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Contributions of PAR1 and PAR4 to thrombin induced GPIIbIIIa activation in human platelets

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Running title: Strategies for targeting PAR4 on human platelets

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Abbreviations: PAR, Protease activated receptor; ACS, acute coronary syndrome; MI, myocardial infarction; TL, tethered ligand; AP, activating peptide; TRA, thrombin receptor antagonists; CRC, concentration response curve; PET, positron emission tomography

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Abstract

Human platelets display a unique dual receptor system for responding to its primary endogenous activator, α-thrombin. Due to the lack of efficacious antagonists, the field has relied on synthetic peptides and pepducins to describe protease activated receptor (PAR)1 and PAR4 signaling. The precise contributions of each receptor have not been established in the context of thrombin. We took advantage of newly discovered PAR antagonists to contrast the contribution of PAR1 and PAR4 to thrombin mediated activation of the platelet fibrin receptor (GPIIbIIIa). PAR1 is required for platelet activation at low but not high concentrations of thrombin, and maximal platelet activation at high concentrations of thrombin requires PAR4. As the concentration of thrombin is increased, PAR1 signaling is quickly overcome by PAR4 signaling, leaving a narrow window of low thrombin concentrations that exclusively engage PAR1. PAR4 antagonism reduces the maximum thrombin response by over 50%. Thus, although still active at higher concentrations of thrombin, the PAR1 response is superseded by PAR4. Truncation of a known PAR4 antagonist and identification of the minimum pharmacophore converted the mechanism of inhibition from non-competitive to competitive, such that the antagonist could be outcompeted by increasing doses of the ligand. Fragments retained efficacy against both soluble and tethered ligands with lower cLogP values and increased free fraction in plasma. These reversible, competitive compounds represent a route towards potentially safer PAR4 antagonists for clinical utility and the development of tools such as radioligands and PET tracers that are not currently available to the field for this target.

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Introduction

Acute Coronary Syndrome (ACS) is the leading cause of death and morbidity in the western world (Grech and Ramsdale, 2003), with up to one-third of patients experiencing secondary events, including myocardial infarction (MI) and unstable angina within 6 months (Collinson *et al*., 2000). The importance of platelet activation in thrombus formation is reflected by the efficacy of anti-platelet reagents in preventing recurrent ischemic events. The advent of dual anti-platelet therapy (aspirin + clopidogrel) resulted in substantial reductions in cardiovascular events (Bowry et al., 2008); however, an increased risk of bleeding has been reported since the first aspirin/clopidogrel combination therapy clinical trials (Harker *et al*., 1999; Sabatine *et al*., 2005; Yusuf *et al*., 2001). Other P2Y12 antagonists (ticagrelor, prasugrel) have emerged showing incremental reduction in the risk of thrombosis but with a concomitant increase in TIMI major and fatal bleeding events (Montalescot *et al*., 2009; Wallentin *et al*., 2009; Wiviott *et al*., 2007). The most challenging aspect of developing new anti-platelet reagents is balancing efficacy with safety.

Thrombin is the terminal enzyme of the coagulation cascade at the center of both thrombosis and hemostasis. Thrombin is generated at the site of vascular insult, cleaving fibrinogen for cross-linking and activating vascular cells through protease activated receptors (PARs). Platelets express a dual receptor system for responding to thrombin, PAR1 and PAR4 (Coughlin, 2000; Kahn *et al*., 1999). Thrombin activates PARs through cleavage of the extracellular domain of the receptor, revealing an encrypted tethered ligand (TL) that binds intramolecularly to activate the receptor (Vu *et al*., 1991). PARs can also be activated artificially with a synthetic soluble “activating peptide” (AP) corresponding in sequence to the naturally derived TL (Vu *et al*., 1991; Xu *et al*., 1998). PAR1 contains a “hirudin-like domain” with high affinity for thrombin and, consequently, is activated by relatively low concentrations of thrombin (Vu *et al*., 1991). PAR4 lacks this domain (Xu *et al*., 1998) and requires more than a full log-order higher concentration of thrombin for activation (Kahn *et al*., 1999). PAR4, the low affinity

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receptor, is engaged after PAR1 in a sequential manner at a 20-70-fold slower rate (Covic *et al*., 2000). A lack of effective small molecule tools has prevented a detailed investigation of the relative roles of PAR1 and PAR4 in thrombin stimulated human platelet activation.

Thrombin receptor antagonists (TRAs) have been eagerly anticipated in cardiovascular medicine; vorapaxar is a PAR1 specific TRA that underwent two phase III clinical trials, TRA•CER (Executive and Steering, 2009) and TRA^{o}2P (Morrow et al., 2012; Morrow et al., 2009; Scirica et al., 2012; Tricoci et al., 2012). After safety review, the TRA•CER trial was halted early, and the TRA^{o}2P secondary prevention trial was partially discontinued due to an alarming increase in bleeding (Bohula et al., 2015; Morrow et al., 2013; Tricoci et al., 2012). Vorapaxar was approved with a black box warning against "use of zontivity® (vorapaxar) in patients with a history of stroke, transient ischemic attack, or intracranial hemorrhage or active pathological bleeding", greatly limiting the scope of its clinical utility. Efforts to target the alternate PAR on platelets, PAR4, were initiated and in 2013 Bristol Myers Squibb published a patent describing a series of efficacious and bioavailable PAR4 antagonists (Lawrence, 2013). We synthesized the lead from this patent as a tool reagent, BMS-3 (internally assigned as VU0652925), an analog of BMS986120, the recently disclosed clinical candidate. In conjunction with an analogue of vorapaxar that was kindly provided by Merck, we interrogated the relative contribution of PAR1 and PAR4 to thrombin mediated platelet activation.

We chose inside-out activation of the platelet integrin complex GPIIbIIIa as a primary readout. Activation of GPIIbIIIa allows interaction with divalent fibrinogen or multivalent vWF linking platelets and leading to aggregation (Savage *et al*., 2001). GPIIbIIIa dependent aggregation is central to thrombosis as evidenced by the effectiveness of GPIIbIIIa inhibitors (Bhatt and Topol, 2000; Coller *et al*., 1983). Both tools are selective for their respective receptors and effective against the TL. We demonstrated that antagonism of PAR1 can be completely overcome by increasing concentrations of thrombin. PAR4, on the other hand, is responsible for the majority of platelet response to higher concentrations of thrombin. PAR4

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antagonists reduce GPIIbIIIa activation by over 50%. Concerned with the safety of a non-competitive antagonist that does not allow full recovery of the platelet thrombin response, we deconstructed the PAR4 antagonist, identified a minimum pharmacophore, and converted the compound's mechanism of inhibition to a classic competitive modality such that it could be outcompeted by reasonable concentrations of thrombin.

Materials and Methods

Materials Activating peptides for PAR1 (PAR1-AP; SFLLRN) and PAR4 (PAR4-AP, AYPGKF) were purchased from GL Biochem (Shanghai, China). α-thrombin and γ-thrombin were purchased from Enzyme Research Laboratories (Southbend, IN). FITC conjugated PAC1 and PE conjugated P-selectin were purchased from Becton Dickinson (Franklin Lakes, NJ).

Blood collection and platelet isolation Human platelets were obtained from healthy volunteers. The studies were approved by the Vanderbilt University Internal Review Board. Informed consent was obtained from all individuals prior to the blood draw. Blood was collected into sodium citrate anticoagulant (final concentration 0.32%) through a 19 gauge needle. Platelet rich plasma is collected after centrifugation at 1,100 rpm (15 min, RT). Acid citrate dextrose is added and incubated for 10 min (RT) before centrifugation at 2,400 rpm for 10 min to isolate platelets. Platelets are washed and equilibrated with Tyrode's Buffer (15 mM HEPES, 0.33 mM NaH_2PO_4, pH7.4, 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM dextrose) with 0.1% BSA. Platelets were collected, counted on a Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA), and diluted in Tyrode's with 0.1% BSA to the indicated concentrations.

Platelet aggregation Platelets were diluted to $2.0x10^8$/mL and aliquoted into glass cuvettes. Antagonists were allowed to equilibrate with platelets for 20 min prior to stimulation with the indicated agonists. Aggregations were recorded for 10 min on a model 700 Optical Lumi Aggregometer (Chrono-log, Havertown, PA).

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Flow cytometry For detection of PAC1 (GPIIbIIIa activation) and CD62p (P-selectin expression) binding, washed platelets at $1.5x10^7$ cells/ml were pre-incubated with PE conjugated CD62P and FITC conjugated PAC1 for 20 min before stimulation with the appropriate agonist for 10 min. Samples were fixed with 1% paraformaldehyde in PBS for 20 min before dilution with PBS. Data was collected on a BD LSRII 5 laser (Becton Dickinson, Franklin Lakes, NJ) and analyzed with Flowjo software (Ashland, OR). Mean fluorescence intensity (geometric) of PE and FITC was determined from 30,000 events after compensation correction. Data were normalized to vehicle controls. EC_{50} values were gleaned from non-linear regression analysis (4-parameter, variable slope) performed with Prism (La Jolla, CA). For Schild analysis, dose ratios (DR) were constructed from PAR4-AP EC_{50} values with and without antagonist conducted on the same day with the same donor.

Plasma protein binding*:* The protein binding of each compound was determined in plasma via equilibrium dialysis employing RED Plates (ThermoFisher Scientific, Rochester, NY). Plasma was added to the 96 well plate containing test compound and mixed thoroughly for a final concentration of 5 µM. Subsequently, an aliquot of the plasma-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with a phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, aliquots from each chamber were diluted 1:1 with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile containing internal standard (50 ng/mL carbamazepine) (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rcf, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. Fraction unbound was determined using the following equation

$$F_u = \frac{Conc_{buffer}}{Conc_{plasma}}$$

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Intrinsic clearance*:* Human or rat hepatic microsomes (0.5 mg/mL) and 1 μM test compound were incubated in 100 mM potassium phosphate pH 7.4 buffer with 3 mM $MgCl_2$ at 37 °C with constant shaking. After a 5 min preincubation, the reaction was initiated by addition of NADPH (1 mM). At selected time intervals (0, 3, 7, 15, 25, and 45 min), aliquots were taken and subsequently placed into a 96-well plate containing cold acetonitrile with internal standard (50 ng/mL carbamazepine). Plates were then centrifuged at 3000 rcf (4 °C) for 10 min, and the supernatant was transferred to a separate 96-well plate and diluted 1:1 with water for LC/MS/MS analysis. The *in vitro* half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (CL_{int}, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (CL_{hep}, mL/min/kg, Eq. 3) was determined employing the following equations:

(1) $$T_{1/2} = \frac{Ln(2)}{k}$$

where k represents the slope from linear regression analysis of the natural log percent remaining of test compound as a function of incubation time

(2) $$CL_{\text{int}} = \frac{0.693}{in\,vitro T_{1/2}} x \frac{mL\ incubation}{mg\ microsomes} x \frac{45 mg\ microsomes}{gram\ liver} x \frac{20^{a}\ gram\ liver}{kg\ body\ wt}$$

[a]scale-up factors: of 20 (human) or 45 (rat)

(3) $$CL_{hep} = \frac{Q_h \cdot CL\,\text{int}}{Q_h + CL\,\text{int}}$$

where Q_h (hepatic blood flow, mL/min/kg) is 21 (human) or 70 (rat).

LC/MS/MS Bioanalysis of Samples from Plasma Protein Binding and Intrinsic Clearance Assays*.* Samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad mass spectrometer (San Jose, CA) via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient elution on a dual column system with two Thermo Hypersil Gold (2.1 x 30 mm, 1.9 μm) columns (San Jose, CA) thermostated at 40 °C. HPLC mobile

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phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 10% B after a 0.2 min hold and was linearly increased to 95% B over 0.8 min; hold at 95% B for 0.2 min; returned to 10% B in 0.1 min. The total run time was 1.3 min and the HPLC flow rate was 0.8 mL/min. While pump 1 ran the gradient method, pump 2 equilibrated the alternate column isocratically at 10% B. Compound optimization, data collection and processing was performed using Thermo Electron's QuickQuan software (v2.3) and Xcalibur (v2.0.7 SP1).

Results

Specificity and potency of PAR tool compounds. We synthesized a PAR4 antagonist (VU0652925) recently presented in a Bristol Myers Squibb patent (Fig 1A) (Lawrence, 2013) and Merck kindly provided an analog of vorapaxar (SCH602539) to study PAR1. Using soluble peptides corresponding in sequence to the TL of each receptor we profiled the specificity of each compound in human platelets. SCH602539 had no effect on PAR4-AP-induced aggregation at concentrations effective against PAR1-AP. Similarly, VU0652925 had no effect on PAR1-AP induced aggregation at concentrations effective against PAR4-AP (Fig 1B and 1C). Flow cytometry analysis of GPIIbIIIa activation (PAC1) and P-selectin expression (P-Sel) was consistent with aggregation demonstrating no major off-target effects at concentrations as high as 1 μM for each compound (Fig 1D and 1E). Both were fully effective against their respective APs and displayed comparable potency against both GPIIbIIIa activation and P-selectin secretion. Against PAR1-AP, SCH602539 had a PAC1 IC_{50} of 26.5 nM (-pIC_{50}±SEM: 7.58±0.05) and P-Sel IC_{50} of 36.5 nM (-pIC_{50}±SEM: 7.44±0.05). Against PAR4-AP, VU0652925 had a PAC1 IC_{50} of 43.0 pM (-pIC_{50}±SEM: 10.4±0.04) and a P-sel IC50 of 39.2 pM (-pIC_{50}±SEM: 10.41±0.04).

Inhibition of PAR1 and PAR4 abolish thrombin mediated signaling on human platelets. In order to dissect the roles of PAR1 and PAR4 in the context of thrombin-mediated platelet activation and accurately determine the relative contribution of each, tool compounds must

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display full efficacy against the TL. Incubation with SCH602539 or VU0652925 alone had only partial effects on thrombin mediated activation (Fig 2A). SCH602539 had no effect on 10 nM thrombin but reduced 2 nM thrombin mediated platelet activation by 27.3±4.73%; VU0652925, on the other hand, reduced activation by up to 74.4±8.72% in the context of 2 nM thrombin and 64.2±7.40% in the context of 10 nM thrombin. When combined at the most effective concentrations, 316 nM VU0652925 and 1 μM SCH602539, thrombin mediated platelet activation (Fig 2B) and aggregation (Fig 2C) was abolished. The fact that there was no residual activation with combined PAR1 and PAR4 antagonism indicates that each antagonist is capable of abolishing TL mediated activation of their respective receptor.

Relative contributions of PAR1 and PAR4 to thrombin mediated platelet signaling. Due to the lack of tool compounds with appropriate attributes, the precise contributions of PAR1 and PAR4 to platelet signaling has never been established in the context of its endogenous ligand thrombin. When exactly is PAR4 engaged as the concentration of thrombin increases? How much of the response at high concentrations of thrombin can be attributed to PAR4? To date these questions about a relevant pharmacological target remain only vaguely defined. To address this, thrombin concentration response curves (CRCs) were challenged with increasing doses of SCH602539 and VU0652925 individually. Displacement of the thrombin activation curve by each antagonist eventually saturated, representing the point at which the response is exclusively driven by the other PAR (Fig 3A and B). However, the nature of displacement was very different. Increasing doses of SCH602539 induced a parallel rightward shift whereas increasing doses of VU0652925 induced a depression of the maximal response. The parallel rightward shift with SCH602539 resembles the shift in agonist potency typically observed with competitive antagonists. As more agonist is added, the antagonist is outcompeted and the cellular response is restored; however, the displacement with SCH602539 saturates. Consistently, in the presence of a fixed concentration of VU0652925, increasing concentrations of SCH602539 lead to full suppression of the thrombin response. Thus, within the timeframe of

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the assay dictated by the kinetics of GPIIbIIIa activation on the human platelet, SCH602539 exhibits the characteristics of a non-competitive antagonist (Fig 3C) (Becker *et al*., 2009; Chintala *et al*., 2010). Since we demonstrated that SCH602539 is capable of abolishing PAR1 mediated GPIIbIIIa activation, the full restoration of the response with increasing concentrations of thrombin can only be attributed to engagement of PAR4. Importantly, these data indicate that PAR1 exclusively contributes to GPIIbIIIa activation at only a very narrow window of thrombin concentrations. VU0652925, on the other hand, induces a progressive and saturable depression of the maximum thrombin response with no rightward shift. The residual response in the presence of VU0652925, at high concentrations of thrombin, is likely PAR1 mediated. When platelets were preincubated with SCH6052965, increasing concentrations of VU0652925 fully suppressed the thrombin CRC, indicating that VU0652925 is also exhibiting non-competitive characteristics in our assay. Thus the residual response at high concentrations of thrombin in the presence of saturating doses of VU0652925 is PAR1 mediated. It is difficult to definitively define mechanism of action with functional assays alone. It is possible that these compounds are reversible but have an extremely slow off-rate. However, we were careful to conduct our assays in human tissue with a physiologically relevant readout allowing us to contrast biological activities of these antagonists and make suggestions about their physiologic implications based on the functional consequences. Importantly, these data indicate that although PAR4 requires slightly higher concentrations of thrombin to be engaged, it is responsible for the majority of thrombin mediated GPIIbIIIa activation on the human platelet.

Identification of the minimum pharmacophore. As shown in Table I (and Fig 4) example 1, BMS-3 (VU0652925) is a large molecular weight compound with implicit plasma binding and toxicology concerns. Given its large size and non-competitive nature in our assay we began an effort to identify the minimum pharmacophore within VU0652925 that retains specificity, activity against AP, and TL mediated activation. Activity against PAR4-AP, γ-thrombin, and PAR1-AP was monitored as compounds representing progressive truncations of VU0652925 were

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synthesized. Figure 4 illustrates the route taken to arrive at the minimum pharmacophore, a 2-methoxyimidazo[2,1-*b*][1,3,4]thiadiazole ring on an otherwise unsubstituted benzofuran core (example 6) and Table I shows biological activity. Although, potency suffered with the deletion of the 2-phenylthiazole and methoxy moieties from the benzofuran core, specificity and activity against AP and TL was retained.

Schild analysis and identification of competitive PAR4 antagonists. We performed Schild analysis to determine the mechanism of action of each compound in the series (Fig 5). Schild analysis of VU0652925 with AP suggests a non-competitive mode of action, consistent with the α-thrombin data (Fig 3D). Increasing concentrations of VU0652925 lead to full depression of the PAR4-AP CRC and log-log plots of DR-1 versus [VU0652925] yield a slope of 2.86±0.53, clearly inconsistent with a competitive mode of inhibition. Replacement of the 2-phenylthiazole group (example 2, VU0661247) leads to an apparent switch in modality. Increasing concentrations of antagonist fail to induce significant depression of the maximum response suggesting the antagonist is fully reversible within the time frame of GPIIbIIIa activation. However, DR-1 versus [antagonist] plot yielded a slope slightly greater than 1 (m=1.23±0.11). Removal of all benzofuran substituents (example 6, VU0806526) resulted in a slope of 1.02±0.02, consistent with a classic competitive mode of inhibition.

Smaller PAR4 antagonists display better drug metabolism and pharmacokinetic dispositions. Initial characterization of VU0652925 revealed a compound with undetectable free fraction and relatively high cLogP values (>5), indicative of potentially poor bioavailability. Therefore, drug metabolism and pharmacokinetic parameters (DMPK) such as plasma protein binding (PPB) and clearance (CL_{hep}, CL_{int}) were also followed during modification of VU0652925. Replacement of the 2-phenylthiazole moiety with a methoxy group (example 3) resulted in a detectable free fraction and the successive truncation of the compound down to example 6 resulted in increasing concentrations of unbound compound in plasma and more

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favorable cLogP values (Table II). Clearance rates increased concomitantly as the fraction unbound increased and cLogP values decreased.

Novel PAR4 antagonists are effective against the tethered ligand and completely reversible. Schild analysis with the BMS-3 fragments indicates that they are reversible within the time constraints of AP mediated GPIIbIIIa activation on human platelets. However, in order for this to be tractable strategy towards novel PAR4 antagonists, compounds should be effective against the TL but reversible as the concentration of thrombin increases. Therefore we compared Schild analysis with VU0652925 and one of our lead compounds developed from the minimum pharmacophore identified here, VU0661224. Platelets were activated with increasing concentrations of γ-thrombin, a product of α-thrombin cleavage which does not interact with or activate PAR1. In the context of γ-thrombin, VU0652925 is able to suppress GPIIbIIIa activation even at extremely high concentrations. In contrast, although effective at lower concentrations of γ-thrombin, antagonism of tethered ligand mediated PAR4 activation with VU661224 is reversed at high concentrations. Clearly these antagonists display distinct pharmacodynamics. It remains to be seen if this will translate to distinct outcomes *in vivo*.

Discussion

With a PAR4 cleavage blocking antibody and a small molecule PAR1 antagonist, Coughlin *et al* initially noted the synergy between PAR1 and PAR4 in the induction of platelet aggregation (Kahn *et al*., 1999). As we also observed (Fig 2) PAR1 is able to substitute for PAR4 and vice versa to induce *ex vivo* platelet aggregation in the context of relatively high concentrations of thrombin. Subsequently Kuliopulous *et al.* described the biphasic kinetics of PAR1 and PAR4 activation noting that the slow signal from PAR4 (20 to 70 fold slower than PAR1) is responsible for the majority of the Ca^{2+} response to thrombin on human platelets (Covic *et al*., 2000); however, the lack of improved pharmacological tools has prevented any further detail of the relationship between PAR1 and PAR4 in the context of thrombin from being revealed. Our data

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with SCH602539 and VU0652925 are in agreement with this historic data. PAR1 is able to substitute for PAR4 and vice versa to induce aggregation, however detailed investigation of the individual contributions of PAR1 and PAR4 to GPIIbIIIa activation suggest that the receptors are not redundant. Using non-competitive antagonists we have determined that over half of the GPIIbIIIa-activation response at higher concentrations of thrombin can be attributed to PAR4 and cannot be substituted by PAR1 activity. The physiologic relevance of such high concentrations of thrombin has not been established as it is currently unfeasible to accurately measure local concentrations of circulating thrombin; however, results presented in the Bristol Myers Squibb patent (Lawrence, 2013) around the efficacy of VU0652925 in reducing thrombus volume in a cynomolgus electrolytic carotid artery injury model of thrombosis with a PAR4 antagonist, and their movement into phase II clinical trials, speaks to the relevance of these higher concentrations of thrombin and the efficacy of inhibiting their action on platelets. Precedent for the strong and superseding PAR4 response comes from work conducted by our group and others (Duvernay *et al*., 2013; Falker *et al*., 2011; Vretenbrant *et al*., 2007).

In our primary assay we observed non-competitive modes of inhibition for both SCH602539 and VU0652925. Vorapaxar (the orally bioavailable analogue of SCH602539) is reportedly a reversible compound but with an extremely slow off-rate. Terminal half-life is 126-269 hours and anti-platelet effects can be expected for 4 weeks beyond discontinuation of dosing, therefore the compound is described by Merck as “essentially irreversible” (Becker *et al*., 2009; Chintala *et al*., 2010). SCH602539 likely has a similarly slow off-rate which contributes to the non-competitive mode of pharmacology that we observed. SCH602539 may not have adequate time to dissociate within the window of platelet activation. The kinetics of platelet activation, once stimulated by thrombin, are expected to be similar *ex vivo* and *in vivo*, making the alteration of thrombin induced GPIIbIIIa activation by SCH602539 that we observed in our *ex vivo* assay physiologically relevant. VU0652925 may have a similarly extremely slow off-rate

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rendering the compound “essentially irreversible” within the kinetics of thrombin induced GPIIbIIIa activation. This would explain the mode of inhibition we observed.

The unanticipated results of the vorapaxar clinical trials indicating significant bleeding risk heed caution in designing new TRAs. The nature of the dual receptor system, as illustrated by these results, suggests distinct safety implications for PAR1 and PAR4 antagonists. The effects of a PAR1 antagonist, no matter if it is competitive or noncompetitive, can be overcome by engagement of PAR4. There are no other lower affinity thrombin receptors on the platelet, and since PAR4 mediates the majority of thrombin induced GPIIbIIIa activation, a non-competitive PAR4 antagonist will permanently depress the thrombin response. Given the role of thrombin mediated platelet activation in hemostasis it may be important that the effects of PAR4 antagonists are surmountable so that they can be effective against thrombosis but overcome in the context of life-threatening bleeding (i.e. surgery or trauma). A competitive PAR4 antagonist should inhibit platelet activation at low concentrations of thrombin but eventually allow reversal of the antagonism and full rescue of the thrombin-mediated platelet response. This would not necessarily render the PAR4 antagonists safer than vorapaxar. However, unless it is competitive and reversible a PAR4 antagonist may present an even greater risk than vorapaxar. Given the bleeding risk noted in the vorapaxar clinical trials with administration of the antagonist on top of the standard of care it is important to compare multiple pharmacological modes of PAR4 inhibition to determine the safest and most efficacious strategy.

A reversible, competitive PAR4 antagonist not only has lower safety concerns but also advantages in terms of its utility as a tool compound. A radiolabeled reversible, competitive antagonist would be capable of defining the binding site of the TL, which until now remains elusive. A competitive antagonist that interacts with the TL binding pocket would also be critical for determining whether or not a novel ligand is an allosteric modulator. Finally, reversibility is a requisite for developing positron emission tomography (PET) tracers. PAR4 expression has been demonstrated to be dynamic and reflective of various pathologic conditions (Dabek *et al*.,

2009; Henrich-Noack *et al*., 2006; Rohatgi *et al*., 2003; Yu *et al*., 2015; Zhang *et al*., 2014a; Zhang *et al*., 2014b) and, therefore, has the potential for developing into a biomarker.

The last example presented in this manuscript (example 6, VU0806526) represents a route forward to developing additional specific PAR4 antagonists that can be fully outcompeted by higher concentrations of thrombin. A companion manuscript to this one will be published in BMCL describing the development of a series of competitive, reversible PAR4 antagonists around the minimum pharmacophore identified in this manuscript. The compound highlighted in Fig 6B (VU0661224) showing efficacy against the TL but full reversibility is the lead presented in the BMCL manuscript. Future efforts will focus on engineering potency into these novel PAR4 antagonist for clinical and basic science research so that we may better understand this important pharmacological target.

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Authorship contributions:

Participated in research design: Duvernay, Lindsley, Hamm

Conducted experiments: Duvernay, Temple, Maeng, Blobaum

Performed data analysis: Duvernay, Temple, Maeng, Blobaum

Wrote or contributed to the writing of the manuscript: Duvernay, Lindsley, Hamm

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Footnotes

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Figure legends

Figure 1 Specificity and potency of PAR tool compounds. A) Chemical structures of PAR1 (SCH602539) and PAR4 (VU0652925) antagonists. B) The effect of PAR1 and PAR4 antagonists on PAR1-AP induced aggregation. Washed human platelets were preincubated for 20 min with 1 μM SCH602539 or 316 nM VU0652925 before initiating aggregation with 20 μM PAR1-AP. Shown are representative tracings of three independent experiments. C) The effect of PAR1 and PAR4 antagonists on PAR4-AP induced aggregation. Platelets were pretreated with antagonist as in B. Aggregation was initiated with 200 μM PAR4-AP. Shown are representative tracings from three independent experiments. D) The effect of SCH602539 on PAR1-AP and PAR4-AP induced platelet activation. GPIIbIIIa inside out activation (PAC1) and P-selectin expression (P-sel) were measured by flow cytometry with PAC1 and CD62p antibodies. Platelets were treated with increasing concentrations of SCH602539 for 20 min prior to activation with 20 μM PAR1-AP or 200 μM PAR4-AP. Data are normalized to vehicle control. Shown are the means±SEM, n=3. E) The effect of VU0652925 on PAR1-AP and PAR4-AP induced platelet activation. Platelets were treated as in D. Data are normalized to vehicle control. Shown are the means±SEM, n=3.

Figure 2. Inhibition of PAR1 and PAR4 abolish thrombin mediated signaling on human platelets. A) The effect of PAR1 and PAR4 antagonists individually on thrombin mediated platelet activation. Platelets were treated with increasing doses of SCH602539 or VU0652925 for 20 min prior to activation with 2 or 10 nM α-thrombin. GPIIbIIIa activation was monitored by PAC1 binding. Shown are the means±SEM, n=4. B) The effect of PAR1 and PAR4 antagonists, combined, on platelet activation by thrombin. Platelets were treated for 20 min with 3.16 μM SCH62539 or 1 μM VU652965 prior to activation with 2 or 10 nM α-thrombin. Shown are the means±SEM, n=3. C) The effect of PAR1 and PAR4 antagonists on platelet aggregation induced by thrombin. Platelets were pretreated for 20 min with antagonist prior to

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activation with 10 nM α-thrombin. Shown are representative tracings of three independent experiments.

Figure 3. Relative contributions of PAR1 and PAR4 to thrombin mediated platelet signaling. A) The effect of increasing doses of SCH602539 on α-thrombin induced GPIIbIIIa activation (PAC1) concentration response curves (CRC). CRCs were constructed with PAC1 binding data measured by flow cytometry. B) The effect of increasing doses of VU0652925 on α-thrombin PAC1 CRCs. C) The effect of increasing doses of SCH602539 on PAR1 isolated thrombin mediated platelet activation. Platelets were pretreated with a fixed concentration of 316 nM VU0652925 and the indicated concentrations of SCH602539 before activation with increasing concentrations of α-thrombin. D) The effect of increasing concentrations of VU0652925 on PAR4 isolated thrombin mediated platelet activation. Platelets were pretreated with a fixed concentration of 1 μM SCH602539 and the indicated concentrations of VU0652925 before activation with increasing concentrations of α-thrombin. Shown are the means, n=3.

Figure 4. Structures of VU0652925 analogs

Figure 5. Schild analysis and identification of competitive PAR4 antagonists. Progressive fold shift experiments and accompanying Schild analysis with VU0652925 fragments. Platelet activation was monitored by PAC1 binding. Platelets were pretreated with increasing concentrations of each antagonist for 20 min prior to activation with increasing concentrations of PAR4-AP. Each curve was constructed from at least three independent experiments. Dose ratios were calculated from the EC_{50}s of each individual experiment ($vehicle_{EC50}/VU\#_{EC50}$) and plotted against the administered concentration of antagonist. Shown on the right are the means±SEMs of log DR-1, n=3. Graph insert: m=slope from linear regression.

Figure 6. Novel PAR4 antagonists are effective against the tethered ligand and completely reversible. Progressive fold shift experiments with A) VU0652925 and novel PAR4

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antagonist B) VU0661224. Platelet activation was monitored by PAC1 binding. Platelets were pretreated with increasing concentrations of each antagonist for 20 min prior to activation with increasing concentrations of PAR4-AP. Each curve was constructed from at least three independent experiments.

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Tables:

Table I	VU#	PAR4-AP, %Max PAC-1	PAR4-AP, IC_{50} PAC-1	γ-thrombin, %Max PAC-1	γ-thrombin, IC_{50} PAC-1	PAR1-AP, %Max PAC-1	MW
1	VU0652925	0%	43 pM	0%	229 pM	94%	460.53
2	VU0661247	0%	210 pM	0%	3.03 nM	51.5%	407.44
3	VU0661245	0%	472 pM	0%	8.42 nM	92.6 %	331.35
4	VU0807074	0.19%	1.68 nM	21.1%	16.2 nM	105%	301.32
5	VU0807081	0.27%	176 pM	0%	7.18 nM	102%	301.32
6	VU0806526	0.20%	1.69 nM	6.99%	58.8 nM	111%	271.04

Table I. Identification of the minimum pharmacophore. Table I. Biological activities and molecular weights of VU0652925 analogs. Platelet activation was induced by 200 μM PAR4-AP, 100 nM γ-thrombin, or 20 μM PAR1-AP. Shown are the means of at least three independent experiments. Experiments were conducted at 10 μM antagonist for % Max values.

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Table II	MW	PPB fu (h, r)	CL_{Int} (h, r) mL/min/kg	CL_{hep} (h, r) mL/min/kg	CLogP
1 (VU0652925)	460.53	<0.001, <0.001	2.17, 2.14	1.91, 16.4	5.44
2 (VU0661247)	407.44	0.001, 0.003	17.7, 37.9	9.62, 24.6	5.09
3 (VU0661245)	331.35	0.008, 0.031	20.7, 61.5	10.4, 32.7	3.32
4 (VU0807074)	301.32	0.007, 0.03	133, 336	18.1, 57.9	3.23
5 (VU0807081)	301.32	0.015, 0.022	65.3, 210	15.9, 52.5	3.23
6 (VU0806526)	271.04	0.01, 0.047	89.4, 285	17, 56.2	3.31

Table II. ***In vitro*** **drug metabolism and pharmacokinetic parameters of VU0652925 fragments.** Values were determined as described in materials and methods. Molecular weight (MW), plasma protein binding (PPB), fraction unbound (fu), intrinsic clearance (CL_{int}), hepatic clearance (CL_{hep}), octanol/water partition coefficient (cLogP).

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Fig 1

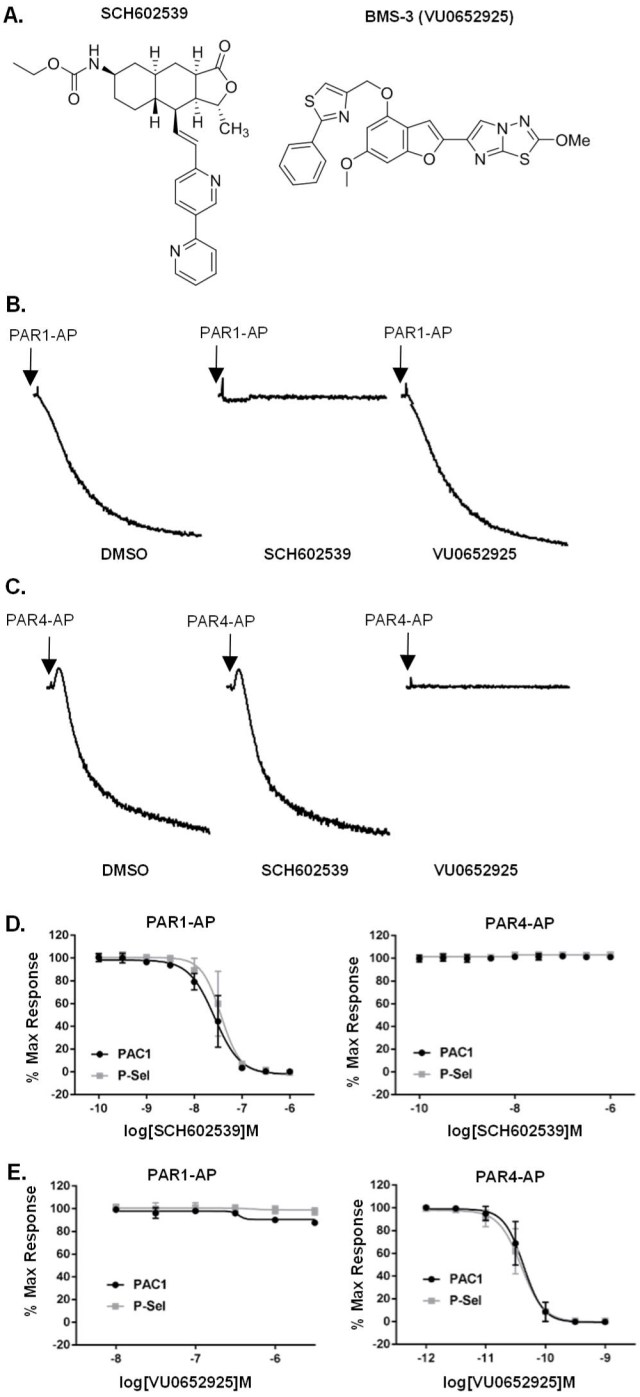

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Fig 2

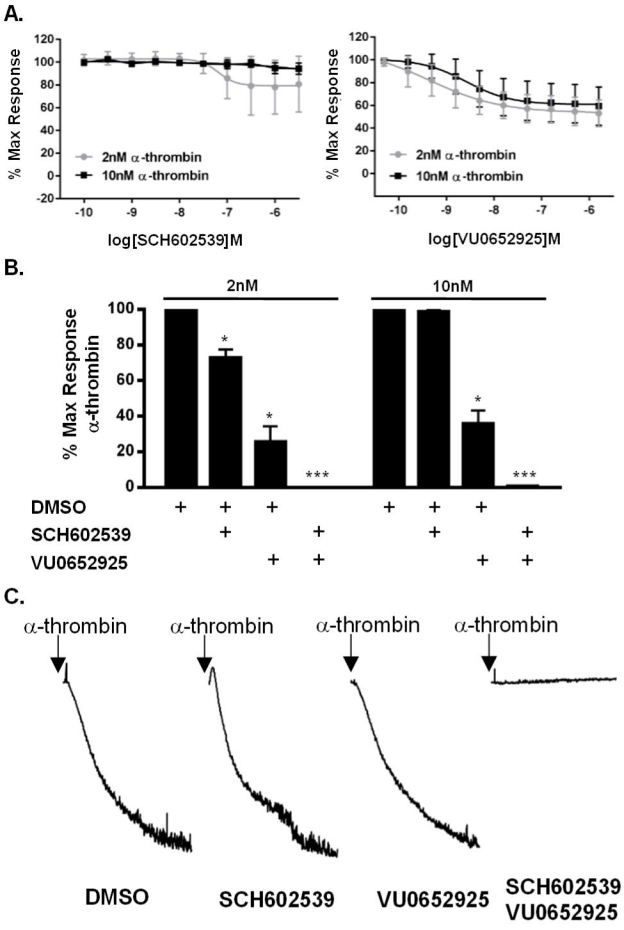

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Fig 3

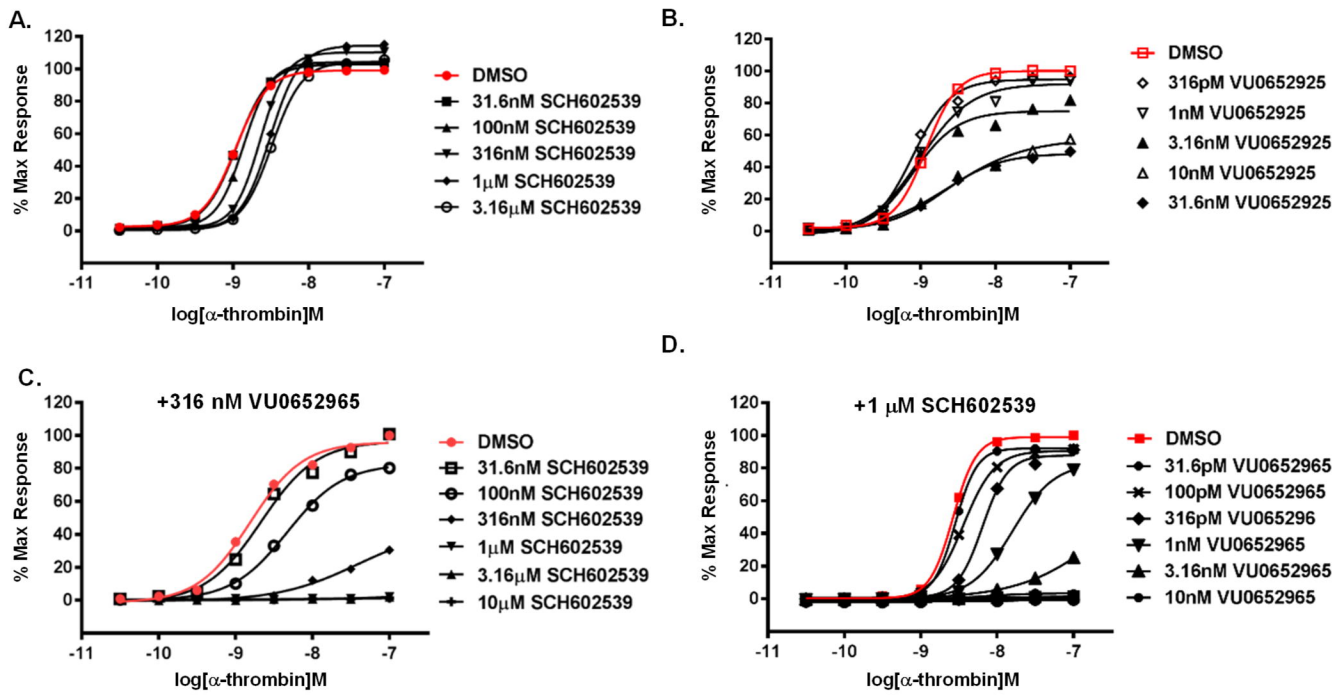

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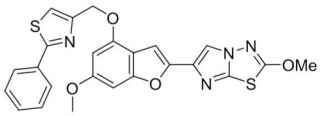
Fig 4



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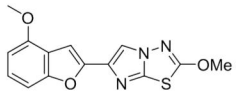

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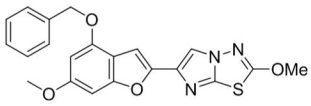

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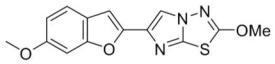

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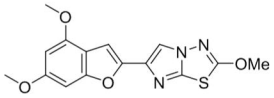

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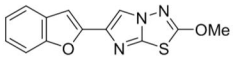

6. VU0806526

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Fig 5

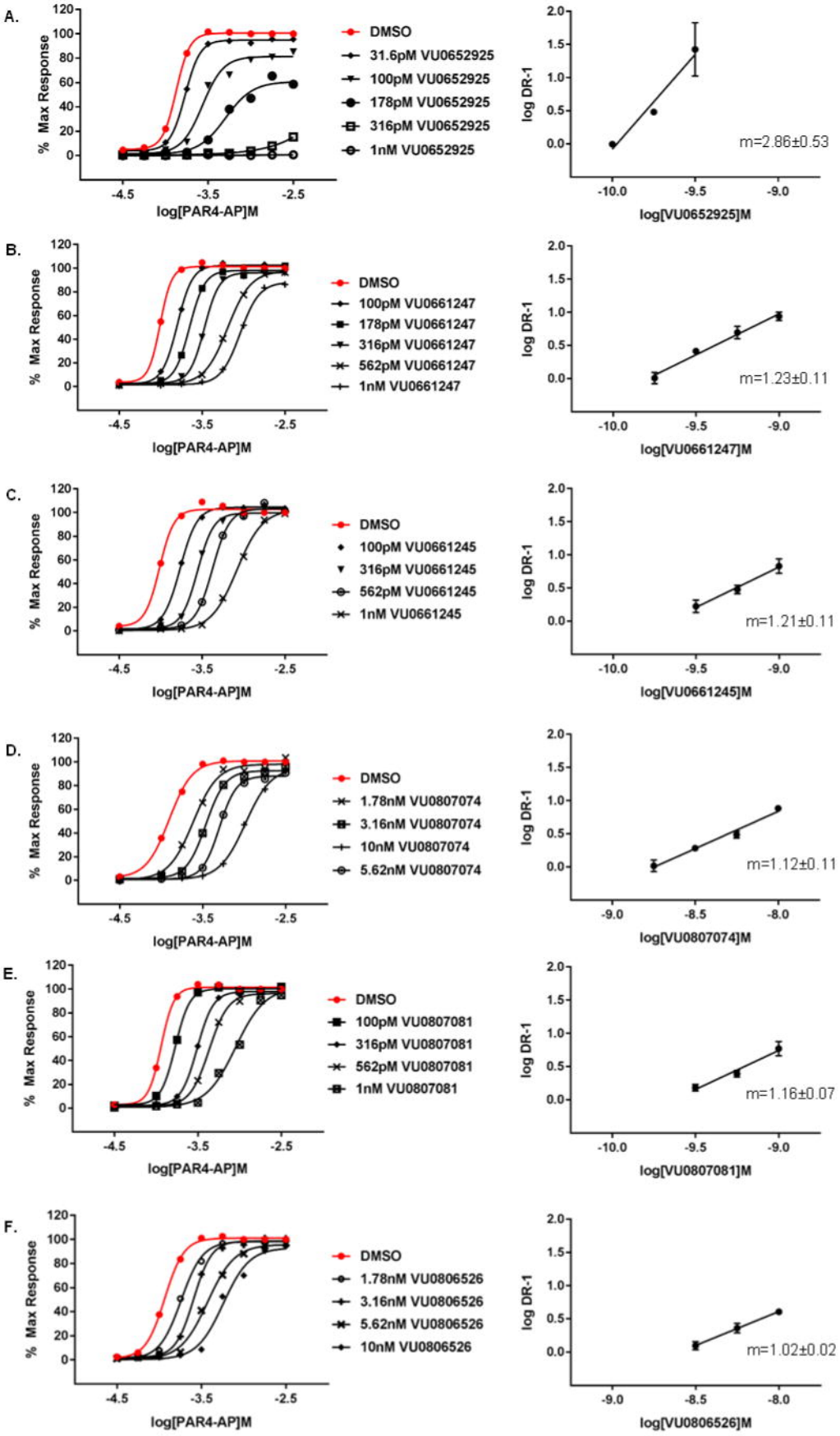

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Fig 6