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Non-invasive imaging and cellular tracking of pulmonary emboli by near-infrared fluorescence and positron-emission tomography

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Functional imaging of proteolytic activity is an emerging strategy to quantify disease and response to therapy at the molecular level. We present a new peptide-based imaging probe technology that advances these goals by exploiting enzymatic activity to deposit probes labelled with near-infrared (NIR) fluorophores or radioisotopes in cell membranes of disease-associated proteolysis. This strategy allows for non-invasive detection of protease activity in vivo and ex vivo by tracking deposited probes in tissues. We demonstrate non-invasive detection of thrombin generation in a murine model of pulmonary embolism using our protease-activated peptide probes in microscopic clots within the lungs with NIR fluorescence optical imaging and positron-emission tomography. Thrombin activity is imaged deep in tissue and tracked predominantly to platelets within the lumen of blood vessels. The modular design of our probes allows for facile investigation of other proteases, and their contributions to disease by tailoring the protease activation and cell-binding elements.

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Proteases are particular target enzymes of interest given their association with disease. Pioneering work on coagulation factors, cathepsins and matrix metalloproteases have found differential activity of these proteases throughout the progression of a number of diseases, including atherosclerosis. Despite our greater understanding of proteases and their contribution to pathological conditions, practical issues have limited more widespread adoption of protease-based imaging in research and in the clinic. Thrombin—the pivotal protease of blood coagulation—enemplifies such issues. Sensitive and specific imaging is below the high copy number required to improve patient diagnosis and clinical outcome. Targeted imaging agents have traditionally relied on a one-to-one stoichiometric binding of probe to the target molecule for visualization. Enzyme-activated probes, developed to overcome this stoichiometric limitation, have typically suffered from low specificity and low signal intensity with high background noise. Proteases are particular target enzymes of interest given their association with disease. Pioneering work on coagulation factors, cathepsins and matrix metalloproteases have found differential activity of these proteases throughout the progression of a number of diseases, including atherosclerosis. Despite our greater understanding of proteases and their contribution to pathological conditions, practical issues have limited more widespread adoption of protease-based imaging in research and in the clinic. Thrombin—the pivotal protease of blood coagulation—enemplifies such issues. Sensitive and specific imaging is below the high copy number required to improve patient diagnosis and clinical outcome. Targeted imaging agents have traditionally relied on a one-to-one stoichiometric binding of probe to the target molecule for visualization. Enzyme-activated probes, developed to overcome this stoichiometric limitation, have typically suffered from low specificity and low signal intensity with high background noise.

### Results

**Identification of RIPS.** A library of potential membrane-interacting peptides, bearing the remnants of a protease cleavage site and absence of their natural post-translational modifications, was analysed to identify 14 peptides capable of binding phospholipid membranes post-proteolytic activation. Sequences were optimized to maintain the endogenous sequence of the AMP while maximizing the thrombin cleavage site (Fig. 1b–d; Supplementary Table 1). Temporin L (TempL) was chosen from this group on the basis of its relatively short sequence, large number of homologues, modest cationic disposition, conformational change into an α-helix on membrane binding and reasonable haemocompatibility to human blood cells (Fig. 1b,g). These factors enable facile synthesis, tunable potency, limited non-specific interactions, robust on and off state definition and the ability to insert into eukaryotic cell membranes. The protease-activated receptor-1 (PAR1) activation site, a natural substrate of thrombin, was chosen to incorporate into the initial design owing to its known high sensitivity to the protease. Extended interaction of this substrate outside of the protease active site is known to mediate efficient recognition by thrombin, yet this has not been incorporated into past imaging agents owing to its size. The chosen cleavage site and extended interaction sequence from PAR1 encompasses P2–P14 in Schechter and Berger's nomenclature and more than 1,000 Å² buried surface area in the enzyme substrate complex. The buried area compares favourably to antibody–antigen complexes and highlights the sensitivity enabled by the RIP architecture. A peptide whose sequence is composed of TempL followed by the sequence of PAR1 was synthesized and is referred to as PAR1–RIP.

**Protease-dependent interactions of PAR1–RIP constructs.** PAR1–RIP has a slight anionic disposition with a predicted pI of 6.2 in its pro-peptide form. When cleaved by thrombin two fragments are produced; one with a pI value of 11.2 that binds to membranes and another with a pI of 3.5 that diffuses away (Fig. 1e,f). The protease-dependent activation mechanism involves two steps. In the first, electrostatics and hydrophilicity hinder the ability of the probe to associate with the phospholipid membrane and penetrate into the hydrophobic core. Second, amino acid residues in the target sequence and the properties of imaging agent hinder the peptide from forming an α-helix at the solvent membrane interface as observed in circular dichroism spectra (Fig. 2a,b). Proteolysis, therefore, aims to enable both membrane association and insertion of the previously restricted probe.

Measurement of changes in the intrinsic fluorescence of the singular tryptophan residue in the membrane-interacting segment demonstrated that the thrombin-cleaved form indeed shows markedly enhanced peptide insertion into lipid compared with the pro-form. A 10-nm decrease in the maximum emission wavelength of the thrombin-cleaved form indicated burial of this residue that resides in the middle of the membrane-binding module into the apolar phosphatidylcholine (PC) liposomes (Fig. 2c). Similar restriction and post-proteolysis binding was observed with both zwitterionic and anionic detergent micelles suggesting that high membrane curvature does not impact probe behaviour and the discrimination is robust (Supplementary Fig. 1). Association with the anionic
phosphatidylinerse (PS) liposomes was also evident by the
decrease in signal intensity presumably reflecting proximity of
the tryptophan to the negative charge. In contrast, the intact pro-
peptide had limited interaction with PC phospholipid membranes
eydient by the shift in the maximum emission
wavelength of its spectral profile in the presence of liposomes
consisting of either PC or PS and quenching was not observed
(Fig. 2d). As anticipated from the use of an AMP with a cationic
disposition in our design, we observed a preference for anionic
lipids, weaker interaction with zwitterionic surfaces and little
affinity for cationic membranes (Fig. 2e). As the maximum
emission wavelength from the pro-peptide does not significantly
decrease at high molar ratios of phospholipid, we infer that the
peptide does not insert into the membrane and the observed
signal reflects non-specific binding and a limit of the experiment
(Fig. 2f). Indeed, when thrombin was directly added to mixtures
of the fluorescent dye DRAQ7 only occurred in the protease-
treated form (Supplementary Fig. 3a) not the pro-peptide alone
of the key role for thrombin in this process. Cells grown on plastic
culture dishes were incubated with 100 nM pro-peptide to confirm that uptake
in vivo

Figure 1 | Mechanism underlying RIP technology. (a) RIP pro-peptides exist in a disordered state in solution with little affinity for phospholipid surfaces owing to the presence of the exosite-targeting sequence (red) and imaging agent (yellow) at the termini of the membrane-binding segment (blue). Following proteolysis, the membrane-binding segment is released and capable of interacting with cellular membranes wherein it undergo conformational change and insertion. (b) Fourteen antimicrobial peptides (AMPs) were synthesized lacking their conventional post-translational modifications while including the Pro–Arg sequence that would be leftover after proteolysis by thrombin. The peptides were found to have various limited degrees of cellular toxicity. (c) Toxicity of these initial peptides weakly correlated with incorporation into PC liposomes or SDS micelles suggesting a variety of AMP mechanisms could be used in the RIP strategy and with different toxicities. (d) Temporin L (peptide 12 in (b) and (c)) in PAR1–RIP belongs to a large peptide family enabling its optimization when coupled to different imaging groups and targeting sequences. (e,f) Helical wheel diagram of the activated form of the peptide highlights the amphiphobic nature of the peptide. Colouring as in (a) with hydrophobic residues in black and hydrophilic residues in white. In contrast, the PAR1–RIP masks this helix with hydrophilic residues that also create an acidic pI for the peptide. Colouring as in (a

discrimination between on and off states in PAR1–RIP observed in vitro translated in vivo. To gauge the interaction of RIP probes with cells, we measured their toxicity at ~6- to 600-fold excess of the intended use in vivo. Human Jurkat cells in suspension tolerated micromolar levels of dye-free PAR1–RIP pro-peptide. However, the purified active form of PAR1–RIP or co-incubation with thrombin, but not the proteases factor Xa (FXa) and activated protein C, mediated membrane-permeabilization and uptake of Trypan blue in the low micromolar range (Fig. 2h). Inclusion of the highly specific thrombin inhibitor hirudin blocked these protease-dependent effects and confirmed the key role for thrombin in this process. Cells grown on plastic were incubated with 100 μM pro-peptide to confirm that uptake of the fluorescent dye DRAQ7 only occurred in the protease-treated form (Supplementary Fig. 3a) not the pro-peptide alone (Supplementary Fig. 3b). Concentrations required to mediate toxicity in cell culture exceed those used in animal studies described below by several orders of magnitude, but provide a useful readout nonetheless. Protease-dependent membrane-bind-

potency of the membrane-binding segment contribute in different ways to the extent of restricted interaction with phospholipid surfaces and can be modified to tune probe properties.
which is non-toxic, and its thrombin-activated form. Blocking proteolysis with the thrombin-specific inhibitor confirms the role of the protease. Data are non-specific association of the peptide. (Fig. 3c,d). The pattern of cellular uptake of fluorophore-conjugated PAR1–RIP, which bears a near-infrared (NIR) fluorophore later used for non-invasive animal imaging in studies described below, could be unleashed by selective proteolysis. Fluorescence resonance energy transfer (FRET) between the peptide and an appropriate lipophilic dye demonstrated probe association with phospholipid membranes in a protease-dependent fashion when carrying a fluorescent dye (Fig. 3a,b). In certain formulations, such as those containing Cy5, membrane binding of the active form was also evident by up to a 50% increase in fluorescence intensity suggesting the activated form of the peptide could drag the fluorophore, at least partly, into the apolar membrane interior and thus form a stable partially buried helix. These data suggest RIP technology is capable of carrying payloads of varying size, charge and complexity to membrane surfaces with limited caveats.

Protease-dependent cell interactions were observed with Cy7–PAR1–RIP, which bears a near-infrared (NIR) fluorophore later used for non-invasive animal imaging in studies described below (Fig. 3c,d). The pattern of cellular uptake of fluorophore-conjugated activated peptide, assessed by live-cell epifluorescence microscopy, followed a typical sequence involving surface labelling in minutes followed by endosomal uptake over the next hour with redistribution into intracellular membranes. Together these studies confirmed the restricted membrane interaction of PAR1–RIP and its selective activation by thrombin extends to cell-based phospholipids leading to probe deposition and internalization.

PAR1–RIP was selective for the target protease thrombin and exhibited an efficient Michaelis constant ($K_M$) for recognition as previously observed with peptides derived from the PAR1 sequence (Fig. 3e). Comparison of the rates of protease-mediated hydrolysis of a tripeptide chromogenic substrate (P3–P1), quenched oligopeptide substrate (P4–P4') and our PAR1–RIP probe (P4–P14) emphasize the value of a larger surface area of interaction between enzyme and substrate in RIP probes. When substrates have limited potential contacts with the protease, and the majority of them are shared with homologous enzymes, their turnover is non-specific. For example, the chromogenic substrate d-Phe-Pro-Arg-paranitroanilide is not only an excellent substrate for thrombin but also for many other proteases (Fig. 3f). As substrate length increases to include four additional residues on the prime side of the scissile bond, catalytic increases the relative specificity eightfold and reduces the $K_M$ for a physiologically relevant value of $0.2 \pm 0.1 \mu M$ (mean ± s.d., n = 3; Fig. 3h). The kinetic advantages gained on exosite inclusion are similar to those reported in other substrate designs suggesting the membrane-binding module does not negatively influence binding characteristics. Catalytic efficiency of thrombin-mediated PAR1–RIP activation, $k_{cat}/K_M$
of 8.4 ± 0.3 × 10^6 M⁻¹ s⁻¹, is 100- to 900-fold greater than previous thrombin-targeted probes that can localize to sites of its activity for in vivo imaging entirely due to its lowered K_M,6,26,27 Notably, proteases included in Fig. 3e were chosen by their ability to cleave PAR1–RIP with any measurable rate. Other proteases tested such as factors IXa, Xa and XIa, activated protein C, plasmin and tissue-type plasminogen activator did not cleave PAR1–RIP after 24 h of co-incubation (Fig. 3e). In isolation these proteases do not cleave PAR1 yet readily cleave small chromogenic substrates that bind the active site alone28. Several of these proteases are thought to signal through PARs in vivo through assistance from a co-factor.29 Together these data indicate PAR1–RIP presents physiologically relevant kinetic parameters for its activation by thrombin even in the presence of a membrane-binding module.

Imaging arterial thrombosis in the mouse carotid artery. Ferric chloride-induced endothelial damage in the carotid artery shows that Cy5–PAR1–RIP deposit intensively at the injury site in comparison to an adjacent healthy vessel. Under similar conditions of vascular damage an uncleavable version of the probe bearing an alanine at the P1 position accumulated with much less intensity at the injury site (Fig. 4a). The normalized signal of damaged/healthy vessel over time indicates that cleavage of the probe, rather than occlusion of the vessel, is the driving force behind PAR1–RIP deposition to the thrombotic lesion (Fig. 4b). Thus, PAR1–RIP probes accumulate at sites of thrombin proteolytic activity, even when no significant occlusion of the vessel can be observed.

Imaging thrombin generation during ex vivo clot formation. PAR1–RIP labelled platelets during ex vivo blood clot formation (Fig. 4c). Murine whole blood was collected in the presence of sodium citrate and clotted by adding coagulation FXa and calcium in the presence of fluorescein–PAR1–RIP on glass slides. The pattern of probe accumulation was compared with fluorescent antibodies against CD41 (anti-mouse CD41 phycoerythrin, MWReg30 clone, eBioscience, 1:100 dilution), a marker of platelets, and TER119 (anti-mouse TER-119 phycoerythrin, TER-119 clone, eBioscience, 1:100 dilution) a marker of red blood cells. Probe accumulation overlapped that from CD41 yet was more heterogeneous in its location and signal intensity. Certain platelets within the clot showed little probe accumulation whereas others were intensely labelled. Under similar conditions of clot formation, an uncleavable variant of PAR1–RIP bearing an alanine at the P1 position accumulated with much less intensity on platelets. When whole murine blood was re-calculated and stimulated with platelet activators such as ADP or the AYPGKF peptide, PAR1–RIP neither accumulated on platelets nor other cell type indicating the insufficiency of P2Y1 and P2Y12 ADP receptors or PAR4 mediated platelet activation for probe accumulation and the need for thrombin to cleave the probe for a maximal response. Normal platelet functions, such as thrombin-induced aggregation (Fig. 4d) and clot retraction (Fig. 4e), were shown to be unaltered by concentrations of PAR1–RIP greater than those used in the imaging studies, × 5 and × 40, respectively. These observations highlight the responsiveness of PAR1–RIP to proteolysis and amplified by PS exposure, without interfering with normal haemostasis.
Figure 4 | Probe deposition at sites of vascular injury as a function of thrombin generation. The generation of an occlusive clot is achieved by exposing the carotid artery of C57/B6 mice (n = 3 per treatment group) with a Whatman disk soaked in FeCl₃ solution (4%) for 3 min. Occlusion of the vessel is obtained as a function of thrombin generation and proteolytic activity within the injury. (a) The Cy5-PAR1-RIP probe accumulates in the injury site in a time-dependent manner, showing a distinct difference against its uncleavable variant (P1 alanine). After total occlusion (1 mm³ clot after 20 min), the clotted artery was removed and the fluorescence signal observed was compared with the adjacent healthy tissue of the artery. (b) Normalized signals from the clot/healthy vessel ratio reveals an increasing difference over time between PAR1-RIP and its uncleavable variant. These data corroborate to the importance of thrombin activity for probe turnover and localization at sites of high thrombogenicity. (c) Clotting of whole murine blood (scale bar, 100 µm) in a protease-dependent manner using coagulation FXa (50 nM) converts prothrombin to thrombin and causes fluorescein–PAR1–RIP (50 nM, red) to become activated and localize on platelets preferentially over red blood cells, which are indicated by the CD41 and TER119 antibody markers (green). Similar probe deposition is not observed with an uncleavable variant of the peptide or when the blood is stimulated by ADP or a PAR4-specific agonist (AYPGKF). (d) As observed by light transmission aggregometry (n = 3), after incubation with PAR1-RIP at 37 °C in either pro-peptide and/or active form, platelet aggregation is not impaired by the probes, even at fivefold higher concentrations than that used for imaging. Late-stage platelet activation events, such as clot retraction (e) are also not altered by the probes, even at 40-fold higher concentrations than that used for imaging (n = 3). Data are mean ± s.d. of three biological replicates. All results are expressed as P < 0.05, one-way ANOVA followed by Dunnett’s post-test.

Non-invasive detection of PE using NIR fluorescence. Preliminary analysis of biodistribution, pharmacokinetics and acute toxicology were assessed before inclusion of RIPs into standard clotting models. In mice, the small peptidic nature of the probe and absence of bulking agents led to a rapid clearance from the circulation and subsequent accumulation in the bladder that plateaus within 30 min (Supplementary Fig. 4). Three routes of clearance were evident—hepatic, renal and digestive. We therefore investigated whether PAR1-RIP had acute toxic effects on liver and kidneys and showed that most of the 21 measured serum biochemical parameters were not different from controls at all time points (Supplementary Table 2). Thus PAR1-RIP does not affect hepatic or renal functions and is not haemolytic at the tested dose. The rapid circulating half-life indicated that the probes would have, in the current formulation, applications for short-term experiments asking direct questions about thrombin generation. We therefore chose the thromboplastin-mediated pulmonary embolism model, which rapidly generates microscopic emboli in the lungs.

Pulmonary emboli (PE) could be detected and quantified non-invasively in a dose-dependent manner by PAR1-RIP conjugated to a variety of fluorescent and NIR dyes. Initial studies applied previously reported doses of thromboplastin at lethal levels and direct injection into the inferior vena cava of mice and showed ATTO 680-PAR1-RIP accumulated proportionally to the severity of the insult (Supplementary Fig. 5). We continued to use this PE model, but lowered the maximal dose of thromboplastin injected to 10-fold below the lethal level and turned to tail-vein injection as the route of administration to obviate signal due to surgical manipulation of the animal.

Non-lethal PE were detected non-invasively within minutes of injection of picomolar amounts of Cy7-PAR1-RIP in mice bearing thromboplastin-induced emboli (Fig. 5a). Probe accumulated predominantly in the lungs, a direct outcome from the increased clotting activity promoted by thromboplastin in this pulmonary embolism model (Fig. 5b), and was excreted via renal and hepatic clearance. Using a non-cleavable version of fluorescein-conjugated PAR1-RIP and the thrombin-specific inhibitor argatroban, we observed reduction in probe accumulation near, but not equal to background levels indicating proteolysis of the probe was necessary, but not required, for its deposition in this model (Fig. 5c). The inability to completely abrogate accumulation presumably reflects both the non-specific accumulation of the probe within the clot as well as the inherent affinity of the uncleavable probe for thrombin. In contrast with ATTO 680-containing PAR1-RIP, the Cy7 variant exhibited more rapid in vivo clearance and presented markedly increased liver uptake. Nevertheless, lung deposition was quantitative and linear reflecting the low and non-lethal amount of thromboplastin injected (Fig. 5d). The intense signal, when viewed non-invasively or with extracted organs, resulted from Cy7-PAR1-RIP accumulation in a small number of intensely labelled emboli in the lung that could be tracked ex vivo (Fig. 5e,f), even in non-occluded vessels (Supplementary Fig. 6). The small number of emboli observed in the lung agrees with the linear response of probe accumulation as a function of thromboplastin, which is
more than 10-fold below its reported LD₅₀ (dose lethal to 50% of animals tested) in mice, because much of the lung tissue is normal and with unobstructed vessels at this dose of coagulant (Fig. 5e). The ability to track protease activity down to a precise cellular location highlights the broader utility of RIP technology beyond non-invasive detection. Signal from the Cy7 dye was detected throughout the emboli, but tended to co-localize with platelet-rich areas based on signal overlap with antiplatelet-CD41 and also haematoxylin and eosin staining (Fig. 5f; Supplementary Figs 6 and 7). These data indicate RIP technology can be used to couple non-invasive imaging of protease activity to its location at the cellular level.

**Real-time measurement of thrombin generation after wounding.**

The non-invasive imaging of a haemostatic process was achieved in a wounding model using a 28-gauge needle to create a small puncture wound in the hind leg of a mouse shortly after tail-vein injection of ATTO 680–PAR1–RIP (Fig. 6a). The signal detection arising from a region of interest defined at this site over time revealed an eightfold gain in signal over sham wounding where probe was injected with the same conditions. Signal intensity saturated within twenty minutes of wounding (Fig. 6b). Initial velocity of probe deposition in this model suggests ~10¹⁰ molecules of thrombin are generated by wounding the saphenous vein of a mouse with a 28-gauge needle. The short half-life of the probe does not enable an accurate assessment of the length of time in which thrombin is acting during this wounding model, and further studies coupling pharmacokinetics with intravital microscopy are required. Unlike the Cy7 variant, ATT0 680–PAR1–RIP showed limited liver accumulation and was primarily cleared via the renal system. These data highlight the utility of RIP technology for real-time quantification of proteolytic events in vivo through non-invasive detection.

**Non-invasive imaging of PE using a PET/CT RIP.**

Encouraged by the ability of PAR1–RIP to accumulate in sufficiently high copy number for the non-invasive imaging of non-lethal PE, a variant of the peptide was created bearing diethylene triamine pentaacetic acid (DTPA)—a broad spectrum metal chelator—for nuclear imaging. DTPA–PAR1–RIP was labelled with the positron-emitting radioisotope ⁶⁸Ga (t½ 68 min) for positron-emission tomography (PET) imaging. The dynamic capabilities of PET were used to image picomolar RIP dosing regimens in the PE model. Similar to that observed with Cy7–PAR1–RIP, the nuclear imaging variant accumulated in the lungs of the mice when co-administered with thromboplastin but not in untreated mice...
(Fig. 6c). RIP technology was used to deposit radioisotopes in high abundance as indicated by a fivefold gain in signal-to-noise and a per cent injected dose per cubic centimetre (%ID cc⁻¹) exceeding 5% to the sites of protease activity. Probe accumulation ceased within the first 4 min of accumulation in the lung (Fig. 6d). The rate of probe deposition cannot quantify thrombin generated in this model, as the liver rapidly took up the metal-chelated probe similar to the Cy7 variant described previously. Nonetheless, non-invasive imaging of clot formation using a relevant imaging modality suggests the potential for clinical translation of our RIP technology.

Discussion
To enable non-invasive real-time imaging of protease activity, we developed a scaffold differing significantly from past approaches and coupled biochemistry more directly to cell biology. Hairpin-structured activatable cell-penetrating peptides have been proposed to mediate intracellular delivery in response to metalloproteases31 and more recently thrombin26. Active site-dominated contacts limit the specificity of these probes and their protease-responsiveness is limited32. Unlike cell-penetrating peptides, our polymers have a defined localization mechanism involving formation of an α-helix on membrane interaction, lack a high density of cations and do not transpose into the cytoplasm. Our thrombin-specific probes exhibit 100-fold lower $K_M$ values than comparable activatable cell-penetrating peptides27 that localize after activation. The lower $K_M$ values in turn enable 100-fold lower dosing amounts, more rapid imaging times and lower background noise. These features are also significantly improved over commercially protease-targeted imaging agents that require 8–24 h delay from time of injection to imaging and are not documented to respond to thrombin. Similarly, approaches for PS-based imaging have been presented, but lacked a mechanism whereby the natural presentation of the phospholipid at low levels is accounted for and the probes have poor pharmacokinetic properties33. In another approach an IgG-based, platelet-targeted sensor was used to reveal thrombin gradients during clot formation. This sensor has specificity restricted to one cell type, since it is based and driven by an antibody directed against the platelet marker CD41 (ref. 34).

Preference for PS provided our technology an unexpected enhancement on the target cells and low non-specific accumulation. PS constitutes between 2 and 10% of total cellular phospholipid yet nearly all of this material is maintained in the inner leaflet of healthy cells35. Translocation of PS to the outer membrane is a hallmark of cell death, cancer, and activated platelets during blood clotting. Probes that detect PS have been documented to respond to thrombin. Similarly, approaches for PS-based imaging have been presented, but lacked a mechanism whereby the natural presentation of the phospholipid at low levels is accounted for and the probes have poor pharmacokinetic properties33. In another approach an IgG-based, platelet-targeted sensor was used to reveal thrombin gradients during clot formation. This sensor has specificity restricted to one cell type, since it is based and driven by an antibody directed against the platelet marker CD41 (ref. 34).

Figure 6 | Real-time detection of thrombus generation using PAR1–RIP formulated for NIR fluorescence or PET imaging. (a) Puncture of the saphenous vein in mice ($n = 3$ per group) creates a submillimeter-sized blood clot whose formation can be directly quantified (b) via a signal-to-noise increase of more than eightfold saturating in ~20 min. Gain in signal is not due to background from the animal as the absence of the injury does not result in a detectable signal. (c) Non-lethal pulmonary emboli via tail-vein injection of thromboplastin were detected non-invasively by the clinically relevant imaging modality PET/CT using $^{68}$Ga-labelled DTPA–PAR1–RIP. Healthy mice ($n = 5$ per treatment group) were tail-vein injected with 150–200 μCi of $^{68}$Ga-DTPA–PAR1–RIP and scanned dynamically for 20 min to detect thrombin generation in the lungs via PET. Accumulation in the liver is more pronounced using this variant of the probe compared with NIR formulations and this appears entirely driven by the radioisotope and its chelator rather than the peptide compound of the RIP. (d) Radioisotope deposition enables similar quantification of thrombin generation. The difference in signal compared with a no thromboplastin control is plotted. %ID cc⁻¹ is the amount of radioisotope injected per cubic centimetre of tissue as sampled through the lung of the mouse. Data are mean ± s.d. of three biological replicates. All results are expressed as $P < 0.05$, one-way ANOVA followed by Dunnett’s post-test. cps, counts per second.
images of disease solely based on the presence of PS and improved probes are sought. RIPv bind cell membranes after proteolytic activation at sites of disease in live animals. In the absence of a proteolytic trigger, they have little affinity for cell membranes and, owing to their small size, are rapidly removed from circulation. Proteolysis unleashes membrane-binding activity of the RIP and this is more pronounced when the associated cells also present PS; a useful pairing for the detection of blood clots and other diseases. Coupling selective proteolysis and PS exposure in the RIP approach creates a useful platform for functional imaging using optical or nuclear modalities by enabling picomolar probe dose sizes, low background signal, real-time non-invasive measurement and signal tracking in sectioned tissues to resolve spatial heterogeneity of biochemical events. The algorithmic nature of AMP structure and function affords the potential for incorporating non-natural amino acids, therapeutic modalities, imaging agents or pharmacokinetic modifiers. The current peptides are not as water soluble as envisaged likely due to the length of peptides but our results suggest the potential for optimization. Improving water solubility and lengthening circulating half-life are tractable problems that could be achieved through pegylation or D-amino acid exchange. These and other improvements such as enhancement of restricted events. The algorithmic nature of AMP structure and function maintenance at either 310 or 298 K by connecting the cuvette holder to an external nitrogen to remove trace solvent. The lipid film was then hydrated at room temperature (RT) in phosphate buffer (pH 7.4) with vortexing. Followed by three evaporator for at one hour. The resulting lipid film was dried under stream molar ratio (80–125 M) and the solvent removed under vacuum in a rotary. The resulting lipid film was dried under stream nitrogen to remove trace solvent. The lipid film was then hydrated at room temperature (RT) in phosphate buffer (pH 7.4) with vortexing. Followed by three freeze-thaw cycles in a dry ice-ethanol bath. Large unilamellar vesicles were preparch by extrusion of the lipid suspension through 100-nm pore-size polycarbonate filters. Liposomes were used within 1 week of their preparation.

**Peptide synthesis and fluorophore labelling.** Peptides were synthesized on an automatic peptide synthesizer by using standard protocols for fluorenlymethoxycarbonyl solid-phase synthesis. All peptides were at least 95% pure by high performance liquid chromatography (HPLC) and further characterized through mass spectrometry.

**Tryptophan fluorescence spectroscopy.** Emission spectra of the intrinsic fluor-excence of tryptophan within the membrane-binding segment of the peptide were collected using a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Longjumeaux, France). Vesicle suspensions were prepared as for solid-state NMR experiments above in the absence of peptide, except that the freeze-thaw cycles were omitted, yielding large multilamellar vesicles. Vesicles containing POPC/POPS (75:25) and POPE/POPS (75:25) were prepared at a concentration of 5 mg ml⁻¹. From these suspensions, 150 μl was added to 0.85 ml PBS, and then peptide in solution (2 mg ml⁻¹ in PBS) was added to produce a final peptide concentration of ~0.01 mg ml⁻¹. A peptide/lipid molar ratio of 1:40 was maintained. Tryptophan emission spectra of the lipid/peptide suspension were acquired by scanning from 310 to 450 nm using an excitation wavelength of 295 nm and a spectral bandwidth of 5 nm for both excitation and emission. A spectrum of the aqueous peptide was acquired at a peptide concentration of 0.1 mg ml⁻¹ in the same buffer. All spectra are an average of three scans. The temperature was maintained at either 310 or 298 K by connecting the cuvette holder to an external water bath.

**Circumbralochism.** Spectra were acquired on a Jasco J-810 spectrometer (Jasco, Tokyo, Japan) with samples maintained at 310 K. Spectra were recorded from 250 to 850 nm using a spectral bandwidth of 1 nm and a scan rate of 100 nm min⁻¹. Samples were prepared as for the fluorescence experiments above, but with the lipid suspension undiluted. From the lipid suspension, 240 μl was added to a 1-mm cuvette and then 12 μl peptide solution (2 mg ml⁻¹) was added and thoroughly mixed. Spectra were treated using Jasco spectra analysis software, where a spectrum of the peptide-free suspension was subtracted and means-moving smoothing with a convolution width of five points was applied.

**Peptide kinetics.** Human clotting factors IIa (thrombin), VIIa, IXa, Xa, Xla, plasmid.macPc were obtained from HPLC Technologies (Vermont, USA). Kinetics studies of probe digestion were measured by HPLC over the course of 1 h at 37 °C, using 10 nM of enzyme and 1 μM of PAR1–RIP in PBS (pH 7.4). Reactions were terminated with 20 μl of 1 M perchloric acid. Time-dependent digestion of PAR1-RIP was confirmed by mass spectrometry.

**Live-cell fluorescence microscopy.** The human cancer cell lines were plated in 35-mm glass-bottom culture dishes (MatTek) and allowed to grow in DMEM supplemented with 1% (vol/vol) GlutaMAX, 10% FBS and 1% penicillin–streptomycin until the cells were 75–90% confluent. Before imaging, the cells were washed with PBS and allowed to grow in serum-free DMEM for 1 h at 37 °C. The cells were then incubated with a 1:11-AF488 with PAR1–RIP probe in DMEM with a 1:250 dilution under 5% CO₂. A11-AF488 in serum-free media was added to a final dye concentration of 100 nmol l⁻¹ (approximate final volume was 2.3 ml). The culture dishes were returned to the incubator for 2 h, after which the plates were washed with PBS to remove any unbound antibody and 2 ml of phenol red-free, serum-free media was added for imaging. The cells were imaged within 10 min of removal from the incubator using a Nikon 6D high throughput epifluorescence microscope at the Nikon Imaging Center at the University of California at San Francisco/QB3.

**Fluorescence-activated cell sorting analysis and microscopy.** Jurkat cells provided by UCSF Cell Culture Facility (clone E6-1, ATCC) were cultured in RPMI medium 1640 plus 10% (vol/vol) FBS to a density of 0.5–1 × 10⁶ cells per ml. The media was refreshed 1 day before assaying with RIPS. Cells were washed with Hanks’ balanced salt solution (HBSS) buffer three times, resuspended in HBSS at 0.5–1 × 10⁶ cells per ml, stained with 1 μM peptide in HBSS at RT for 10 min, washed three times with cold HBSS and analysed by flow cytometry at 530-nm emission for fluorescein-labelled peptides or at 675 nm for Cy5-labelled peptides. We collected 10,000 events from cells judged to be healthy by their forward and side scatter. Peptide association with HT-1080 cells was similarly quantified by flow cytometry after release from adherence with trypsin. For microscopic imaging, HT-1080 cells grown to 70% confluence were washed with HBSS three times, stained with 1.25 μM peptide and 1 μg ml⁻¹ Hoechst 33258 (a nuclear stain), rinsed twice, trypsinized and replated on polylysine-coated dishes and, imaged for Cy5 content (excitation 625–645 nm; emission 665–695 nm) and Hoechst 33258 (excitation 375–385 nm; emission 420–460 nm).

**Animals.** Adult wild-type C57BL/6/j mice and Foxn1nu mice from both genders, weighting 10–30 g were obtained from The Jackson Laboratory (Bar Harbor, ME). Experiments were in compliance to the UCSF Institutional Animal Care and Use Committee (IACUC) who approved all animal care and experimental procedures. The number of mice chosen to confirm our hypotheses was 128 and were derived from three mice groups: (A, n = 44) controls where no procoagulant is injected, or a procoagulant may or may not be injected and tested with an uncleavable RIP; (B, n = 50) RIP probe is injected over a time course following thrombus formation; and (C, n = 34) RIP is injected over a time course in the presence of a specific inhibitor; nude mice, n = 33; C57BL/6, n = 95. An error threshold of 10% relative to the injection volume was maintained as adopted as sample exclusion criteria (mice not directly injected with <90% of initial volume were excluded from statistical analysis.

**Imaging arterial thrombosis in the mouse carotid artery.** The ferric chloride (FeCl₃)-induced vascular injury was performed as described elsewhere. Briefly, Cy5–PAR1–RIP or its Cy5-uncleavable variant was administered by tail-vein injection as previously described for the pulmonary embolism model and a midline incision made through the skin from sternum to chin of anaesthetized C57BL/6J mice to expose the carotid artery. After exposure, a Whatman filter saturated in 4% FeCl₃ solution was placed on the carotid artery for 3 min. Complete occlusion was observed within 20 min after contact with FeCl₃ generating clots of ~1 mm² in size. Fluorescence signal was obtained from the damaged area and compared with the contralateral upstream hemisphere which further没啥上小的信号-to-noise ratio. Fluorescence capture was performed using the IVIS imaging system with excitation of 640 nm and emission 700 nm.
Thromboplastin-induced pulmonary embolism. We used a model similar to that described previously. Thromboplastin (Sigma-Aldrich) used for these studies was from rabbit brain. Each vial of thromboplastin (3–4 mg) was resuspended in 4 ml saline and 100 μl of this solution was injected per mouse as below. The dose range examined had a 100% survival rate. As described previously female mice were used as preliminary studies revealed a sex difference in sensitivity to thromboplastin. Briefly, anaesthesia was induced in F1 (C57BL/6j × C3H/10T1/2) mice (3–4 weeks old) with a combination of intraperitoneal injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹). After a 10-min recovery period, mice were placed in a ventilator (Shanghai Ventilator Co., Ltd) with an endotracheal tube and connected to the ventilator system. The respiratory parameters were set to a minute ventilation of 750 ml min⁻¹ and a tidal volume of 2 ml. Continuous monitoring of the ECG and respiratory rate was performed. Experimenters were blinded to the treatment groups.

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In addition to the ketamine and xylazine, the anesthetic mixture contained isoflurane having appropriate oxygen flow. Once animals reached deep anaesthesia, they were maintained at 2% isoflurane with continuous monitoring. With the animals under deep anaesthesia, thromboplastin (4 mg ml⁻¹) isolated from rabbit brain (Sigma-Aldrich) was administered via tail-vein injection to reach sub-lethal doses in order to trigger the formation of micro embol. After 10 min from the initial injection, PAR1−RIP (10 μM kg⁻¹) was administered in PBS (pH 7.4) containing polyethylene glycol 16,000 and no more than 15% dimethylulphoxide (DMSO). The sera were centrifuged at 25,000 × g for 10 min before use. Three different doses were used: 100 μl of 100 μl of 50 μl and 25 μl.

In order to examine the effect of thromboplastin on the microcirculation of the lung, we used a model similar to that described elsewhere. Briefly, blood was collected from healthy donors using a BD Vacutainer Safety-Lock blood collection set with a 23-gauge needle. Platelet-rich plasma was isolated by centrifugation at 120 g for 15 min at RT and the platelet pellet was obtained by further centrifugation at 1,100 g. Platelets were washed with Heps–Tyrode albumin buffer (10 mM HEPES, 0.2738 M NaCl, 5.4 mM KCl, 24 mM NaHCO₃, 0.72 mM NaH₂PO₄, 2 mM MgCl₂, 4 mM CaCl₂, 10 mM dextrose and 0.7% BSA) at pH 6.5 in the absence of CaCl₂ and resuspended in the complete buffer at pH 7.4. Cells from the washed platelet suspension were counted in a Hemavet 950 cell counter (154 ± 4. ± 10⁶ cells ml⁻¹). Platelets were incubated with the probes, DMSO or argatroban (1.6 μM) for 2 min at 37 °C before addition of human thrombin (30 nM) and aggregometry using a Chrono-log aggregometer model 700. Informed consent was obtained from all human subjects and experiments performed in compliance to the protocol approved by the HUAP Research Ethics Committee, Brazil—ID number: 241.284. The usage of anti-inflammatory drugs, anticoagulants or hormones was adopted as sample exclusion criteria (donors under medical regimen that could affect normal haemostasis were not included in the study).

Histology. Histological staining was conducted on lung tissue sections embedded with paraffin by the UCFS Tissue Core. Lung tissue sections were deparaffinized with xylene and alcohol, and were then subjected to standard haematoxylin and eosin staining. Fluorescent staining of the tissues was performed with standard protocols and the FITC-anti-platelet CD41 (WReg30-PE clone, eBioscience) solution was prepared as a 1:100 dilution. Briefly, frozen slices were rehydrated in PBS for 1 h with buffer exchange each 20 min. Excess PBS was removed from the samples, which were then overlaid with CAS-Block reagent and FITC-anti-platelet CD41 (WReg30-PE clone, eBioscience) in PBS for 1 h at RT. Excess blocking reagent was removed and the samples overlaid with 125 μl PBS (0.4% bovine serum albumin (BSA)) containing 5 μl of FITC CD41 and incubated overnight at 4 °C in a sealed container. After incubation the samples were washed twice in cold PBS for 10 min each. Next, the samples were overlaid by 125 μl of (0.2% BSA) containing 0.2 μl 4,6-diamidino-2-phenylindole (DAPI) (10 μg ml⁻¹), 5 μl WGA Texas Red (1 mg ml⁻¹) and 1 μl of FITC CD41 and incubated at RT for 10 min. After washing three times in cold PBS, buffer was removed and samples overlaid with 35 μl Mowiol 4–88 (90%) mixed with ProLong Gold antifade (10%). A coverslip was added to the mount and the slide incubated at 37 °C for 10 min before imaging.

NIR optical imaging. Nude mice for the optical studies (n = 5 per treatment) were fed an alfalfa-free diet of Harlan Teklad Global 2018 to minimize background fluorescence. The mice were anaesthetized with 2% isoflurane. PAR1−RIP conjugated to 68Ga was diluted in PBS containing 0.1% PEG 16,000 and no more than 15% DMSO and administered at a final concentration of 300 picomoles per mouse. Mouse was injected via the tail vein of the mice at a volume of 150 μl ten minutes after the injection of thromboplastin or 10 min before wounding. Images were collected in fluorescence mode on an IVIS spectrum using Living Image software. Region of interest measurements were made, the fluorescence emission images were normalized to reference images and the radiant efficiency was computed.

Positron-emission tomography. 68Ga for the radiolabeling reaction was obtained by the fractioned elution of a commercially available 68Ge/68Ga generator in 1 ml of 0.1 M HCl. The acidic elution solution was removed and the 68Ga was resuspended in a 25 mM sodium acetate solution (pH 4.0). The labelling of the DTPA-conjugated peptide with 68Ga was accomplished using standard chelation conditions. Briefly, 100 μl of 128 mCl/C2 68Ga (10 mCi) and 10 μl of DTPA–PAR1–RIP in a 100 μl volume of 25 mM sodium acetate (pH 4.0) was added to an equal volume of 68Ga (10 mCi) and incubated at 37 °C for 60 min. Labelling efficiency was determined by radioactive thin-layer chromatography to be >99.9%. After labelling, the pH was adjusted to neutrality by the addition of 1.25 M sodium acetate and the samples were diluted with PBS for injection without any further purification. PET scans were performed on a PET/ computed tomography (CT) scan (Inveon, Siemens Healthcare, Malvern, PA). Mice (n = 5 per treatment) fasted overnight and were injected intravenously with 150–200–μCi 68Ga-labelled DTPA–PAR1−RIP corresponding to a peptide mass of 150–200–μCi 68Ga–PAR1–RIP. PET/CT imaging was performed from 2 to 24 h post-injection. Images were acquired in 120 min frames and reconstructed using the available software.

Clot retraction assays. Clot retraction assays were performed as previously described. Briefly, human blood was drawn from healthy donors and clotting was triggered in re-calculated (10 mM CaCl₂) whole blood by bovine thrombin (2.5 U ml⁻¹), in the presence of probes, DMSO or tirofiban (100 μM). Clotting and subsequent retraction proceeded in glass tubes kept at 37 °C in a water bath for 2 h. Clots were imaged and weighted using a Mettler Toledo precision balance NewClassic MS. Informed consent was obtained from all human subjects and experiments performed in compliance to the protocol approved by the HUAP Research Ethics Committee, Brazil—ID number: 241.284. The usage of anti-inflammatory drugs, anticoagulants or hormones was adopted as sample exclusion criteria (donors under medical regimen that could affect normal haemostasis were not included in the study).

Statistical analysis. Toxicology results are presented as mean ± s.e.m. Data were analysed using GraphPad Prism (GraphPad Software Inc., San Diego, CA) and statistical differences among experimental groups of animals were detected using one-way analysis of variance (ANOVA) followed by Dunnett’s post-test; P < 0.05 was considered statistically significant.

References


