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Targeting a Proteinase Activated Receptor-4 (PAR4) carboxyl terminal motif to regulate platelet function

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Text Details:
Text Pages:
Tables: 1
Figures: 5
References: 52
Abstract: 118 words
Introduction: 592 words
Results 1213 words
Discussion: 806 words

ABBREVIATIONS USED
Amino acids are abbreviated by their one-letter codes, e.g. L = leucine, S = serine.
HEK: Human embryonic kidney-derived cells
HBSS: Hank’s Buffered salt solution

rLUC: Renilla luciferase

PAR: Proteinase-activated receptor (PAR1, PAR2)

YFP or eYFP: Yellow fluorescent protein
Abstract

Thrombin initiates human platelet aggregation by coordinately activating proteinase activated receptors (PARs)-1 and -4. However, targeting PAR1 with an orthosteric tethered ligand binding site antagonist results in bleeding, possibly due to the important role of PAR1 activation on cells other than platelets. Because of its more restricted tissue expression profile, we have therefore turned to PAR4 as an antiplatelet target. We have identified an intracellular PAR4 C-terminal motif that regulates calcium signaling and β-arrestin interactions. By disrupting this PAR4 calcium/β-arrestin signaling process with a novel cell-penetrating peptide, we are able to inhibit both thrombin-triggered platelet aggregation \textit{in-vitro} and clot consolidation \textit{in-vivo}. We suggest that targeting PAR4 represents an attractive alternative to blocking PAR1 for antiplatelet therapy in humans.
Introduction

Platelet activation and the formation of a multiplatelet thrombus subsequent to blood vessel injury underlie the pathophysiology of a number of cardiovascular diseases. Therapeutic intervention to prevent thrombosis involves the use of anti-coagulants or anti-platelet agents. Anti-coagulants include direct coagulation pathway inhibitors (coumarins, heparins and thrombin catalytic site inhibitors). While clinically effective, these agents present with significant challenges and require stringent patient monitoring. Thus, much effort has been expended to develop anti-platelet agents including the widely used salicylates, GPIIb/IIIa inhibitors and ADP receptor antagonists. These agents are also clinically very useful and used widely. However there are many drawbacks and side effects that limit their use (McKee et al., 2002; Wiviott and Antman, 2004).

Thrombin is the most potent activator of platelets, and in humans it acts on two G-protein coupled receptors, proteinase activated receptors (PARs)-1 and 4 (Coughlin, 2000), to trigger multiple responses including platelet shape change, granule secretion and aggregation. These actions have prompted the search for drugs that target the thrombin-activated receptor, PAR1 (Andrade-Gordon et al., 1999; Bernatowicz et al., 1996; Chackalamannil et al., 2005; Serebruany et al., 2009). Thrombin activates PARs 1 and 4 by proteolytically unmasking an N-terminal motif that acts as a tethered ligand (TL) to initiate signaling. This TL mechanism has made development of antagonists challenging. Notwithstanding, the small molecule PAR1 peptidomimetic antagonists that have been developed (Chackalamannil et al., 2008; Chackalamannil et al., 2005; Derian et al., 2003) have resulted in clinically useful agents like Vorapaxar (SCH530348, trade name Zontivity) (Baker et al., 2014; Goto et al., 2010; Morrow et al., 2012a; Morrow et al., 2012b) that block thrombin-mediated platelet aggregation whilst retaining thrombin’s hemostatic clotting activity. Unfortunately, some unexpected side effects have become evident in the course of the clinical studies of Vorapaxar, including incidences of
intracranial bleeding (Morrow et al., 2012a; Morrow et al., 2009; Pierluigi Tricoci, 2011; Tricoci et al., 2012). While not fully understood, one explanation for the bleeding diatheses could come from the relatively widespread expression of PAR1, particularly on endothelial cells (Ramachandran, 2012), where it has an important role in maintaining the integrity of the endothelial barrier (Bae and Rezaie, 2008; Feistritzer et al., 2006; Schuepbach et al., 2009). Endothelial barrier protective responses through PAR1 are triggered by activated protein-C, as opposed to thrombin activation of PAR1, which can disrupt the endothelial barrier (Riewald and Ruf, 2005; Schuepbach et al., 2012). More recently, compounds that target PAR1 on the intracellular face of the receptor have been developed in order to target pro-thrombotic signaling specifically through this receptor while sparing the cytoprotective signaling pathways (Aisiku et al., 2015; Dowal et al., 2011). Thus, compounds that bind to the third intracellular loop of PAR1 inhibit $\alpha_{13}$ dependent signaling (Aisiku et al., 2015), while compounds that interact with helix 8 of PAR1 block $\alpha_{4}$ dependent signaling (Dowal et al., 2011).

Given the challenges in selectively blocking the detrimental effects of activating PAR1 on platelets while maintaining PAR1 cytoprotective responses in endothelial cells, we turned to the other platelet thrombin receptor - PAR4 (Xu et al., 1998). PAR4 has a more restricted tissue expression profile and could be a more tractable therapeutic target for antiplatelet therapy in humans. Our initial studies were aimed at trying to understand the intracellular receptor motifs regulating signal transduction through PAR4. We identified an intracellular C-terminal sequence in PAR4 which regulates calcium signaling and is critical for PAR4- $\beta$ -arrestin interactions. We followed on with the development of a cell penetrating peptide based on that sequence that blocks PAR4 signaling and platelet aggregation \textit{in-vitro} and prevents clot consolidation \textit{in-vivo}. 
Materials and Methods

Chemicals and Other Reagents. Thrombin from human plasma (catalogue number 605195; 2800 NIH units/mg), verified to be free of trypsin-like activity using soya trypsin inhibitor, was from EMD Biosciences (San Diego, CA). A concentration of 1 unit/ml was calculated to be 10nM thrombin.

Design and synthesis of agonist and antagonist peptides. All peptides, (>95% purity: HPLC/Mass spectrum) were purchased from the Peptide Synthesis Facility, University of Calgary (peplab@ucalgary.ca) or EZbiolabs (Carmel, IN). The PAR4 antagonist targeting the C-terminal intracellular face of PAR, corresponding to the receptor’s putative arrestin-interacting site, was prepared as an N-terminal palmitoylated peptide to facilitate its insertion into the plasma membrane. The antagonist peptide, palmitoyl-RAGLFQRS-NH₂ (RAG-8) and the control reverse-sequence peptide, palmitoyl-SRQFLGAR-NH₂ (SRQ-8: Reverse-RAG8) were custom synthesized by EZbiolabs (Carmel, IN) using standard solid phase synthesis, purified by reverse-phase high-performance liquid chromatography and verified by mass spectrometry analysis.

Cell lines and culture conditions. All media and cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Human embryonic kidney-derived HEK-293 cells (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate and 2.5µg/ml plasmocin (Invivogen, San Diego CA). Cells stably transfected with Wild Type (wt) or mutated PAR4-expressing vectors were routinely cultured in the above media supplemented with 600µg/ml G418. Since trypsin activates the PARs, cells were routinely subcultured using enzyme-free isotonic phosphate-buffered saline, pH 7.4, containing 1 mM EDTA and plated in appropriate culture plates or glass bottom slides for further experimentation.
Molecular cloning and constructs. The plasmid encoding human PAR4 (wt-hPAR4) cloned in pCDNA3.1 (GenBank: AY431102.1) was obtained from the cDNA Resource Center (University of Michigan, Rolla, MO; currently hosted at Bloomsburg University, Bloomsburg, PA). A C-terminal eYFP (enhanced YFP) tag was fused in-frame with the PAR4 sequence by mutating the PAR4 stop codon to tyrosine and insertion of the eYFP fragment with flanking XhoI and XbaI restriction enzyme sites at the C terminus of the PAR4 coding sequence. Plasmid DNA mutations in the C-terminus of PAR4 were created using the QuikChange Lightning Multi Site-directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON) to generate all mutants described in this study. All constructs were verified by direct sequencing (University of Calgary DNA sequencing facility). Cells were transfected with FuGENE 6 (Promega) in 6-well multiplates (Nunc). Transfected cells were subcultured under G418 (600 µg/ml) selection, sorted by flow cytometry, and cell stocks were maintained in liquid nitrogen for future experiments.

Calcium signaling. Calcium signaling experiments were performed essentially as described before (Mihara et al., 2013; Ramachandran et al., 2011). Cells cultured to approximately 80% confluency in a T75 flask were detached in enzyme-free cell dissociation buffer, re-suspended in 18 ml of serum-containing growth medium, and 100 µl/well of the cell suspension was plated in black-walled cell culture-treated clear bottom 96-well plates (Corning) and cultured overnight. The next day, adherent cells were washed with PBS and placed in 100µL of Fluo-4-AM no wash (NW) calcium indicator dye (Thermo Fisher Scientific). Intracellular fluorescence (excitation 480 nm; emission recorded at 530 nm) was monitored for 2 min after the injection of agonists (enzymes or PAR-activating peptides) into each well, using a Victor X4 plate reader (PerkinElmer Life Sciences). The increase in fluorescence emission monitored at 530 nm was used as an index of increases in intracellular calcium. In some experiments calcium signaling was also monitored in Fluo4 loaded cells in suspension on an AB2 spectrofluorometer (Thermo Life Science). Where appropriate, responses were normalized to the calcium signal generated.
by 2 μM of A23178 (Sigma), a calcium ionophore.

**MAPKinase assay.** Agonist-stimulated MAPKinase signaling in HEK-293 cells expressing wt-PAR4-YFP or dRS-PAR4-YFP was monitored by western blot analysis as described previously (Mihara et al., 2013; Ramachandran et al., 2011). In brief, cells were rinsed with PBS and placed in serum free media. Cells were then stimulated with agonists for varying time periods, rapidly rinsed with ice-cold isotonic phosphate-buffered saline and placed on ice. Total protein was extracted by adding ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.5 % NP40, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 25 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF and 1mM DTT) and cleared by centrifugation (15,000Xg for 10 minutes). The protein samples were heat-denatured at 92°C for 10 min in denaturing Laemmli buffer and resolved on 4-20% gradient Novex Tris-Glycine gels (ThermoFisher). The resolved proteins were transferred to PVDF membrane, blocked in PBST buffer [PBS with 0.1% (v/v) Tween-20] supplemented with 1% ECL Advance Blocking Agent (GE Healthcare, Waukesha, WI) for 1 h at room temperature. p42/44 (Thr202/Tyr204), p38 (Thr180/Tyr182) and AKT (Ser 473) phosphorylation was detected with specific antibodies (Cell Signaling Technology) (diluted 1/2,000 in PBST with 1% ECL Advance Blocking Agent) overnight at 4°C. phospho-P42/44 immunoreactivity was detected using the horseradish peroxidase (HRP)-conjugated anti mouse or rabbit secondary antibody (Cell Signaling Technology) (1/10,000 in PBST/1 % ECL Advance Blocking Agent for 1h). After washing the membrane with PBST, the peroxidase activity was detected with the chemi-luminescence reagent ECL-Advance (GE Healthcare, Waukesha, WI) on a KODAK Image Station 4000MM. PVDF membranes were then stripped with stripping buffer (ThermoFisher) at room temperature, blocked in PBST with 1% ECL Advance Blocking Agent before incubation with the appropriate Total-P42/44 (t-P42/44), T-p38 or T-AKT antibody (1/2000 in PBST with 1% ECL Advance Blocking Agent) overnight at 4°C and incubation with
the horseradish peroxidase conjugated anti-rabbit secondary antibody (1/10,000 in PBST/1 % ECL Advance Blocking Agent for 1 h). Membranes were washed with PBST, and imaged using the chemiluminescence reagent (ECL-Advance) and a Kodak Image Station 4000MM. Band intensities representing activated MAPKinase were quantified using the ImageJ quantification software (http://rsbweb.nih.gov.ezproxy.lib.ucalgary.ca/ij/). Phospho-kinase levels were normalized for differences in protein loading by expressing the data as a percentage of the corresponding total-kinase signal. MAPKinase activation was also monitored using the Proteome Profiler Human Phospho-MAPK Array ARY002 (R&D systems, Minneapolis, MN) according to the manufacturer’s instructions. Platelet AKT activation was monitored following extraction of proteins from 100µL of a washed platelet suspension (3X10^8 cells/ml, see below) using the same protein extraction and western blotting procedures described above. Cell were stimulated with agonists for 10 minutes and all antagonist pre-treatments was done for 20 minutes.

**Confocal microscopy.** HEK-293 cells transfected with wt-PAR4-YFP or dRS-PAR4-YFP with FuGENE 6 in six well plates were sub-cultured into 35 mm glass bottom culture dishes (MatTek) and placed in growth medium for an additional 16 hours. Cells were stimulated with agonists for specified times, fixed with 10% buffered formalin and receptor trafficking was monitored by imaging eYFP expression with an Olympus FV1000 confocal microscope system on an Olympus IX70 microscope using the fluoview system software.

**BRET detection of β-arrestin recruitment.** Resonance Energy Transfer-based detection of β-Arrestin-1 and -2 interaction with wt-PAR4-YFP and dRS-PAR4-YFP was monitored in HEK-293 cells as described (Ramachandran et al., 2011; Ramachandran et al., 2009). PAR-YFP (1 µg) and Renilla luciferase-tagged β-arrestin-1 (Rluc-β-arr1 and 2) (0.1µg) were transiently transfected for 48 hours. Cells were plated in white 96-well culture plates (Brand plates), and interactions between the receptors and β-arrestin-1/2 were detected by measuring BRET at
timed intervals over 20 min following the addition of 5 µM coelenterazine (Nanolight Technology, Pinetop, AZ) on a Mithras fluorescence plate reader (Berthold) in luminescence mode using the appropriate filters.

**Platelet isolation and aggregation assay.** Blood was drawn under informed consent (REB14-0367) from healthy volunteers who denied taking any anti-platelet drugs by standard venipuncture into 4 ml Vacutainer tubes containing 0.45 mL of 0.1 Molar Sodium Citrate Solution. Platelet aggregation studies were done in washed platelets. The blood was centrifuged at 300g for 15 min and the platelet rich plasma was transferred to 50 ml conical tubes containing 10% acid citrate dextrose (ACD) solution (39mM citric acid, 75mM sodium citrate, 135mM glucose, pH 7.4). Following another centrifugation step at 800g the pelleted cells were resuspended in tyrode’s buffer (12mM NaHCO3, 127mM NaCl, 5mM KCl, 0.5mM NaH2PO4, 1mM MgCl2, 5mM glucose and 10mM HEPES), counted on a coulter counter, and adjusted to a concentration of 3X10^8 cells/ml. 400 µL of this cell suspension was used for light transmission aggregometry on a platelet aggregometer (Chrono-Log Corp, Havertown, PA).

**Confocal intravital imaging in-vivo.** Mice (8-10 week old male C57BL/6) were injected intraperitoneally with 20 mg/kg RAG8 or the control reverse-RAG8 in 100µl of isotonic phosphate-buffered saline pH 7.4. Mice were then anesthetized (10 mg per kg body weight xylazine hydrochloride and 200 mg per kg body weight ketamine hydrochloride), and body temperature was maintained using a heating pad. The right jugular vein was cannulated to administer additional anesthetic and fluorescent labels. Platelets were labeled with a CD49b antibody coupled to Alexa 647 (Biolegend, 5µg/mouse) and blood vessels were labeled with an anti-CD31 (PECAM) antibody coupled to PE (ebioscience, 2µg/mouse). The femoral artery and vein were surgically exposed, baseline readings were taken and a 1-2 mm² filter paper soaked in 10% FeCl3 was placed on the blood vessels for 3 min. The injury site was rinsed thoroughly.
by perfusing with isotonic saline and development of the thrombus was imaged using spinning
disc confocal microscopy. Imaging was performed using an Olympus BX51W1 (Olympus)
upright microscope equipped with a 4×/0.16UPlanSApo or 10x/0.30 UPlanFLN objective. The
microscope was equipped with a confocal light path (WaveFx, Quorum) based on a modified
Yokogawa CSU-10 head (Yokogawa Electric Corporation). Laser excitation at 488, 561, 649
and 730 nm (Cobalt) was used in rapid succession, and fluorescence in green, red and blue
channels was visualized with the appropriate long-pass filters (Semrock). Exposure time for all
wavelengths was constant at 1s. Sensitivity settings were kept the same for all experiments. A
512 X 512 pixel back-thinned EMCCD camera (C9100-13, Hamamatsu) was used for
fluorescence detection. Volocity Acquisition software (Improvision) was used to drive the
confocal microscope. Images captured using the spinning disk were processed and analyzed in
Volocity 4.20. Thrombus area was quantified over the entire field of view for each treatment
using the Volocity software.

**Measurement of tail vein bleeding times (TVBTs).** Mice were injected with 20mg/kg RAG8 or
control reverse-RAG8 and anesthetized with an i.p. injection of Ketamine/Xylazine (10 mg per
kg body weight xylazine hydrochloride and 200 mg per kg body weight ketamine hydrochloride).
The tails were transected 0.5 cm from the tip and placed in a cuvette containing saline at 37°C.
The time taken for the cessation of blood flow was monitored. Once blood flow had stopped, the
vein was monitored for a further 60 seconds to confirm stable occlusion. If blood flow resumed
within the one-minute time-frame, the time taken for cessation of continued blood flow was
monitored again up to 800 seconds.

**Statistical Analysis.** Statistical analysis of data and curve fitting were done with Prism 5
software (GraphPad Software, San Diego, CA). Statistical significance was assessed using the
Student’s t-test or Anova with Tukey’s post-test.
**Results**

**Impaired calcium signaling via C-terminal mutant PAR4 receptors.** Based on sequence alignment, we initially observed that human PAR4 lacked a cysteine residue that is present in the rodent PAR4 (C368 in mouse PAR4) C-terminus, in the human PAR1 (C387-388) and in PAR2 (C361). Since C-terminal cysteine residues serve as sites of receptor palmitoylation and regulate signaling (Qanbar and Bouvier, 2003), we generated mutant PAR4 receptors QC-PAR4 where the glutamine is mutated to a cysteine, as well as a variant which had an 8 amino acid deletion flanking this region (dRS-PAR4) (Table 1).

In order to examine the functional consequence of such mutations, HEK-293 cells transiently expressing wt-PAR4-YFP, QC-PAR4-YFP and dRS-PAR4-YFP (Table 1; Figure 1A) were loaded with Fluo-4AM NW and calcium signaling triggered by the PAR4-selective activating peptide, AYPGKF-NH$_2$, was monitored. Since HEK-293 cells do not endogenously express PAR4, the calcium signal detected originated entirely from the transfected constructs. Equal levels of cell surface expression were observed for all constructs when transiently expressed at 48 hours and we further generated cell lines permanently expressing equivalent levels of wt-PAR4-YFP and dRS-PAR4-YFP by flow cytometry assisted cell sorting. In HEK-293 cells AYPGKF-NH$_2$ triggered concentration-dependent calcium signaling in wt-PAR4-YFP expressing cells (EC$_{50}$ 10µM). The dRS-PAR4 expressing cells did not generate a significant calcium signal (Figure 1B and C), while in QC-PAR4-YFP expressing cells, AYPGKF-NH$_2$ acted as a partial agonist with responses seen at 100 and 300µM concentrations which were 50% of the maximum elicited in the wt-PAR4-YFP expressing cells (Supplementary figure 1).

**MAPK signaling in C-terminal mutant PAR4 receptors.** Since in other studies we have observed that PARs can trigger MAPK signaling in the absence of calcium signaling (Ramachandran et al., 2011; Ramachandran et al., 2009) we investigated MAPK activation in
the HEK-293 cell lines expressing wt-PAR4-YFP or dRS-PAR4-YFP. Following activation of the cells with 100µM AYPGKF-NH₂ for 10 minutes, significant elevation of p42/44 and p38 MAPK was detected using Proteome Profiler Human Phospho-MAPK Array Kit ARY002 (R&D systems) for both wt and dRS-PAR4 (Supplementary figure 2A). In contrast, no activation-phosphorylation of RSK, GSK, JNK and AKT was observed following PAR4 activation. Further, using a pepducin targeting the third intracellular loop (ICL3) of PAR4 (p4Pal10)(Covic et al., 2002), we were able to inhibit the AYPGKF-NH₂ triggered p38 MAPK activation (Supplementary figure 2B).

**Loss of β-Arrestin interactions with dRS-PAR4 receptors.** Since previous studies with other GPCRs have shown that MAPK activation can occur in the absence of G-protein coupling through an arrestin-dependent mechanism (Luttrell and Gesty-Palmer, 2010), we investigated β-arrestin recruitment to wt-PAR4 and dRS-PAR4 using a BRET-based assay monitoring the interaction between YFP tagged receptor and Rluc-βarrestins. We found robust β-arrestin recruitment to wt-PAR4 in response to both thrombin and AYPGKF-NH₂ stimulation of cells (Figure 2A). However, the dRS-PAR4 expressing cells did not recruit β-arrestin in response to either stimulus (Figure 2A) while AYPGKF-NH₂ and thrombin triggered β-arrestin-1 and 2 recruitment to the QC-PAR4-YFP mutant receptor was not impaired (Supplementary figure 3A and B). Together with the inhibition of p38 MAPK by an intracellular loop 3 targeted pepducin, we conclude that PAR4 dependent MAPK signaling is β-arrestin independent and is dependent on PAR4 intracellular loop 3, and not C-terminus, interaction with signaling effectors.

**Impaired trafficking of dRS-PAR4 receptors.** Following activation with AYPGKF-NH₂, wt-PAR4 internalized into vesicular structures as is expected for activated GPCRs while the dRS PAR4 mutant did not internalize (Figure 2B), consistent with the observation that it did not recruit β-arrestins upon activation. Upon activation with AYPGKF-NH₂, the QC-PAR4 mutants
also internalized like wt-PAR4 (Supplementary figure 3C). Thus we conclude that the C-terminal 8-amino acid PAR4 motif that we have identified regulates calcium signaling, β-arrestin interaction with PAR4 and the internalization of activated receptors.

**Targeting signaling involving the PAR4 C-terminal RAG8 domain with cell penetrating peptides.** Having identified a key regulatory sequence in the PAR4 C-terminus, we investigated if we could pharmacologically target this motif to modify receptor function using a cell-penetrating palmitoylated peptide (a pepducin) in keeping with the approach developed for other GPCRs (Tressel et al., 2010). Therefore, to target the C-terminus of PAR4 we synthesized a peptide with a sequence corresponding to the identified 8-amino acid motif in PAR4 (RAGLFQRS), coupled to an N-terminal palmitoyl moiety (RAG8) and C-terminal NH₂ (Figure 3A). A control peptide had an identical architecture but with the reverse amino acid sequence (SRQFLGAR: SRQ8). We then investigated the ability of these peptides to regulate PAR4 function in various assays. At 10µM, RAG8 attenuated calcium signaling triggered by 10, 30 and 100µM of the PAR4 agonist peptide AYPGKF-NH₂ (Figure 3B). Similarly, both β-arrestin-1 and β-arrestin-2 recruitment to AYPGKF-NH₂ activated PAR4 were significantly attenuated by 10µM RAG8 (Figure 3C and D). RAG8 had no effect on the related GPCR, Proteinase Activated Receptor-2 recruitment of β-arrestin1 and 2 (Supplementary Figure 4).

**Targeting PAR4 to regulate platelet function.** Previous work has shown that β-arrestin-2 supports PAR4 signaling in murine platelets, enabling platelet fibrinogen binding and thrombus formation (Li et al., 2011). We therefore hypothesized that by interdicting the PAR4-arrestin interaction with our RAG8 pepducin, we would affect PAR4-mediated platelet aggregation and clot consolidation. Indeed, in assays done with washed human platelets, RAG8 inhibited platelet aggregation stimulated by the PAR4 agonist peptide (AYPGKF-NH₂), but not by the PAR1
agonist peptide (TFLLR-NH$_2$) (Figure 4A). Of note, RAG8 (Figure 4B), but not the control peptide SRQ8 (not shown), also inhibited platelet aggregation triggered by thrombin.

Since AKT signaling is known to be involved in murine platelet activation (Woulfe et al., 2004), we investigated whether PAR4 activation could trigger AKT phosphorylation in human platelets and whether RAG8 modulates this process (Figure 4C). In washed human platelets, the PAR4 agonist peptide and thrombin triggered an increase in phosho-AKT. RAG8 alone did not trigger a significant elevation of p-AKT, while RAG8 inhibited the AYPGKF-NH$_2$ and thrombin triggered elevation of AKT activation in a concentration-dependent manner (Figure 4C and D).

**Targeting PAR4 to regulate thrombus formation in-vivo.** Because we were able to block platelet aggregation with our RAG8 pepducin *in-vitro*, we next tested its ability to affect thrombosis *in-vivo* in a rodent model where platelet function is regulated by PAR4 in the absence of PAR1. We used spinning disc confocal microscopy to monitor murine thrombus formation following FeCl$_3$ blood vessel injury of blood in the presence and absence of either RAG8 or the control peptide, SRQ8. In keeping with our results *in-vitro* with washed platelets, we observed a significant inhibition of thrombosis at 15-30 minutes after FeCl$_3$ injury (Figure 5A and B) in RAG8-treated mice but not in SRQ8-treated animals. Upon analysis of the video recording of thrombus formation in real time after treatment with RAG8, we observed that at early time points following FeCl$_3$ injury, the platelet trombus does develop, but then very rapidly disintegrates and is washed away. This result suggested that RAG8 interferes with stabilization and consolidation of the platelet thrombus (Supplementary Video 1). This conclusion was supported by our observations using a tail-bleeding assay. We observed that in control mice, bleeding from the tail vein ceased completely at the 3-minute time point. However, in RAG8 treated mice, whilst bleeding also ceased at the 3-minute mark, blood flow then resumed, in some instances up to the 15-minute time point when the experiment was concluded (Figure 5C). This result indicated that the thrombus failed to stabilize.
Discussion

Our main finding was that a specific motif in the C-terminus of PAR4 (RAGLFQRS) coordinately regulated calcium signaling and interactions of the receptor with β-arrestins 1 and 2. Yet, the receptor missing this domain was nonetheless able to trigger activation of MAPKinase. Further, by targeting this sequence with a cell-penetrating peptide (RAG-8), we were able to block PAR4-dependent platelet activation in-vitro and to attenuate clot stabilization in-vivo without affecting signaling by PAR1. The impact of the RAG-8 peptide on platelet aggregation and clot consolidation can be linked to its inhibition of Akt activation, which has been identified as a key arrestin-dependent signaling event for platelet activation (Li et al., 2011; Woulfe et al., 2004). Our work thus underscores the utility of targeting PAR4 as an antithrombotic strategy, as an alternative to blocking platelet activation by PAR1. Our finding that deletion of a C-terminal motif disrupts Gαq dependent calcium signaling in addition to β-arrestin recruitment was unexpected since mutational and structural studies have shown that G-protein-receptor interactions occur through contacts at the intracellular loops and transmembrane helices (TM) of GPCRs (Chung et al., 2011; Rasmussen et al., 2011; Venkatakrishnan et al., 2013). While further work is required to understand the exact mechanisms, it is possible that the loss of signaling could come from a disruption of the network of receptor ionic and hydrogen-bond interactions, that may form a so called ‘ionic lock’ in the PAR4 carboxyl tail (Audet and Bouvier, 2012).

It is also interesting that dRS-PAR4 activation could still trigger MAPK signaling, suggesting that this occurs in a β-arrestin and Gαq independent manner, likely through Gα12/13 activation (Voss et al., 2007). In human platelets PAR4 does not interact with Gαi (Voss et al., 2007), however PAR4 coupling to Gαi is reported in endothelial cells (Hirano et al., 2007). Using the P4pal10 pepducin that targets the intracellular loop 3 (ICL3) of PAR4 we were able to attenuate p38 MAPK signaling, pointing to ICL3 as the Gα12/13 or Gαi interacting domain. Thus, our data
point to the C-terminus of the PARs as motifs for developing pepducin-like receptor regulators in addition to the intracellular loop sequences that have been used to date for the design of GPCR-inhibiting agents (O'Callaghan et al., 2012).

Our approach to blocking PAR4 activation complements the development of presumed orthosteric inhibitors of the receptor that are thought to target the tethered ligand docking site, like YD-3 (Wu et al., 2000; Wu et al., 2002) and substituted indoles based on the YD-3 structure such as ML354 (Wen et al., 2014; Young et al., 2013). The precise mechanism of antagonism of the indole-related compounds has not yet been determined. Further, whether the RAG-8 sequence we have identified interacts directly with effectors like Goq and the arrestins to modulate signaling or rather regulates the conformation of the C-terminal domain so as to change those interactions remains to be determined. Since we could not detect any inhibition of PAR1 or PAR2 signaling, RAG8 does appear to be a PAR4 specific inhibitor. The exact mechanism of action however remains to be elucidated. Nonetheless, the mechanisms whereby the indole-related compounds and the RAG-8 pepducin block PAR4 activation will undoubtedly differ. Since PAR4 subserves many physiological functions apart from regulating platelet reactivity, it will very likely be of value to block its actions in a pathway selective manner, as per the RAG-8 antagonist, rather than blocking all of its actions.

The mechanisms by which PAR4 stabilizes the platelet thrombus are not yet fully worked out, but PAR4 acts upstream of other factors that act to stabilize the platelet thrombus including the activation of the P2Y12 receptors (Cornelissen et al., 2011; Holinstat et al., 2006; Kim et al., 2002; Li et al., 2011) and may serve as an important node for multiple thrombin dependent responses in platelets. Thus, inhibition of PAR4 could prevent the formation of large occlusive thrombi, without preventing the formation of the small juxtamural thrombus that develops as a result of thrombin activation of PAR1. This selective inhibition of PAR4 that enables perivascular
homeostasis may be of particular relevance in individuals with a PAR4 mutation that renders their platelets more susceptible to PAR4 activation (Edelstein et al., 2014; Edelstein et al., 2013).

In conclusion, we have uncovered a novel molecular determinant of PAR4 signaling involving its C-terminal domain and have developed a cell-penetrating peptide based on this sequence that blocks PAR4-stimulated platelet activation and thrombus consolidation. This inhibition of PAR4 provides an attractive alternative to blocking PAR1 for dealing with vascular occlusive disease. We believe that this signaling mechanism may also be a therapeutic target in other settings where PAR4 can be involved and propose that the use of RAG8 could also be of benefit in pathologies such as pain and inflammatory disease (Mao et al., 2010; McDougall et al., 2009) where PAR4 is thought to play an important role.
Acknowledgements. The authors would like to thank Michael Dicay for help with the platelet aggregation assays. This work was supported by the Snyder Mouse Phenomics Resources Laboratory and Live Cell Imaging Facility, both of which were funded by the Snyder Institute for Chronic Diseases at the University of Calgary.

Authorship Contribution.

Participated in research design: Ramachandra, Hollenberg, Bouvier

Conducted experiments: Ramachandra, Mihara, Thibeault, Vanderboor, Petri, Saifeddine

Performed data analysis: Ramachandra, Mihara, Petri

Wrote or contributed to the writing of the manuscript: Ramachandra, Petri, Hollenberg, Bouvier
References:


Footnotes. This work was funded by grants from the Canadian Institutes of Health Research to RR, MDH and MB.
**Table 1:** Amino acid sequence of the human PAR4 C-terminus highlighting the different mutant constructs described in this study.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>WT-PAR4-YFP</td>
<td>SAEFRDKVRAgFLQPgDTvASKASAE-YFP</td>
</tr>
<tr>
<td>QC-PAR4-YFP</td>
<td>SAEFRDKVRAgFCRgDTvASKASAE-YFP</td>
</tr>
<tr>
<td>dRS-PAR4-YFP</td>
<td>SAEFRDKV---------PGDTvASKASAE-YFP</td>
</tr>
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Figure Legends:

Figure 1. PAR4 c-terminal domain regulates calcium signaling. (A) Scheme depicting the location and amino acid sequence for the PAR4 regulatory c-terminal motif. (B) In HEK cells transfected with wt-human PAR4 (wt-PAR4) or a PAR4 mutant with a deletion of the 8 amino acid motif (dRS-PAR4). Deletion of the c terminal motif abolishes PAR4 dependent calcium signaling. Data are expressed as the mean data ± SEM relative to calcium ionophore A23187 from at least three separate experiments. (C) Representative traces showing dose dependent AYPGKF-NH₂ triggered calcium signaling in wt-PAR4 expressing cells. The signal observed in dRS-PAR4 expressing cells treated with 100µM AYPGKF-NH₂ (red line) is also depicted. For clarity traces for 0.3 and 1µM AYPGKF-NH₂ in wt-PAR4 expressing cells which lie on top of the dRS-PAR4 trace are not shown.

Figure 2. PAR4 c-terminal domain is critical for PAR4 dependent β-arrestin interaction and trafficking. (A) HEK cells were co-transfected with Rluc tagged β-arrestin-1 or 2 and wt-PAR4-YFP or dRS-PAR4-YFP. Thrombin or AYPGKF-NH₂ triggered recruitment of β-arrestin to the receptor was monitored. wt-PAR4 expressing cells show a concentration dependent recruitment of β-arrestin which is abolished in the dRS-PAR4 expressing cell. Data are expressed as the mean ± SEM from three independent experiments. (B) PAR4-YFP or dRS-PAR4 YFP expressing HEK cells were stimulated with the PAR4 activating peptide AYPGKF-NH₂ and internalization of the receptors was monitored by confocal microscopy. dRS-PAR4 failed to internalize following agonist treatment. Arrows indicate internalized receptor containing vesicles. The scale bar is 10µm. Data are representative of the internalization seen in at least 4 independent experiments.

Figure 3. RAG8 inhibits PAR4 dependent calcium signaling and β-arrestin recruitment. (A) Scheme depicting structure of RAG-8 and proposed mechanism of action. (B) Pretreatment
of cells with 10µM RAG8 (20 minutes) abrogates PAR4 dependent calcium signaling. Data are expressed as the mean response relative to calcium ionophore A23187 from at least three separate experiments. * indicates a significant difference (p<0.05) in the RAG8 treated cells compared to the corresponding control cells and are the mean data from at least three independent experiments. Pretreatment of cells with 10µM RAG8 (20 minutes) abrogates PAR4 dependent (C) β-arrestin-1 and (D) β-arrestin-2 recruitment to PAR4. * indicates a significant difference (p<0.05) in the RAG8 treated cells compared to the corresponding control cells and are the mean data ± SEM from at least three independent experiments.

Figure 4. Targeting the PAR4 intracellular motif to regulate human platelet function. Representative light transmission aggregometry traces for platelet responses showing that (A) RAG8 inhibits platelet aggregation responses to PAR4 agonist peptide (AYPGKF-NH₂), but not the PAR1 agonist peptide (TFLLR-NH₂) (B) RAG-8 inhibits thrombin dependent platelet aggregation in platelets that express both PAR1 and PAR4 as the thrombin receptors. (C) Representative western blot showing RAG-8 inhibition of thrombin (1U/ml) and AYPGKF-NH₂ (100µM) stimulated AKT activation in human platelets. 100µL of a 3X10⁸ cells/ml platelet cell suspension was treated with agonists for 10 minutes. Samples were pre-treated with RAG8 for 20 minutes prior to agonist stimulation. (D) Densitometry analysis of phospho-AKT expression in human platelets normalized to total-AKT relative to the untreated control (NT). Data are mean ± SEM from three independent experiments. * indicates significant difference (p<0.05) from untreated samples (NT) as calculated by an ordinary one way ANOVA with a Tukey’s multiple comparisons test. # indicates significant difference (p<0.05) from the corresponding non RAG8 treated thrombin or AYPGKF-NH₂ treated sample.

Figure 5. RAG-8 inhibits FeCl₃ triggered thrombosis in vivo. (A) Mice were injected i.p. with RAG8 or the reverse control peptide SRQ8 (control). Platelets are labeled with Alexa 647-
CD49b antibody injected i.v., the femoral artery and vein are exposed and thrombosis is induced by placing a filter paper soaked in 10% FeCl₃. Images are taken over 30 min and image shown in Fig A is at the 15 minute time point. (B) The total area of the platelet thrombus was calculated over time. Data are the mean ± SEM from at least three mice. * indicates significant difference (p<0.05) from corresponding control (C) Mice injected with RAG8 or SRQ8 (control) had tails cut 0.5 cm from the end of the tail, placed in a cuvette with warm saline and time to cessation of bleeding monitored. Stable occlusion was determined to have occurred if the bleeding ceased for a full minute.
Figure 1
**Figure 2**

A. **Thrombin Activation**

![Graph showing Thrombin Activation](image-url)

- **PAR4 wt Arr1**
- **PAR4 wt Arr2**

B. **AYPGKF-NH₂ Activation**

![Graph showing AYPGKF-NH₂ Activation](image-url)

- **PAR4 dRS Arr1**
- **PAR4 dRS Arr2**

**B. wt-PAR4**

- **NT**
- **AYPGKF-NH₂**

**B. dRS-PAR4**

- **NT**
- **AYPGKF-NH₂**
Figure 3

Graph A: Comparison of Calcium ES50 (% of 2μM A23187) with different concentrations of AYPGKF-NH2 (100μM, 30μM, 10μM) in the presence of wt-PAR4-YFP and RAG-8 10μM.

Graph B: BRET Ratio for β-arrestin rLUC/PAR4-YFP showing a decrease with increasing concentrations of AYPGKF-NH2 (100μM, 30μM, 10μM).

Graph C: BRET Ratio for β-arrestin-1 with wt-PAR4-YFP, AYP 100μM, and RAG-8 10μM, showing a decrease with RAG-8.

Graph D: BRET Ratio for β-arrestin-2 with wt-PAR4-YFP, AYP 100μM, and RAG-8 10μM, showing a decrease with RAG-8.
Figure 4
Figure 5