding model.** The saphenous vein bleeding model was performed in rats as follows: the right saphenous vein was exposed and covered with normal saline to prevent drying. Five minutes after an i.v. bolus injection (saline, variegin, ultraviegin, or UFH) or 15 min after initiation of continuous i.v. infusion (bivalirudin), a 23-G needle was used to pierce a hole in the right saphenous vein followed by a longitudinal incision of ~1 mm. Blood was gently wicked away every 15 s until haemostasis occurred. The clot was formed and gently removed using a 30-G needle to restart bleeding. The process of clot disruption was repeated after every incidence of haemostasis. The number of haemostasis events (i.e., clot formation) after repeated clot disruptions within a 30 min period were recorded. The average time per bleeding event was calculated by dividing 30 min with the number of clots (i.e., bleeding time).

**Pigs.** Both male and female SPF pigs (40–70 kg) were locally farmed for research purpose and obtained from the SEMC (SingHealth Experimental Medicine Centre, Singapore). All pigs were initially sedated with a mixture of ketamine (12 mg/kg), midazolam (0.5 mg/kg) and atropine (44 μg/kg). Endotracheal intubation was performed and anaesthesia was initiated with 5% isoflurane for induction followed by 2% isoflurane for the duration of the experiment. Animals were ventilated using an Aspire ventilator machine (GE Healthcare, UK). Plasma replacement (6% volume) was given as a continuous infusion for the duration of the experiment. For the establishment of the extracorporeal loop, the carotid artery and jugular vein were surgically isolated and cannulated with 8 and 9 French percutaneous sheath introducers, respectively. Saline, variegin, ultraviegin, UFH, or bivalirudin, was administered through the cephalic vein cannula and blood were collected from the femoral artery. Real-time monitoring of ECG, heart rate, body temperature, respiration rate, mean arterial blood pressure, blood oxygen saturation and carbon dioxide levels was performed on a LifeWindow Lite multiparameter physiologic monitor (Digicare Biomedical Technology Inc., FL, USA).

**Preparation of the ex vivo perfusion chamber.** The descending aorta was removed from each terminated pig. The fascia and connective tissue were removed from the tunica externa. The aorta was cut into rectangular strips of 7 mm width × 27 mm length. Sharp forceps were then used to peel the smooth endothermal layer away from the rest of the vessel, exposing the tunica media. The thickness of the aortic strip was maintained at 0.5–0.6 mm, as measured using a calliper.

Perfusion chambers and stainless-steel connectors were custom manufactured (Sunway Precision Engineering, Singapore, Singapore). The perfusion chamber consists of two Plexiglas blocks, a bottom block (9 × 16 × 50 mm) with a 2-mm diameter tubular channel, and a top block (5 × 16 × 50 mm). A 28-mm section of the tubular channel was exposed in the middle section of the bottom block. A cobalt-chromium coronary stent (2.5 mm diameter × 23 mm length) was expanded to an outer diameter of 2 mm, pre-weighted and placed inside the tubular channel. A 7 × 27 mm strip of endothelium-denuded porcine aorta was placed on top to cover the exposed surface of the coronary stent. This simulated the contact area between the coronary stent and the coronary artery during PCI. The top block was then used as a cover for the bottom block and secured with a clamp. To connect the perfusion chambers to the tubing used in the extracorporeal loop, stainless-steel connectors (with a 2-mm diameter tubular channel) were screwed into the bottom Plexiglas block, directly in line with the tubular channel of the Plexiglas perfusion chamber.

**Porcine ex vivo stent thrombosis model.** For UFH, 100 U/kg was injected initially and 30 U/kg UFH top-up was used if needed to maintain the activated clotting time between 250s and 350s as per clinical practice guidelines. Bivalirudin was administered through an i.v. bolus injection of 0.73 mg/kg followed by a continuous i.v. infusion (DAPT) or 0.1 mg/kg (with DAPT) was injected as a single bolus without additional doses. For ultraviegin, either 0.25 mg/kg (without DAPT) or 0.025 mg/kg (with
DAPT were injected as a single bolus. DAPT (300 mg aspirin and 180 mg ticagrelor) was administered orally 16 h prior to surgery. The extracorporeal loop was set up by connecting the patient to an extracorporeal circuit (Pall ForteBio, CA, USA) and data analysis performed using ForteBio Data System (Pall ForteBio, CA, USA). The remaining data generated in this study are provided in the Article, Supplementary Information or Source Data file. Source data are provided with this paper.

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**References**

1. Davies, M. J. The pathophysiology of acute coronary syndromes. *Heart* 83, 361–366 (2000).
2. Luscher, T. F. et al. Drug-eluting stent and coronary thrombosis: biological mechanisms and clinical implications. *Circulation* 115, 1051–1058 (2007).
3. Franchi, F., Rollini, F. & Angiolillo, D. J. Antithrombotic therapy for patients with STEMI undergoing primary PCI. *Nat. Rev. Cardiol.* 14, 361–379 (2017).
4. Bonaca, M. P. et al. Antithrombotics in acute coronary syndromes. *J. Am. Coll. Cardiol.* 54, 969–984 (2009).
5. Hillegass, W. B. & Bradford, G. S. Risk guided use of the direct thrombin inhibitor bivalirudin: insights from recent trials and analyses. *J. Thorac. Dis.* 8, E1034–E1040 (2016).
6. Widimsky, P. Is bivalirudin just an expensive heparin? *Eur. Heart J.* 37, 1321–1324 (2016).
7. Mackman, N., Bergmeier, W., Stouffer, G. A. & Weitz, J. I. Therapeutic mechanisms and clinical implications. *Nat. Rev. Cardiol.* 9, 361–379 (2012).
8. Stone, G. W. et al. Antithrombotic strategies in patients with acute coronary syndromes undergoing early invasive management: one-year results from the ACUTITY trial. *J. Am. Med. Assoc.* 298, 2497–2506 (2007).
9. Stone, G. W. et al. Bivalirudin during primary PCI in acute myocardial infarction. *N. Engl. J. Med.* 358, 2218–2230 (2008).
10. Lincoff, A. M. et al. Bivalirudin and provisional glycoprotein IIb/IIIa blockade compared with heparin and planned glycoprotein IIb/IIIa blockade during percutaneous coronary intervention: REPLACE-2 randomized trial. *J. Am. Med. Assoc.* 289, 853–863 (2003).
11. Shahzad, A. et al. Unfractionated heparin versus bivalirudin in primary percutaneous coronary intervention (HEAT-PPCI): an open-label, single centre, randomised controlled trial. *Lancet* 384, 1849–1858 (2014).
12. Hui, Y. et al. Bivalirudin vs heparin with or without tirofiban during primary percutaneous coronary intervention in acute myocardial infarction: the BRIGHT randomized clinical trial. *J. Am. Med. Assoc.* 313, 1336–1345 (2015).
13. Gargiulo, G. et al. Bivalirudin or heparin in patients undergoing invasive management of acute coronary syndromes. *J. Am. Coll. Cardiol.* 71, 1231–1242 (2018).
14. Elinge, D. et al. Bivalirudin versus heparin monotherapy in myocardial infarction. *N. Engl. J. Med.* 377, 1132–1142 (2017).
15. Neumann, F. J. et al. 2018 ESC/EACTS guidelines on myocardial revascularization. *Eur. Heart J.* 40, 87–165 (2019).
16. Secemsky, E. A. et al. Use and effectiveness of bivalirudin versus unfractionated heparin for percutaneous coronary intervention among patients with ST-segment elevation myocardial infarction in the United States. *JACC Cardiovasc. Inter.* 9, 2376–2386 (2016).
17. Verheugt, F. W. A., Ten Berg, J. M., Storey, R. F., Cuisset, T. & Granger, C. B. Antithrombotics: from aspirin to DOACs in coronary artery disease and atrial fibrillation (Part 3/5). *J. Am. Coll. Cardiol.* 74, 699–711 (2019).

**Statistical analysis.** All statistical analyses and curve-fitting by non-linear regression were performed on Prism 6.0 (GraphPad, CA, USA). Dose–response fits for Table 1 and Fig. 7a, b were calculated by fitting Eq. (3) to the log-log line for Supplementary Table 2 were used ascertained with goodness-of-fit analyses as implemented in Prism 6.0. The Pearson correlation coefficient, r, was used to calculate the correlation between bleeding times obtained in the tail bleeding and saphenous vein bleeding models (Fig. 6c, inset). One-way analysis of variance (ANOVA) and post hoc Tukey’s multiple comparisons tests were used to compare the quantitative end-points between treatment groups in Figs 5 and 6. Two-tailed, unpaired t test was performed for comparison between treatment (Ab1283) and control (saline) groups in Fig. 7d. Multiplicity-adjusted P values for each comparison were reported.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Transcriptomic data utilised have been previously published and not generated in this paper. The sequence data used to inform the design of ultravariegin as depicted in Fig. 1 is publicly available in UniProt under accession number P85800 and in GenBank under accession number BAD29729.1, DAA34688.1, DAA34258.1 and DAA34160.1. Sequences of antibodies tested as reversal agents are available upon reasonable request under cover of a non-disclosure agreement until the application for intellectual property is completed and published. The remaining data generated in this study are provided in the Article, Supplementary Information or Source Data file. Source data are provided with this paper.
18. Kumbhari, D. J. et al. 2020 ACC expert consensus decision pathway for anticoagulant and antiplatelet therapy in patients with atrial fibrillation or venous thromboembolism undergoing percutaneous coronary intervention or with atherosclerotic cardiovascular disease. J. Am. Coll. Cardiol. 77, 629–658 (2021).

19. Koh, C. Y. & Kini, R. M. Molecular diversity of anticoagulants from haematophagous animals. Thromb. Haemost. 102, 437–453 (2009).

20. Rydel, T. J. et al. The structure of a complex of recombinant hirudin and human α-thrombin. Science 249, 277–280 (1990).

21. Figueiredo, A. C. et al. Unique thrombin inhibition mechanism by anophelin, an anticoagulant from the malaria vector. Proc. Natl Acad. Sci. USA 109, E3649–E3658 (2012).

22. Francischetti, I. M., Valenzuela, J. G. & Ribeiro, J. M. Anophelin: kinetics and mechanism of thrombin inhibition. Biochemistry 38, 16678–16685 (1999).

23. Waranga, S. et al. Identification and characterization of novel salivary thrombin inhibitors from the ixodide tick, Haemaphysalis longicornis. Eur. J. Biochem. 270, 1926–1934 (2003).

24. Cappello, M. et al. Isolation and characterization of the tsste thrombin inhibitor: a potent antithrombotic peptide from the saliva of Glossina morsitans morsitans. Am. J. Trop. Med. Hyg. 54, 475–480 (1996).

25. Calisto, B. M. et al. Sulfotyrosine-mediated recognition of human thrombin by thrombin fly anticoagulant mimics physiological substrates. Cell Chem. Biol. 28, 26–33 e28 (2021).

26. Thompson, R. E. et al. Tyrosine sulfation modulates activity of tick-derived thrombin inhibitors. Nat. Chem. 9, 909–917 (2017).

27. Agnet, S. M. et al. Sulfation-mediated binding of thrombin through hybridization of salivary sulfoproteins from haematophagous arthropods. Angew. Chem. Int. Ed. Engl. 60, 5348–5356 (2021).

28. Maragone, J. M., Bourdon, P., Jablonski, J., Ramachandran, K. L. & Fenton, J. W. 2nd Design and characterization of hirudins: a novel class of bivalent peptide inhibitors of thrombin. Biochemistry 29, 7995–7991 (1990).

29. Koh, C. Y. et al. Inactivation and degradation of active site serine of thrombin inhibitor from the tropical broad tick. J. Biol. Chem. 282, 29101–29113 (2007).

30. Iyer, J. K. et al. Avathrin: a novel thrombin inhibitor derived from a multiprecursor in the salivary glands of the ixodid tick, Amblyomma variegatum. PASEB J. 31, 2981–2995 (2017).

31. Koh, C. Y., Kazimirnova, M., Nuttall, P. A. & Kini, R. M. Noncompetitive inhibitor of thrombin. Chembiochem 10, 2155–2158 (2009).

32. Koh, C. Y. et al. Crystal structure of thrombin in complex with S-variegin: insights of a novel mechanism of inhibition and design of tunable thrombin inhibitors. PLoS ONE 6, e26367 (2011).

33. Shih, N. et al. Development of bioanalytical assays for variegin, a peptide-based bivalent direct thrombin inhibitor. Bioanalysis 9, 693–705 (2017).

34. Ribeiro, J. M. et al. Thrombin inhibitors from the ixodidae tick, Haemaphysalis longicornis. Amblyomma variegatum. BMC Genomics. 6, 2076 (2005).

35. Witting, J. L., Bourdon, P., Breznia, D. V., Maragone, J. M. & Fenton, J. W. 2nd Thrombin-specific inhibition by and slow cleavage of hirudin-1. Biochem. J. 371, 137–147 (2002).

36. Suzuki, K. et al. Usefulness of the APTT waveform for the diagnosis of DIC and prediction of the outcome or bleeding risk. Thromb. J. 17, 12 (2019).

37. Wada, H., Matsumoto, T., Ohishi, K., Shiraki, K. & Shimaoka, M. Update on the clot waveform analysis. Clin. Appl Thromb. Hemost. 26, 10766029620912027 (2020).

38. Sammond, T., Scholz, U., Schweb, R. & Siegemund, A. Clot waveform analysis in patients with haemophilia A. Haemostasis 34 Suppl 1, S48–S52 (2014).

39. Ay, C., Hisada, Y., Cooley, B. C. & Mackman, N. Factor XI-deficient mice exhibit increased bleeding after injury to the saphenous vein. J. Thromb. Haemost. 15, 1829–1833 (2017).

40. Monroe, D. M. & Hoffman, M. A mouse bleeding model to study oral anticoagulants. Thromb. Res. 133 Suppl 1, S6–S8 (2014).

41. Becker, E. M. et al. Effects of rivaroxaban, acetylsalicylic acid and clopidogrel as monotherapy and in combination in a porcine model of stent thrombosis. J. Thromb. Haemost. 10, 2470–2480 (2012).

42. Vilahur, G., Segales, E., Salas, E. & Badimon, L. Effects of a novel platelet nict. agonist donor (LABA) on platelet aggregation, clopidogrel, and combined therapy in inhibiting flow- and lesion-dependent thrombosis in the porcine ex vivo model. Circulation 110, 1668–1693 (2004).

43. Zafar, M. U., Santos-Gallego, C. G., Badimon, L. & Badimon, J. J. Badimon perfusion chamber: an ex vivo model of thrombosis. Methods Mol. Biol. 1816, 161–171 (2018).

44. Wilson, S. J. et al. Esox 1 thrombin inhibition with [N]-64179375 inhibits thrombus formation in a human translational model of thrombosis. Cardiovasc. Res. 115, 669–677 (2019).

45. Wilson, S. J. et al. PAR4 (Protease-activated receptor 4) antagonism with BMS-986120 inhibits human ex vivo thrombus formation. Arterioscler. Thromb. Vasc. Biol. 39, 448–456 (2019).

46. Bowes, J. et al. Reducing safety-related drug attrition: the use of in vitro pharmacological profiling. Nat. Rev. Drug Discov. 11, 909–922 (2012).
