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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Functional imaging is an increasingly common approach to measure biological processes in vivo and offers the potential 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 proteases are particular target enzymes of interest given their specificity and low signal intensity with high background noise. Visualizations are often impaired by the high background noise. Enzyme-activated probes, developed to overcome this stoichiometric limitation, have typically suffered from low specificity and low signal intensity with high background noise. Past thrombin-targeted imaging agents have significant limitations that illustrate the need for alternative strategies. Long circulating half-lives and limited signal-noise ratios combine to hinder their utility with an invasive procedure often being required for probe detection. Previous designs also borrow heavily from historically successful in vitro approaches using synthetic peptide substrates whose specificity hinges on a few amino acid residues. These synthetic peptide substrates were created to assist in protein purification or to answer questions of mechanistic enzymology, and therefore, their in vitro applicability is limited. Proteolytic turnover of short oligopeptide substrates is dominated by generic interactions in the protease active site, leading to considerable non-specific hydrolysis. Similar energetics underlie inhibitors of proteases, and inhibitor designs now aim to minimize contacts with the generic protease active site architecture to achieve selectivity that is meaningful in vivo. As proper registration within the active site is a prerequisite for catalysis, we sought to create probes that could incorporate larger peptide targeting elements to maximize selectivity for individual proteases.

To build sensitivity for probe detection in vivo, we coupled cleavable substrates with probe-containing peptides that localize in cell membranes only after proteolytic release. We turned to peptides that prefer spontaneous interaction with phospholipid membranes that prefer, but do not require, negatively charged surfaces as a scaffold. Often described as antimicrobial peptides (AMPs), with varying degrees of haemolytic activity, these polymers represent rich physicochemical diversity for agent modification. We reasoned that such peptides would provide a substrate, yet established localization mechanism that likely could be influenced by proteolysis. AMP properties differ from previously described designs that incorporate highly hydrophobic groups or significant electrostatics for agent localization, which can lead to non-specific accumulation. In particular, our agents aim to have limited non-specific interaction with healthy cells by permeabilizing membranes, the compounds have a good safety profile and do not affect hepatic or renal functions (Supplementary Fig. 4; Supplementary Table 2). We term these protease-responsive constructs restricted interaction peptides (RIPs) and found them capable of the non-invasive imaging of thrombin activity in vivo and also at monitoring this activity at the cellular level.

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 nomenclature and more than 1,000Å² 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
phosphatidylserine (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 evident by the <3-nm 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 containing the pro-peptide and PC liposomes, proteolysis of the substrate was observed indicating that the pro-peptide does not associate with the membrane and is available for proteolytic activation (Fig. 2g). Other peptide compositions such as TempL, coupled with the corresponding sequence of PAR2 (pI 10.6) bound constitutively to lipid evident by their maximum emission wavelength decrease in the presence of sodium dodecyl sulphate (SDS) micelles (Supplementary Fig. 2). These alternate designs indicate overall charge, position of charge and the 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.

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-binding properties with lipids and cells suggested PAR1–RIP fit our design criteria and would bind eukaryotic membranes after proteolytic activation with a preference for PS.
Kinetics and properties of dye-conjugated PAR1–RIP. PAR1–RIP bearing a N-terminal cysteine was labelled with a variety of fluorescent dyes (Supplementary Fig. 8) using maleimide chemistry and found to retain restricted membrane interaction that 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. 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, f, g). 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, specificity increases 10-fold (Fig. 3g). Inclusion of the 10 amino acids responsible for exosite binding to thrombin further increases the relative specificity eightfold and reduces the K_M to a physiologically relevant value of 0.2 ± 0.1 μM (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, was measured as a function of RIP insertion time into 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. These data suggest RIP technology is capable of carrying payloads of varying size, charge and complexity to membrane surfaces with limited caveats.

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of $8.4 \pm 0.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, 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 $K_M^{\text{ex vivo}}$, $26,27$. 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 alone. Several of these proteases are thought to signal through PARs in vivo through assistance from a co-factor. Together these data indicate PAR1–RIP possesses 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 deposits intensely 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-mouse anti-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, $\times 5$ and $\times 40$, respectively. These observations highlight the responsiveness of PAR1–RIP to proteolysis and amplified by PS exposure, without interfering with normal haemostasis.
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...
Figure 5 | Non-invasive functional imaging of thrombin-mediated clots in a murine model of pulmonary embolism and its tracking to the cellular level. (a) Injection of Cy7–PAR1–RIP (500 pmol) with thromboplastin to Foxn7™ mice (n = 3 per treatment group) produces pulmonary emboli that can be detected non-invasively via near-infrared fluorescence within 15 min post injection. (b) Probe accumulates predominantly in the lungs in this model and not other organs. (c) Fluorescein-conjugated probe lung accumulation is correlated to proteolysis as an uncleavable variant accumulates significantly less and can be reduced by direct thrombin inhibitors like argatroban (indicated by − or +). (d) Non-invasive signal correlates with the amount of clotting agent and confirmed by ex vivo analysis of organ labelled normalized by its weight. The linear response curve reflects the use of non-lethal doses of thromboplastin more than 10-fold below the LD50. 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. (e) The Cy7 signal observed in non-invasive images and that of the extracted organs results from few microscopic emboli (×) generated through non-fatal doses of thromboplastin, whose fluorescence radiates from individual emboli rather than widespread probe deposition throughout the lung. Healthy pulmonary alveoli (■) are preserved, as well as the (○) circulation in bigger vessels (scale bar, 50 μm). (f) Cy7–PAR1–RIP (purple) accumulates within the lumen of the vessel and on or near platelets (CD41, red; scale bars, 25 μm white, 0.5 mm yellow). Cell nuclei revealed by DAPI (blue) and their membranes with the lipophilic dye WGA (green). RE, relative equilibrium; TF, tissue factor (thromboplastin).

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 \( \sim 10^{10} \) 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 \(^{68}\)Ga (t_{1/2} 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.
PET/CT using 68Ga-labelled DTPA–PAR1–RIP. Healthy mice (n=3 per group) were tail-vein injected with 150–200 μCi of 68Ga-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 RIP technology. Non-lethal pulmonary emboli via tail-vein injection of thromboplastin were detected non-invasively by the clinically relevant imaging modality PET/CT using 68Ga-chelated DTPA–PAR1–RIP. Healthy mice (n=5 per treatment group) were tail-vein injected with 150–200 μCi of 68Ga-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. 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.

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 transverse 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).
images of disease solely based on the presence of PS and improved probes are sought. RIPS 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 PSI 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 activity (for example, adding different polycationic sequences to the protase target domain) may eventually facilitate RIP technology applications in non-invasive clinical diagnostics and prognostics.

**Methods**

**Reagents**. The lipids, e.g. 1-2-PC, brain 1-2-PS, 1-2-phosphatidylethanolamine and dodecylphosphocholine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Polycarbonate filters for extruded lipid vesicle preparation using the LiposFast system from Avestin, Inc. (Ottawa, Ontario, Canada). Other chemicals were of analytical grade and of the highest purity and were purchased from Sigma-Aldrich (St. Louis, MO). Wheat-germ agglutinin (WGA) conjugated with Oregon Green 488 was from Invitrogen (Carlsbad, CA). DRAQ7 was from BioStatus (Shepshed, UK). Procoagulant and anticoagulant proteases were from HaematoLogic Technologies (Essex Junction, VT).

**Liposome preparation**. Liposomes were prepared by rehydration and extrusion. PS, PC or phosphatidylethanolamine were dissolved in chloroform at a desired molar ratio (80–125 μmol) and the solvent removed under vacuum in a rotary evaporator for at one hour. The resulting lipid film was dried under stream of nitrogen and 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 prepared 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 fluorescent of tryptophan within the membrane-binding segment of the peptide were acquired using a Fluorolog-3 spectrophotometer (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. From these suspensions, 150 μl was added to 0.85 ml PBS, and then peptide in solution (2 mg/ml in PBS) was added to produce a final peptide concentration of ~0.01 mg/ml. A peptide/lipid molar ratio of 1:40 was maintained. Tryptophan emission spectra of the lipid/peptide suspension were acquired using a previously reported protocol38. The filter set for this experiment was 330/6, 595/20, and 670/20, and spectra were obtained with 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. Samples were treated using Lasco spectroscopy 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, XIa, plasmin and aPC were obtained from HA 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 18 h at 37°C overnight. PAR1–RIP probes were prepared by extrusion of the lipid suspension through 100-nm pore-size polycarbonate filters. Liposomes were used within 1 week of their preparation. The filter set for this experiment was 350/6, 380/6, and 670/20, and spectra were obtained with a scan rate of 100 nm min⁻¹. The resulting lipid film was dried under stream of nitrogen and 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 prepared by extrusion of the lipid suspension through 100-nm pore-size polycarbonate filters. Liposomes were used within 1 week of their preparation.

**Animals**. Adult wild-type C57BL/6 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 after 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 elsewhere39. Briefly, C57-–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/6 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 signal upstream from the site of occlusion. Further analysis was performed using the IVIS imaging system with excitation of 640 nm and emission 700 nm. 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.26 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.26, Brieﬂy, anaesthesia was induced in F04 and C57BL/6j subjects with 3–4% 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 inducing TRAP-1-RIP probe was administered in C57BL/6j mice at a concentration of 100 picomoles per injection of 150 μl 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.26, Brieﬂy, anaesthesia was induced in F04 and C57BL/6j subjects with 3–4% 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 inducing pulmonary thrombosis—pulmonary embolism.

Histology. Histological staining was conducted on lung tissue sections embedded with parafﬁn by the UCGF 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) in each mouse.

NIR optical imaging. Nude mice for the optical studies (n = 5 per treatment) were fed an aﬂal-flea-free diet of Harlan Teklad Global 2018 to minimize background ﬂuorescence. The mice were anaesthetized with 2% isoflurane. PAR1–RIP conjugated to ATT0680 or Cy7 were diluted in PBS containing 0.1% PEG 16,000 and administered at a final concentration of 500 picomoles per injection without any further purification. PET scans were performed on a PET/CT imaging platform, PAR1–RIP probe was administered in C57BL/6j mice at a concentration of 300 picomoles per injection of 150 μl 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.26, Brieﬂy, anaesthesia was induced in F04 and C57BL/6j subjects with 3–4% 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 inducing pulmonary thrombosis—pulmonary embolism.

Clot retraction assays. Clot retraction assays were performed as previously described.24 Brieﬂy, blood was collected from healthy donors using a BD Vacutainer Safety-Lok 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, 0.1738 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 (154.3 ± 14 ± 103 g−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 aggregation monitored at 37 °C in a Warner 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 HUAPE 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).

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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.

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