MODULATION OF THROMBIN

RECEPTOR SIGNALING

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CHAPTER I

INTRODUCTION

Coagulation

The formation of blood clots, coagulation, is an essential process for preventing blood loss following blood vessel injury. Coagulation is initiated by tissue or cellular damage which activates numerous plasma proteases, called factors (F), in serial fashion deemed the coagulation cascade. The coagulation cascade is traditionally divided into the extrinsic or tissue factor pathway and the intrinsic or contact factor pathway.

Following blood vessel injury damaged endothelial and subendothelial cells expose, or decript, tissue factor (TF) a transmembrane procoagulant protein. TF then binds circulating FVII forming the TF-FVIIa complex. The TF-VIIa complex can proteolytically activate FIX and FX into FIXa and FXa. Activated FX can then associate with FVa and the FXa-FVa prothrombinase complex can convert FII to FIIa, also known as thrombin[1-3]. For activation, FXa proteolytically cleaves FII at two distinct sites, Arg273-Thr274 and Arg323-Ser324. Single cleavage events give rise to intermediates prethrombin or meziothrombin, and dual cleavage of prothrombin results in formation of FIIa [4,5].

Additionally, the intrinsic pathway of the coagulation cascade, also referred to as the contact factor pathway, can be initiated through FXII contact with negatively charged cell surfaces, proteins or even microparticles[6]. FXIIa then activates FXI which subsequently activates FIX. FIXa in complex with FVIIIa can facilitate the activation of FX and subsequent FIIa. The culmination of both pathways is the production of localized elevated concentrations of thrombin, which can then convert fibrinogen into fibrin forming

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a hemostatic network. Additionally, thrombin activates platelets, which subsequently adhere to fibrin, forming the clot.

Platelets

Platelet biogenesis and structure

Platelets are unique anuceate cellular particles derived from the megakaryocyte and are present only in mammals. Megakaryocytes are polyploidy cells of hemotapoetic origin that reside in bone marrow[7]. The prevailing theory of the mechanism by which platelets are formed involve megakaryocyte cytoplasmic maturation, migration to a blood vessel, followed by proplatelet formation and release. First megakaryocytes repeatedly undergo endomitosis, a process in which the spindle poles do not pull apart during anaphase thus creating a single but polyploid nucleus (4n, 16n, etc…). Endomitosis provides the nucleic acid, protein, and lipid necessary for the formation of the demarcation membrane system (DMS)[8]. The DMS is a complex network of deeply invaginated membrane continuous with the megakaryocyte plasma membrane and is the source of proplatelet and platelet membrane[9]. Proplatelets are filopodia-like extensions of the megakaryocyte DMS which break through bone marrow sinusoidal vascular walls, and once in the blood, the proplatelets are released and mature into platelets[10].

The mature platelet is an average of 3 μm in size and has an invaginous membrane much like it's parent cell called the canalicular system. Platelets are disposable cells and the whole population turns over every 7-10 days. Platelets possess cellular organelles such as mitochondria and lysosomes however, they also contain specialized organelles including, glycosomes, alpha granules, dense granules, and the dense tubular system (DTS). Glycosomes are intracellular vesicles filled with glycogen particles but, to date, have no associated function. Alpha granules, the must abundant

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organelle, harbors procoagulant proteins including: FV, P-selectin, and Von Willebrand Factor. Defects in packaging alpha granules in the megakaryocyte give rise to Grey Platelet Syndrome, a condition where alpha granules are essentially empty leading to reduced platelet aggregation upon stimulation with a number of agonists and manifests as moderate bleeding in patients[11,12]. Dense granules contain several second messengers and ions including, ADP, ATP, 5-HT, Mg^{2+}, Ca^{2+} and polyphosphates which are released into the blood vessel upon agonist induced vesicle secretion[13-15]. Secretion of both types of granules is dependent on a number of cellular processes most notably, G_q activation of Ca^{2+}, DAG and PKC, and platelet SNARE machinery specifically, syntaxin 4, VAMP-3, and VAMP-8[16-21]. The release of intracellular calcium is controlled primarily by the DTS, a megakaryocyte endoplasmic reticulum derived organelle[22-24]. The DTS is also the location of protein disulfide isomerase, adenylate cyclase and prostaglandin synthesis[25-27].
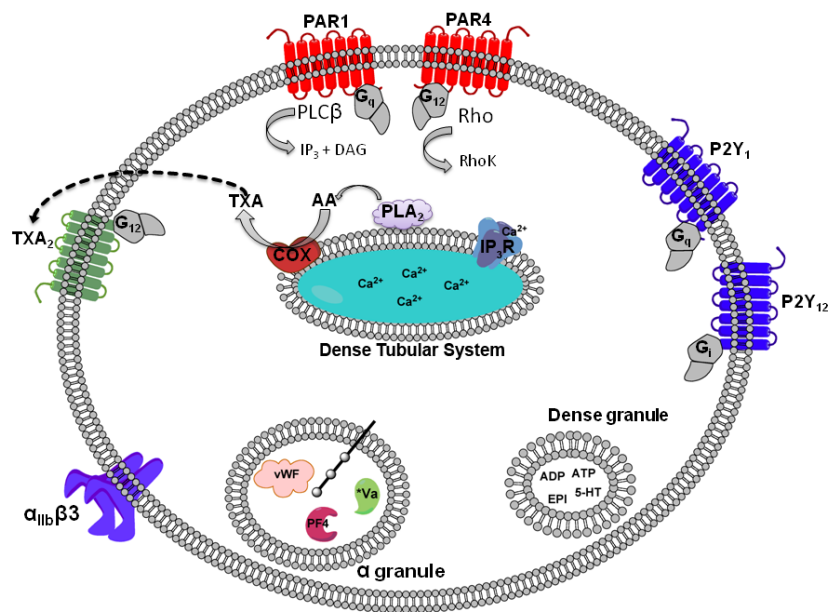
Figure 1. Platelet structure and signaling

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Platelet signaling

The two primary and most potent activators of platelets are collagen and thrombin. Platelets contact collagen as a consequence of subendothelial extracellular matrix exposure following vascular injury. Collagen signals through collagen receptors present on the platelet surface. Collagen can also interact with GPIb and $\alpha_{IIb}\beta3$ integrin receptors through binding Von Willebrand Factor though GPIa/IIa and GPVI are the main platelet collagen receptors[28]. GPIa/IIa is an integrin receptor and mediates adhesion to collagen and activates Src, Syk, FAK, and PLA$_2$ signaling downstream of the receptor. GPVI, an immunoglobin family receptor, can initiate collagen adhesion and signaling to PLCγ causing calcium mobilization and subsequent platelet shape change[29-33]. Thrombin is introduced to the platelets through coagulation cascade initiation following vascular injury. Thrombin binds at least 3 receptors on the platelet cell surface to elicit cellular responses including PAR1, PAR4, and GPIb.

Upon stimulation platelet intracellular calcium, released mostly from the DTS, increases 10 fold[34]. Lysosomal-like calcium stores and store-operated calcium entry also significantly contribute to agonist induced elevations in intracellular calcium[35-37]. Calcium mobilization activates PKC leading to degranulation and release of several feed-forward molecules. G$_q$ signaling activates PLCβ leading to thromboxane (TXA) generation in a cyclooxygenase, arachadonic acid-dependent manner. Upon granule release and TXA synthesis, several receptors on the platelet surface are subsequently activated in an autocrine, paracrine feed-forward manner to provide full platelet activation. ADP, released by dense granule secretion, activates the P2Y$_1$ and P2Y$_{12}$ purinergic receptors on the platelet which are coupled to G$_q$ and G$_i$ respectively[38,39]. Basal levels of cAMP provide an inhibitory tone to the platelet. For example, PKA activates Rap1GAP2 which regulates the small GTPase Rap1, a protein essential to fibrin receptor activation and platelet-fibrin interactions in a clot [40]. G$_i$ signaling releases the inhibitory tone of cAMP

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and helps prime the platelet for activation.   Along with ADP, 5-HT is released from platelet dense granules and can activate platelet 5-HT_{2A} receptors. However, 5-HT_{2A} agonism alone does not significantly aggregate platelets, rather it serves as a potentiator for other platelet agonists[41].

TXA activates the thromboxane A2 receptor which is coupled to G_q and G_{12}[42,43]. The G_{12} family of heterotrimeric G-proteins activates Rho-GEFs, which subsequently activate Rho and Rho kinase. In platelets, Rho mediated activation of Myosin Light Chain kinase is essential for platelet shape change, especially filopodial extensions that increase surface area for fibrin and platelet contact[44].

The multiple pathways required for inside-out activation of the platelet fibrin receptor, $\alpha_{IIb}\beta3$, have not been fully elucidated.   However, G_i signaling in conjunction with G_{12} or G_q is sufficient to fully activate $\alpha_{IIb}\beta3$. Additionally, Rap1 plays a major role in $\alpha_{IIb}\beta3$ activation[45,46].   Thrombin as a primary mediator of platelet activation is unique from collagen as thrombin signaling can activate both G_q and G_{12} to initiate granule secretion, shape change, and inititate feed forward signaling to activate G_i thus fully engaging the platelet. Though platelet activation is essential to hemostasis following vessel injury, platelets also significantly contribute to thrombosis. Several therapeutics inhibit platelet activation with the goal of reducing blood vessel occlusion and thus subsequent tissue injury.

<center>Anti-platelet therapeutics</center>

Aspirin

Aspirin, or acetylsalicylic acid, targets cyclooxygenase (COX) and irreversibly inhibits the enzyme by acetylating serine 530 which blocks arachadonic acid access to the enzyme active site.   COX is responsible for the biogenesis of all eicosanoids

<center>5</center>

including inflammatory prostaglandins and leukotrienes. The potent anti-platelet nature of aspirin, arising from aspirin consumption, is derived from inhibiting the formation of the eicosanoid thromboxane and thus feed forward signaling to TXA_2 receptor. A regular aspirin regimen is a standard of care for the prevention of myocardial infarction and secondary stroke.

Purinergic receptor antagonists

The G_i coupled $P2Y_{12}$ receptor together with the G_q coupled $P2Y_1$ receptor mediate feed forward platelet activation by ADP. The theinopyridine compounds, ticlopidine, clopidogrel (Plavix), and prasugrel are produgs which the active metabolites irreversibly bind to and inhibit $P2Y_{12}$. In recent clinical trials, prasugrel was comparable in safety to clopidogrel but had a faster onset to therapeutic range, and was more consistent in regards to compound mediated inhibition of ADP mediated aggregation[47,48]. Thienopryidine compounds alone and in conjunction with aspirin are commonly used after percutaneous coronary intervention and the placement of stents for the prevention of secondary cardiovascular events.

Integrin $α_{IIb}β3$ inhibitors

The clinically used $α_{IIb}β3$ inhibitors all block fibrin binding to the receptor but have unique pharmacophores. Abciximab is the fragment antigen-binding (F_{ab}) region of a monoclonal antibody directed towards $α_{IIb}β3$. Abciximab must be administered intravenously and interestingly displays a very short plasma half-life due to irreversible target binding and rapid clearance of un-bound drug. Abciximab also binds vitronectin receptors, which are present not only on platelets but also on endothelial cells and smooth muscle. Eptifibatide is a cyclic peptide mimetic of barbourin, a rattlesnake venom peptide that exhibited high selectivity for $α_{IIb}β3$ inhibitory action over other integrin

receptors in a venom screen[49]. Eptifibatide is a readily reversible, high affinity $\alpha_{IIb}\beta3$

blocking drug that is also administered intravenously. A recent trial comparing

eptifibatide to abciximab demonstrated the two drugs were equally efficacious as an

adjunct to percutaneious intervention as measured by ST-segment resolution[50]. Tirofiban

is a non-peptide small molecule inhibitor of $\alpha_{IIb}\beta3$ discovered through virtual screening[51].

Although tirofiban is much cheaper than abciximab, there are conflicting reports as to the

efficacy and safety profile compared with that of abciximab[52,53].

Thrombin: The hemostasis enzyme

Enzyme overview

Hemostasis is literally blood (hemo), standing still (stasis), the cessation of blood

loss while blood continues to flow. Thrombin, or Factor IIa, is the seminal protease

formed during coagulation and is essential for the process of hemostasis. The primary

mechanism for hemostasis is the formation of a platelet-fibrin plug that prevents

continued blood loss at a site of vessel injury. Thrombin is a 36 kDa vitamin K dependent,

trypsin-like serine protease that cleaves many protein substrates C-terminal to arginine

(R) at a D-P/L-R-S consensus site[54]. Thrombin plays two essential roles in hemostasis.

Thrombin enzymatically processes fibrinogen at four distinct arginine-glycine bonds to

form fibrin monomers. Fibrin monomers subsequently crosslink and form a fibrin clot to

which platelets can adhere to at a site of vessel injury. Thrombin also directly activates

platelets via protease-activated receptors (PARs) on the platelet surface. Thrombin

mediated platelet activation is robust and induces shape change, secretion, activation of

integrin $\alpha_{IIb}\beta3$ and aggregation[55,56]. As the central component to clot formation, thrombin

activation is tightly regulated by anti-coagulants. Insufficient thrombin activity can cause

severe hemorrhage, however, excess thrombin activity (or insufficient activity of

regulators of thrombin) results in hypercoaguable states. Thrombosis (vessel occlusion) caused by intra-vessel clot formation is often a result of hypercoaguable states and is the root cause of stroke, myocardial infarction, and thromboembolism. Since clot formation is dependent on thrombin formation *in vivo*, thrombin is an obvious target for anticoagulants.

Thrombin regulation

Thrombin is regulated by a number of direct and indirect mechanisms. As small amounts of thrombin are generated, it can directly activate FV and FVIII in a feed forward manner to produce additional thrombin[57,58]. Thrombin-mediated activation of platelets initiates platelet FVa, release which participates in the prothrombinase complex, forms more thrombin but is resistant to anti-coagulant proteins[59-61]. Thrombin also negatively regulates its own production using thrombomodulin. Thrombomodulin is a single transmembrane protein that when bound by thrombin inhibits thrombin-mediated fibrin formation and activation of factor V[62]. Thrombomodulin-bound thrombin activates Protein C (PC) a potent anti-coagulant that inhibits both FVIIIa and FVa, preventing activation of FX and thus thrombin formation[63]. Non clot-bound thrombin is also susceptible to circulating anti-thrombin (AT)[64]. AT, a serpin family protease inhibitor, binds heparin co-factor and the AT-heparin complex directly inhibits thrombin's active site in an exosite II-dependent manner[63].

Thrombin inhibitors

Warfarin

Warfarin is often the standard of care for secondary prevention of stroke or myocardial infarction. Warfarin is also used to treat recurrent venous thrombosis and

venous thromboembolism. Warfarin is of the coumadin chemical class which are rodent poisons and are effective as such using the same anticoagulant mechanism. Coagulation factors require gamma-glutamate residues near their N-terminus to bind calcium, which is required for binding procoagulant surfaces and thus efficient coagulation. Glutamate is post translationally modified mainly in the liver by gamma-glutamyl carboxylase using vitamin K in the process, which subsequently becomes oxidized. Warfarin inhibits vitamin K epoxide reductase, which reduces vitamin K after oxidation effectively recycling the molecule. Thus, warfarin depletes the available vitamin K and renders coagulation factors incapable of binding procoagulant surfaces. The severe bleeding effects of Warfarin, through this mechanism, are readily reversible by administering vitamin K.

Factor Xa inhibitors

A new class of anti-coagulants, FXa inhibitors, indirectly inhibits thrombin by preventing prothrombinase complex-mediated thrombin formation. Rivaroxiban and Apixaban are both orally bioavailable with comparable dosing regimens and reversibly bind FXa. Apixaban is approved for the prevention of stroke in patients with atrial fibrillation, excluding those caused by heart valve defects and was demonstrated as more efficacious than warfarin in preventing stroke with decreased prevalence of bleeding side effects[65]. However, unlike warfarin, there are no antidotes for rivaroxiban or apixaban, and an overdose would prove lethal.

Direct thrombin inhibitors

Several inhibitors of thrombin are clinically useful for the management or prevention of thrombosis. Hirudin, the first identified non-endogenous thrombin inhibitor, is an enzyme secreted by leeches and is responsible for the anti-coagulant properties of

leech saliva[66]. Hirudin is a bivalent direct thrombin inhibitor, binding both the active site and exosite I to inhibit thrombin mediated fibrin formation[67]. Several direct thrombin inhibitors were designed with the same mechanism of action as hirudin including bivalirudin, lepirudin, and desirudin and are used when heparin is contraindicated. Other direct thrombin inhibitors such as Argatroban and Dabigatran are univalent and only bind to the thrombin active site. Dabigatran is a novel univalent direct thrombin inhibitor that is orally bioavailable and has similar endpoints for stroke and systemic embolism as warfarin for the treatment of atrial fibrillation[68].

Though thrombin inhibitors are an effective way to prevent thrombosis, all anti-thrombotic agents carry an increased risk of bleeding. A target downstream of thrombin in the coagulation cascade would be ideal since thrombin is involved in fibrin formation and without fibrin, hemorrhage occurs[69]. The platelet, the other major clot constituent, harbors two thrombin receptor subtypes responsible for the cross-talk between coagulation and platelet activation. These receptors may serve as better anti-thrombotic targets since even if inhibited, thrombin can continue to act on fibrinogen and preserve the fibrin network and collagen can continue to signal to the platelet at the site of vessel injury.

Platelet thrombin receptors

Introduction

Davey and Luscher provided the first evidence that thrombin was a potent platelet activator capable of initiating platelet aggregation and nucleotide release[70]. Thrombin's mechanism of action on the platelet was later described by Detwiler et. al. as initiating calcium mobilization in a time, and dose-dependent manner consistent with

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agonist-receptor interaction[71-73]. Furthermore, hirudin competitively inhibited I^{125}-thrombin binding to human platelets providing additional evidence for thrombin acting as a ligand for an unknown receptor on the platelet surface[74].

The first thrombin receptor, Protease-Activated Receptor-1 (PAR1), cloned in the lab of Dr. Shaun Coughlin using *Xenopus laevis* oocyte expression cloning, revealed a unique mechanism of thrombin-mediated receptor activation[75]. PARs are activated by proteolytic cleavage of the N-terminus, resulting in a new N-terminus which acts as the endogenous agonist or 'tethered ligand' for the receptors. The 'tethered ligand' mechanism creates essentially irreversible activation of PARs by proteases and thus unique challenges for drug development of PAR antagonists. The PARs are a family of type 1, or family A, G-protein coupled receptors comprising PAR1, PAR2, PAR3, and PAR4. Thrombin activates PAR1, PAR3, and PAR4 whereas PAR2 is activated by trypsin, tryptase, FVIIa, or FXa. The human platelet expresses PAR1 and PAR4, which are responsible for thrombin mediated platelet activation. Ergo, the platelet thrombin receptors are attractive targets for anti-platelet/anti-thrombotic therapeutics.

PAR1

PAR1 is ubiquitously expressed in human tissues and is frequently referred to as the high affinity thrombin receptor due to the presence of a hirudin-like domain on the N-terminus of the receptor which interacts with thrombin's exosite I and allows thrombin to signal at picomolar concentrations[75,76]. The thrombin cleavage site of PAR1 (LDPR/S) is located 41 amino acids from the N-terminus and shares homology with the thrombin cleavage site of Protein C (LDPR/I)[75]. In addition to thrombin, PAR1 can be proteolytically activated by TF-FVIIa-Xa complex, FXa, Trypsin, Plasmin, Granzyme, and Matrix Metalloprotease-1[77-82]. Experimentally, PAR activation, in the absence of receptor cleavage, is accomplished by the use of a synthetic peptide corresponding to the first 6

amino acids following the cleavage site (SFLLRN) [83,84].

PAR1 is a promiscuously coupled GPCR and signals through both G_{12} and G_q, to exert cellular effects[43,84,85]. Though it has been proposed that PAR1 couples to Gi, it is indirect coupling, as evidence shows that without ADP secretion G_i signaling is not engaged downstream of PAR1. Additionally, it is well established that PAR1 activation causes ADP release via dense granule secretion[86]. In platelets, G_{13} (which is of the G_{12} family) is required for optimal platelet shape change, aggregation, and the formation of stable thrombi *ex vivo*[87]. Similarly, it has been demonstrated that G_{12} in platelets, like in other cells, activates Rho kinase and myosin light chain phosphorylation, which canonically produces contractile events[44]. G_q deficient mice provide evidence that G_q signaling is required for thrombin induced secretion and aggregation but not shape change[16]. PAR1 mediated G_q coupling produces rapid rises in intracellular calcium followed by rapid shut off. G_q signaling downstream of PAR1 coupled with G_i signaling downstream of ADP receptors is sufficient to activate the integrin $\alpha_{IIb}\beta3$ receptor so that platelets can bind a fibrin clot[88].

In platelets, PAR1 rapidly desensitizes and remains unresponsive indicating a lack of a readily accessible pool of PAR1 in platelets[89]. PAR1 is desensitized via phosphorylation by the GRK family of kinases on serine and threonine residues in the C-terminal tail of the receptor[90,91]. Due to the irreversible nature of PAR activation, PAR1 is not recycled back to the cell surface post desensitization, rather it is directed to the lysosome for degradation in order to terminate receptor signaling[92,93]. Interestingly, clatherin, rather than β-arrestin, is required for lysosomal sorting in a phosphorylation dependent manner though β-arrestin recruitment to PAR1 occurs and results in signal termination[94,95].

Since PAR1 is absent from mouse platelets, knockout models fail to provide full insight into the function of human PAR1 in thrombosis and hemostasis. In rodents, PAR1

plays a significant role in embryonic development as 50% of PAR1 null mice expire during gestation, a phenotype that can be reversed by endothelial specific expression of PAR1 using the Tie2 promoter, indicating that PAR1 function in the endothelium is critical for embryonic development[96,97]. PAR1 systemically is integral in mediating cross talk between coagulation and inflammation and it has even been suggested that PAR1 activation can exacerbate viral infection[98]. In addition an emerging role for PAR1 in metastasis is being appreciated. Given the physiological roles of PAR1, it is an ideal therapeutic target not only for the prevention of thrombosis but possibly inflammation as well.

Two PAR1 antagonists in clinical trials, Vorapaxar and Atopaxar, provide critical insight to the role of PAR1 in humans. Atopaxar, also called E5555, has recently completed phase II clinical trials with intriguing results. Atopaxar is an orally bioavailable, reversible PAR1 antagonist that competes for PAR1 agonist peptide binding. Preclinical experiments in animal models demonstrated anti-thrombotic properties without an increased risk for bleeding[99]. In safety and tolerability studies, Atopaxar had a 23 hour half-life and the parent molecule was not subject to efficient renal clearance (less than 0.2%)[100]. In the J-LANCELOT studies, Atopaxar administration inhibited PAR1-AP and collagen-mediated platelet aggregation but not ADP-mediated platelet aggregation *ex vivo* in healthy patients, patients with coronary artery disease, or patients with acute coronary syndrome. There was no significant difference in the incidence of major TIMI bleeding compared with placebo treated group however, Atopaxar treatment was associated with a dose-dependent increase in the incidence of hepatic dysfunction and QT prolongation[101]. It is questionable whether Atopaxar will continue forward to phase III clinical trials[102].

Vorapaxar has completed phase III clinical trials with confounding results. Vorapaxar is a natural product analogue derived from Himbacine, a compound produced

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in the bark of the Australian magnolia. Vorapaxar is orally bioavailable, demonstrates irreversible PAR1 antagonism, and apparently binds at the PAR1 peptide binding site[103]. The half-life of Vorapaxar ranges from 2.5 days to 6 days, which like aspirin, correlates with platelet turnover. In accordance with the half-life, Vorapaxar inhibited greater than 50% PAR1-AP mediated platelet aggregation *ex vivo* for up to 4 weeks after a single dose. There were no adverse effects reported on liver function or ECG readings during safety and tolerability studies[104]. The TRA-2P-TIMI 50 trial, excluding patients presenting with previous stroke, was completed and showed that PAR1 antagonism is effective in reducing cardiovascular death and ischemic events[105-107]. The TRA-CER trial was terminated due to an association of Vorapaxar treatment, in conjunction with standard of care (aspirin or clopidogrel), with an increased risk for intracranial hemorrhage. However, the bleeding side effect may be due to aspirin and clopidogrel exacerbating what is a mild bleeding effect of Vorapaxar on its own[107].

PAR3

PAR3 is proteolyzed by thrombin and activates G_q however, any major role it plays in humans remains undetermined. A few studies describe a possible role for PAR3 in pulmonary inflammation, though it may be a vestigial receptor in humans[108,109]. PAR3 null mice are protected against thrombosis however, these results are not directly translatable to human since mouse platelet PAR expression and signaling are not similar to human. On the mouse platelet PAR3 does not induce intracellular signaling and serves as a cofactor for PAR4, the signaling thrombin receptor. PAR3 knockout mice thus display prolonged bleeding times, and protection against ferric chloride-induced thrombosis of mesenteric arterioles and against thromboplastin-induced pulmonary embolism[110-112].

PAR4

PAR4 together with PAR1 mediates thrombin signaling in platelets. PAR4 expression is restricted to the megakaryocyte lineage, tumor cells, and perhaps specific populations of vascular cells[113,114]. PAR4 lacks the hirudin-like domain present in PAR1 and is considered the low affinity thrombin receptor[115]. PAR4 as the low affinity thrombin receptor is consequently engaged by high concentrations of thrombin and thus subject to differential temporal engagement by thrombin. PAR4 shares just 33% amino acid sequence identity with other PAR members and has virtually no homology with any other PAR family members in the N or C-terminus[116].

Reports detail signaling differences between PAR1 and PAR4 indicating that although they are activated by the same endogenous agonist and couple to the same G-proteins, the two platelet thrombin receptors signal in fundamentally distinct ways[60,117,118]. Whereas PAR1 mediated calcium signaling is rapidly desensitized via phosphorylation and internalization, PAR4 mediated calcium mobilization is prolonged[119]. There are also clear differences in the utilization of PI3K downstream of platelet thrombin receptors. PAR4 mediated aggregation is resistant to PI3K inhibition whereas PAR1-mediated aggregation is reversed by PI3K inhibition. Furthermore, activation of $\alpha_{IIb}\beta3$ is partially dependent on PI3K downstream of PAR1 but not PAR4. In addition, PI3K mediates PAR1 but not PAR4-induced calcium entry in human platelets, indicating interesting signaling differences independent of PAR-G-protein coupling[118]. Platelets harbor, and upon stimulation, release FV from granules as well as produce platelet derived microparticles. PAR4-stimulated platelets release more FV and produce more microparticles than PAR1-stimulated platelets. Additionally, PAR4 but not PAR1-mediated microparticle production is highly dependent on Rho kinase. PKC appears to play a dual role in regulating microparticle production downstream of thrombin receptors. PKC inhibition enhances PAR1-mediated microparticle formation but inhibits PAR4-

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mediated microparticle production[60]. Together these findings present PAR4 as the more robust platelet activator in that they suggest a requirement for feed-forward signaling or inhibition of negative regulators for PAR1 to achieve full platelet activation, whereas PAR4 signaling alone is sufficient for full platelet activation.

As the primary mediator of thrombin signaling on the mouse platelet, PAR4 null mice display markedly longer bleeding times than wild type and even PAR3 null mice. Similarly, PAR4 null mice are protected against ferric chloride-induced thrombosis of mesenteric arterioles and carotid arteries, and in a laser injury model of thrombosis[111,120,121]. However, there are no data indicating a role for PAR4 in thrombosis and hemostasis in human as there are no clinical candidate molecules. YD-3 is the sole non-peptide, selective, PAR4 receptor antagonist and it's utility as such has been demonstrated in platelet activation assays *ex vivo* as well as a mouse model of angiogenesis, but not thrombosis[122-126]. YD-3 is highly lipophilic and thus is not a suitable *in vivo* probe. Therefore the understanding of role of PAR4 in thrombosis and hemostasis remains far behind that of PAR1 due to the lack of potent, pharmacological tool compounds possessing favorable physiochemical properties and clinical candidates.

Specific Aims

The subsequent specific aims were followed in an effort to identify and design potent and selective PAR4 antagonists:

Aim I – Improve the synthetic route of YD-3 for ease of future derivitization

   a. Shorten the synthetic route of YD-3

   b. Eliminate the inactive isomer of YD-3

Aim II – Optimize indole-derived compounds for enhanced potency and selectivity

   a. Construct small molecule libraries around an indole scaffold

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b. Determine structure activity relationships by high-throughput screening

c. Determine the potency of lead molecules

d. Determine the mechanism of action of lead molecules

Aim III – Characterize the role of PAR4 in platelets

a. Determine the role of PAR4 in models of thrombus formation

CHAPTER II

APC MODULATION OF PAR1

Introduction

In the endothelium, PAR1 is the primary target of thrombin and activation of the receptor causes disruption of endothelial barrier integrity as well as production of pro-inflammatory mediators[127]. Since PAR1 is ubiquitously expressed and mediates several inflammatory and coagulant responses, PAR1 antagonism may provide effective protection against cardiovascular disease, inflammation, and cancer. Activated protein C (APC) is an important regulator of coagulation. Protein C (PC), like coagulation factors, is a vitamin-K dependent zymogen glycoprotein that is proteolytically activated by thrombin. Activation of PC by thrombin is most efficient in the presence of thrombomodulin (TM). TM forms a complex with PC bound to the endothelial protein C receptor (EPCR) to which thrombin then binds TM and activates PC with high efficiency[128-130]. APC binds Protein S and the complex proteolytically degrades coagulation factors Va and VIIIa, thus inhibiting the prothrombinase complex. The importance of APC in maintaining hemostasis is underscored by the fact that individuals with heterozygous PC deficiency carry an increased risk of deep vein thrombosis and individuals presenting with homozygous PC deficiency develop fatal complications from sepsis if left untreated with exogenous APC[131].

It has been reported that APC not only has anticoagulant functions, it also has anti-inflammatory properties. APC inhibits nuclear factor-κB, tumor necrosis factor-α production, and apoptosis in an EPCR-and PAR1-dependent manner[132]. EPCR is a type 1 single pass transmembrane protein possessing a tripeptide C-terminal tail (Arg-Arg-Gly). Extracellularly, EPCR structurally resembles the major histocompatibility complex

class I (MHCI). EPCR is highly expressed in vascular endothelium but EPCR is also found in other hematopoetic cells. APC binds EPCR using the vitamin-k dependent γ-carboxyglutamic acid (Gla) domain[128]. The APC-EPCR interaction is dependent on Ca^{2+} ion binding to the APC Gla domain likely to facilitate stabilization of the hydrophobic loop and aide interaction with EPCR. The crystal structure of EPCR revealed a phospholipid binding site in the MHCI region, though the functional consequence of phospholipid binding is unknown[133]. EPCR is essential for anticoagulant functions evident as EPCR null mice are embryonic lethal and die before embryonic day 11 in part due to fibrin clot formation leading to placental thrombosis[134]. Accordingly, overexpression of EPCR in mice is protective against thrombosis and sepsis[135].

APC-EPCR Modulation of PAR1 signaling

PAR1 activation elicits potent disruption of the endothelial monolayer. However, if EPCR is occupied by APC and PAR1 is subsequently activated, barrier protection occurs. PAR1-mediated barrier disruption depends on calcium mobilization, Rho activation, and activation of myosin leading to cellular contractile events[136]. G_i signaling is inhibitory to contraction and therefore is barrier protective although exact mechanisms remain unknown. Barrier enhancement may occur through PI3K-dependent activation of the Rac-GTPases, Tiam1 or Vav1, shown to mediate sphingosine-1-phosphate, G_i, dependent barrier protection[137]. Interestingly, it has recently been shown that thrombin activation of PAR1 causes Rho activation, whereas APC activation causes Rac activation[138].

The seminal observation of APC-protective signaling came from microarray data showing recombinant human APC inhibited gene transcription of a number of inflammatory mediators, including NFκb, while increasing anti-apoptotic mediators including eNOS[139]. EPCR dependent activation of PAR1 by APC accounts for all

protective signaling, and APC signaling differs from that of thrombin[140,141]. Protective effects of APC were recapitulated using protease-dead APC and PAR1 synthetic peptide providing evidence that receptor occupancy, not enzymatic function, is necessary for producing protective effects[142]. Since the concentration of APC required to elicit PAR1 cleavage *in vitro* is 10,000 times higher than thrombin, a major criticism of APC-mediated PAR1 activation[143]. However, an occupancy model of APC-mediated PAR1 activation nullifies said argument since theoretically PC (4 μg/mL plasma concentration) can substitute for APC.

Other differences between thrombin and APC-mediated activation of PAR1 have recently been demonstrated at the receptor level. APC, unlike thrombin, does not evoke internalization of PAR1 upon receptor activation though both agonists induce receptor phosphorylation[138]. Caveolin is also essential to the protective phenotype of APC/PAR1, demonstrated by knockdown of CAV-1 in cells. During APC mediated protective signaling, EPCR migrates out of caveolin which suggesting caveolin has an inhibitory role in protective signaling[138,144]. The APC-EPCR complex has been shown to interact with PAR1, and sphingosine-1-phosphate receptor-1 ($S1PR_1$). $S1PR_1$ activation is essential for PAR1 protective responses[145]. APC signaling has been shown in the brain by induction of calcium flux in human brain endothelial cells. Furthermore, in mice, APC offers neuroprotection after ischemic injury independent of APC anti-coagulant activity[132,146].

APC has proven to be an effective treatment of sepsis and may have additional benefits due to reported anti-inflammatory properties of APC. Together these data suggest a receptor conformation unique to APC activated PAR1, which biases signaling to a protective phenotype through an unknown mechanism. Understanding the mechanism by which APC confers anti-inflammatory effects on cells, primarily the endothelium, will provide insight into designing novel therapeutic agents. Attempts were

made to delineate APC modulation of PAR1 in endothelial cells however, it appears that APC, PAR1, and thrombin produce similar rather than distinct cellular phenotypes.

Materials and Methods

Reagents

EA.hy926 cells were a kind gift from Dr. Cora-Jean S. Edgell. Human umbilical vein endothelial cells (HUVEC) were isolated within 48 hours after delivery and all data were collected from passages 1-5. Mouse Anti-ICAM-1 antibody and ATAP2 were purchased from Santa Cruz (Santa Cruz, CA, USA). PAR1 activating peptide (PAR1-AP, TFLLRN), PAR2 activating peptide (PAR2-AP, SLIGKV) and PAR4 activating peptide (PAR4-AP, GYPGKF) were purchased from GL Biochem (Shanghai, China). APC and DEGR-APC were purchased from Haematologic Technologies (Essex Junction, Vermont, USA). The cyclic-AMP assay kit was from GE Healthcare (Connecticut, USA). Fluo4-AM is manufactured by life technologies (Grand Island, NY, USA). Rabbit anti-mouse horseradish peroxidase (HRP) conjugated antibody was purchased from Perkin Elmer (Waltham, MA, USA). TMB substrate was from Thermo (Rockford, IL, USA).

Endothelial calcium mobilization assay

HUVEC (P1-P4) or EA.hy926 cells were seeded at a density of 50,000 cells/well on a 96 well Greiner (Monroe, NC, USA) black/clear bottom tissue culture treated assay plate 18-24 hours prior to assay. Cells were dye loaded for 1 hour with 2.5 µg/mL Fluo4-AM in calcium assay buffer (1X HBSS with calcium and magnesium without phenol red, 20 mM HEPES, 2.5 mM probenecid, 1 mM EGTA) 50 µL/well. Dye loading buffer was exchanged for 90uL of calcium assay buffer without Fluo4-AM. Fluorescence measurements were recorded on a Molecular Devices Flexstation II 384 (Downingtown,

PA, USA) at 37 ºC. Agonists were added at 10X in calcium assay buffer into V-bottom compound plates and 10 µL of agonist was injected by the Flexstation into each well of the assay plate. Experiments reported were performed in triplicate, on the same plate. 485:520 (ex:em) was measured each 1.55 seconds for a maximum of 4 minutes. Data represented as raw fluorescent intensity units (RFU) or as a percent of the maximum agonist response (value*100/max).

Cyclic-AMP assay

Cells were seeded at a density of 200k cells/well in a 24 well cell culture dish. Cells were serum starved for 1 hour followed by pretreatment with 1 mM IBMX and 10 µM forskolin for 5 minutes. Cells were subsequently stimulated with the indicated agonist for 5 minutes. Media was aspirated to terminate signaling, the cell culture plate was wrapped in parafilm and stored at -80 ºC until assay. 147 µL lysis reagent 1B was added to each well and the plate was agitated for 10 minutes at room temperature. The 7.4 protocol was followed exactly as recommended by the manufacturer.

Evans blue albumin assay

Either HUVEC or EA.hy926 cells were plated on Corning (Corning, NY, USA) 24 well Transwell culture dishes containing 3.0 µm pore size at a density of 10k cells/well and allowed to grow to confluence (optimized as 3 days). Cells were serum starved for 1 hour prior to stimulation with the indicated agonist. After stimulation, medium was removed from the top and bottom using the 'flick and slam' method or aspiration. Fresh growth medium containing serum was added to the lower chamber and Evans blue albumin medium (4% w/v Evan's blue dye in DMEM supplemented with 10% fetal bovine serum) was added to he top chamber. After 10 minutes the transwells were removed and discarded. The medium from the lower chambers were removed, diluted 1:3 and

transferred to a 96 well black/clear bottom assay plate. The optical density at 650 for each well was determined via Molecular Devices SpectraMax 190 plate reader (Downingtown, PA, USA).

ICAM-1 ELISA

Either HUVEC or EA.hy926 cells were seeded at a density of 10,000 cells/well on a 96 well Greiner black/clear bottom tissue culture treated plate 48 hours prior to assay. Fresh medium with serum was added to the cells 24 hour prior to assay. Cells were serum starved for 1 hour prior to assay. Indicated agonists were added at 10X in serum free medium and cells were stimulated for the indicated period of time. Stimulation was terminated by washing each well 3 times with 200 µL of 1X PBST (0.1% triton X-100) pH 7.4 and fixing in 100 µL/well 1% paraformaldehyde for 30 minutes. Cells were washed again 3 times in 200 µL PBST and 100 µL 5% milk blocking solution was added for 45 minutes followed by another wash. 50 µL of 1 µg/mL mouse anti-ICAM-1 antibody was incubated with the cells for 45 min followed by a wash. Secondary anti-mouse HRP antibody was added at 1:2000 dilution for 40 minutes followed by another wash. 50 µL of TMB substrate was added allowed to incubate with the cells for 5 min and the optical density at 650 nm was read immediately on a plate reader. Data reported as fold baseline/non-stimulated response.

Results

PAR1 is the primary thrombin receptor in endothelial cells

In order to determine which protease activated receptors signal in endothelial cells, tethered ligand peptides directed towards PAR1, PAR2, or PAR4 were used to stimulate cells and calcium mobilization was measured. HUVEC mobilized calcium in response to

PAR1-AP but not PAR4-AP (Figure 1A). Similarly, EA.hy926 cells responded to PAR1-AP and PAR2-AP but not PAR4-AP (Figure 1B). These data indicate that endothelial cells have functional PAR1 but not PAR4. Furthermore, in EA.hy926 cells the PAR1 cleavage blocking antibody ATAP2 is able to abolish thrombin (Figure 1C) but not PAR1-AP (Figure 1D) mediated calcium mobilization. These data indicate that in endothelial cells PAR1, and not PAR4, is the primary receptor responsible for thrombin receptor signaling. Alternatively, PAR4 may be present in endothelial cells but may not couple to G_q[147]. As expected, PAR1-AP is far less potent (EC_{50}=46.9 ± 0.1 µM) than thrombin (EC_{50}=2.3 ± 0.4 nM) at eliciting calcium mobilization.

APC elicits calcium mobilization

Allosteric modulation of GPCRs may alter receptor coupling to heterotrimeric G-proteins and PAR1 reportedly to couples to G_q, G_i, and G_{12}, thus if APC is allosterically modulating PAR1, signaling effectors downstream of these G-proteins may also be altered. EA.hy926 cells were stimulated with increasing concentrations of thrombin or APC. Both APC and thrombin initiate calcium mobilization alone, though APC is significantly less potent and less efficacious than thrombin by greater than 100-fold (Figure 2). To ensure that the calcium response from APC was not due to contaminating traces of thrombin from the manufacturers APC preparation, 0.4 nM bivalirudin was added to this and all subsequent experiments using APC as an agonist.

APC does not significantly desensitize PAR1

To determine the effectiveness of PAR1 agonists in desensitizing PAR1-initiated calcium mobilization, thrombin, APC, and PAR1-AP were tested. Thrombin (Figure 3A) and PAR1-AP (Figure 3C) were capable of desensitizing PAR1 calcium mobilization. APC did not efficiently desensitize PAR1-AP at 20 nM concentration (Figure 3B).

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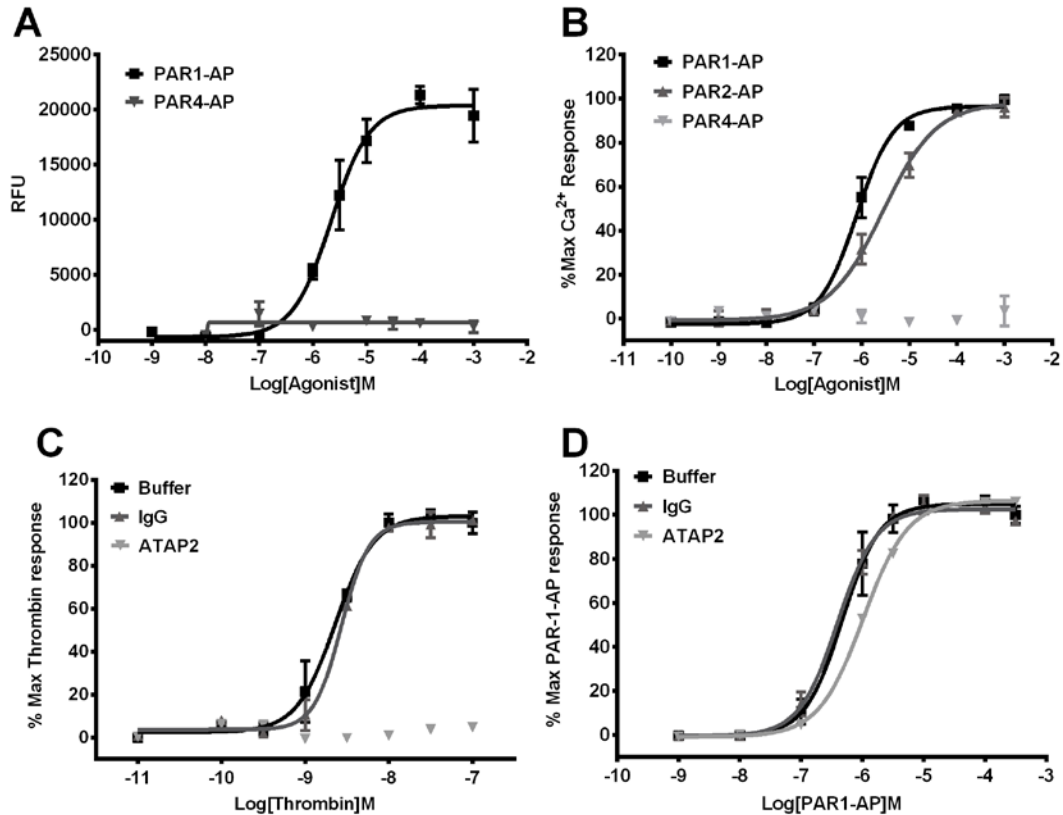
Figure 1. PAR1 is the primary thrombin receptor in endothelial cells. A. HUVEC were treated with increasing concentrations of either PAR1-AP (black square) or PAR4-AP (grey triangle) and calcium mobilization was measured. Data represented as raw fluorescence intensity. mean±S.E.M, n of 1 in triplicate. B. EA.hy926 cells were treated with either PAR1-AP (black square), PAR2-AP (grey triangle), or PAR4-AP (open triangle) and calcium mobilization was measured. Data presented is an n of 1 mean±S.E.M, performed in triplicate. C. EA.hy926 cells were pretreated with assay buffer (black square), 2.5 µg/mL mouse IgG (dark grey triangle) or ATAP2 antibody (light grey triangle) for 20 minutes prior to stimulation with the indicated concentrations of thrombin. D. EA.hy926 cells were pretreated with assay buffer (black square), 2.5 µg/mL mouse IgG (dark grey triangle) or ATAP2 antibody (light grey triangle) for 20 minutes prior to stimulation with the indicated concentrations of PAR1-AP. Data represented is mean±S.D. n of 1 in triplicate.

These data indicate that 20 nM APC is ineffective at desensitizing PAR1-AP either acutely or over time. APC is only weakly efficacious at mobilizing calcium at supraphysiological concentrations. To test the aforementioned EPCR occupancy theory, protease dead APC, DEGR APC, which is chemically modified at the active site and thus

proteolytically inactive, was used in place of APC. DEGR APC, was added to the cells and allowed to bind for 1 hour. Control tests were previously performed with purified DEGR APC to confirm there was no enzyme activity (data not shown). PAR1 was subsequently stimulated with PAR1-AP and calcium mobilization was measured. DEGR APC treatment does not alter the potency or efficacy of PAR1-AP mediated calcium mobilization in HUVEC or EA.hy926 cells (Figure 3D). These data indicate either the G_q pathway is not modulated by APC or APC-EPCR does not modulate PAR1 signaling.
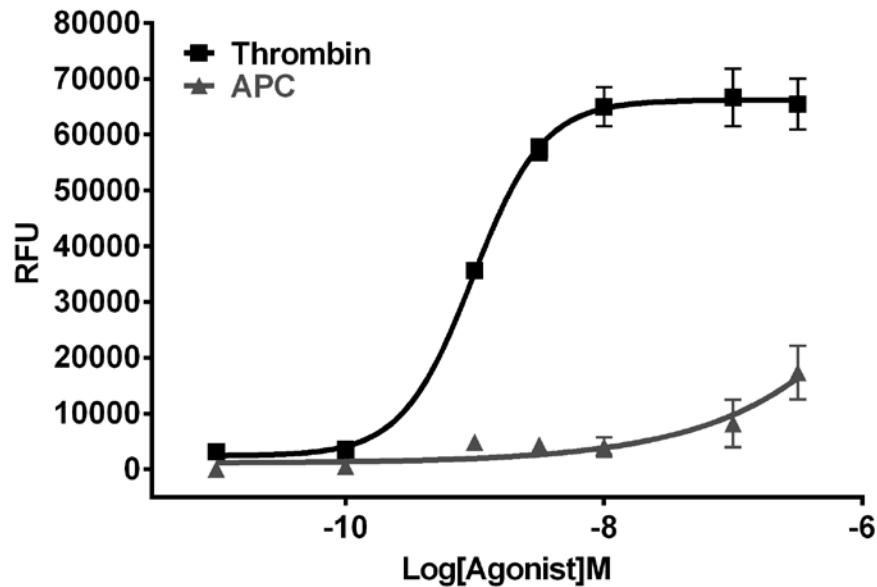
Figure 2. APC elicits calcium mobilization. EA.hy926 cells were stimulated with the indicated concentrations of either thrombin or APC and calcium mobilization was measured. Data represented is raw relative fluorescent intensity units, n of 1 performed in triplicate mean±S.D.

PAR1 activation does not reduce cellular cAMP

To investigate whether G_i signaling was altered downstream of PAR1, total cellular cAMP levels were measured. Forskolin increased cAMP levels approximately 3-fold over basal (Figure 4). Thrombin and APC non-significantly decreased forskolin

stimulated cAMP in endothelial cells at the highest concentration. These data indicate

possible weak G_i signaling downstream of PAR1 activation, although agonist induced

fluctuations in forskolin induced cAMP levels were not significant. Again, APC signaling

does not differ from that of thrombin.
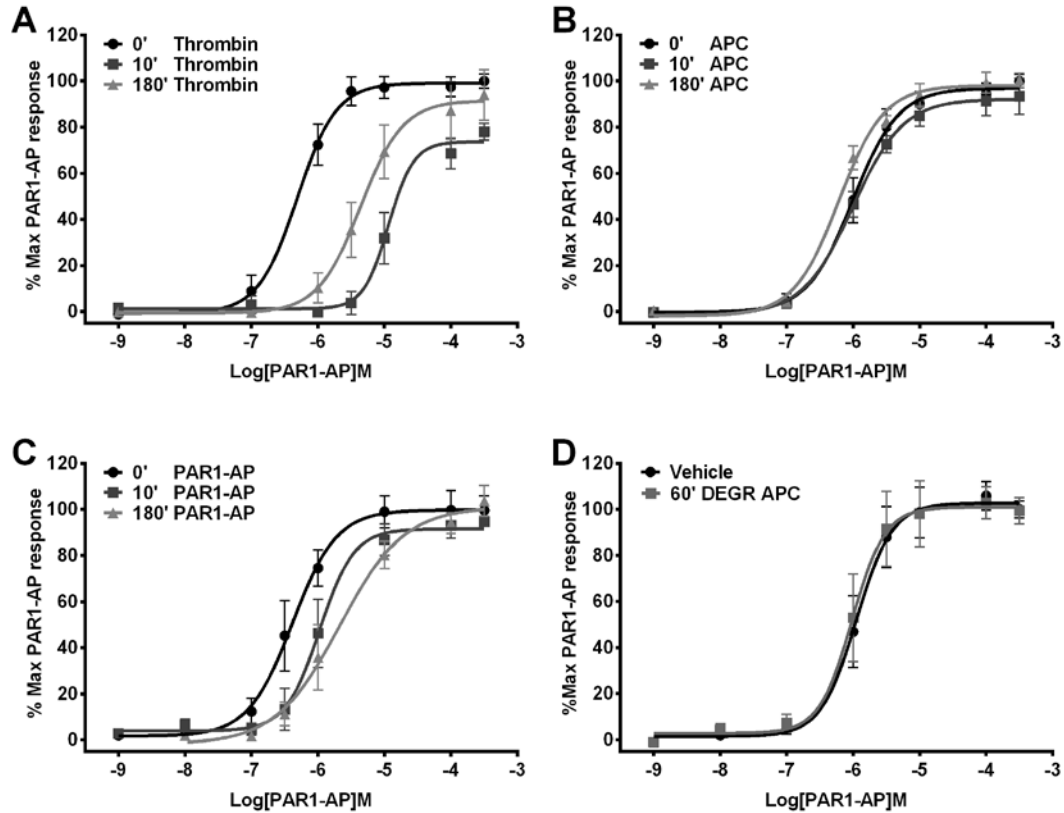
Figure 3. Desensitization of PAR1 calcium mobilization. EA.hy926 cells and Fluo4-AM were used to test calcium desensitization. A. Cells were pretreated for either 10 minutes (black squares) or 3 hours (grey triangles) with 20 nM thrombin followed by stimulation with indicated concentrations of PAR1-AP. Data represented is an n of 2 in triplicate mean±S.D. B. Cells were treated for 10 minutes (black squares) or 3 hours (grey triangles) with 20 nM APC prior to stimulation with increasing concentrations of PAR1-AP. Data presented as n of 1 in triplicate mean±S.D. C. Cells were treated for 10 minutes (black squares) or 3 hours (grey triangles) with 20 µM PAR1-AP prior to stimulation with increasing concentrations of PAR1-AP. Data represented is n of 2 in triplicate mean ±S.D. D. Cells were incubated for 1 hour with 50 nM DEGR APC followed by treatment with increasing concentrations of PAR1-AP. Data is an n of 2 performed in triplicate mean±S.D.

PAR1 agonists induce barrier permeability

Barrier permeability is a cellular contractile event dependent on calcium and Rho, a G_{12} effector. It is reported that barrier permeability induced by PAR1 agonism is counteracted by pretreatment with APC[142]. Attempts to reproduce barrier protection by APC failed to produce similar results to the literature. The data in Figure 5 add controls that establish the acute effects of any PAR1 agonist on barrier permeability. APC (non-significantly), PAR1-AP, and thrombin each induced barrier permeability within 10 minutes of stimulation (Figure 5). Since APC shares the same cellular phenotype as PAR1-AP and thrombin, these data support the hypothesis that APC is a low efficacy PAR1 agonist.
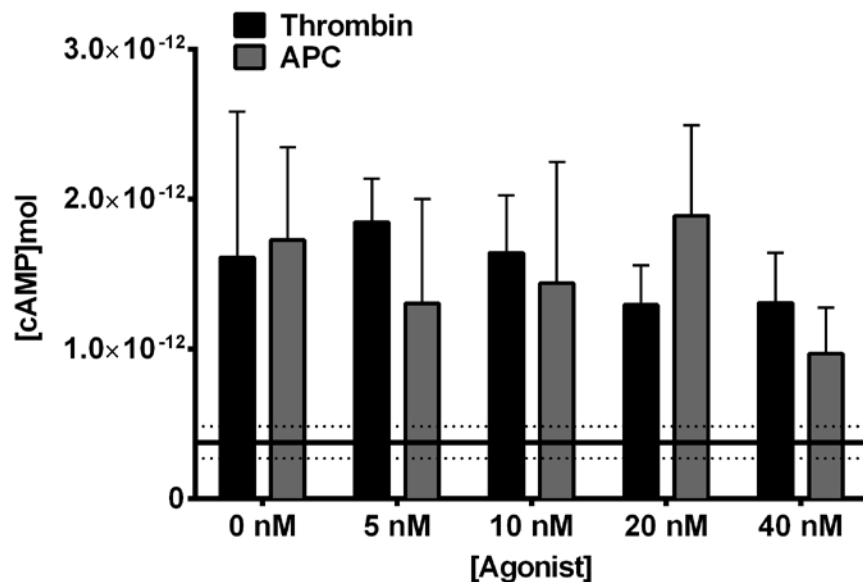
Figure 4. PAR1 activation by APC or thrombin does not effectively reduce cellular cAMP. 1 mM IBMX treated EA.hy926 cells were stimulated with either 10 µM forskolin alone (0 nM) or 10 µM forskolin and the indicated concentration of thrombin and APC. Basal cAMP levels are represented as mean±standard deviation (solid horizontal line±dotted horizontal lines). Bar graph presents data as an n of 2 performed in duplicate mean±S.D.

APC and thrombin inhibit TNFα induced ICAM-1 surface expression

In an attempt to reproduce findings from a recent manuscript, TNFα induced cell surface ICAM-1 expression was measured in EA.hy926 cells and HUVEC [148]. Both APC (Figure 6A) and thrombin (Figure 6B) significantly decreased TNFα-mediated ICAM-1 expression. Both APC (Figure 6A) and thrombin (Figure 6B) significantly decreased TNFα-mediated ICAM-1 expression. When TNFα and thrombin were added together (Figure 6B, 0') thrombin prevented TNFα from up-regulating ICAM-1 over basal levels. To ensure that the differences in the time dependence was not due to the enzymes becoming inactivated, APC and thrombin were refreshed by media exchange every hour prior to TNFα stimulation. These data do not support the hypothesis that APC is cytoprotective and thrombin is not. Rather, these data are in agreement with APC being a low potency, low efficacy PAR1 agonist.
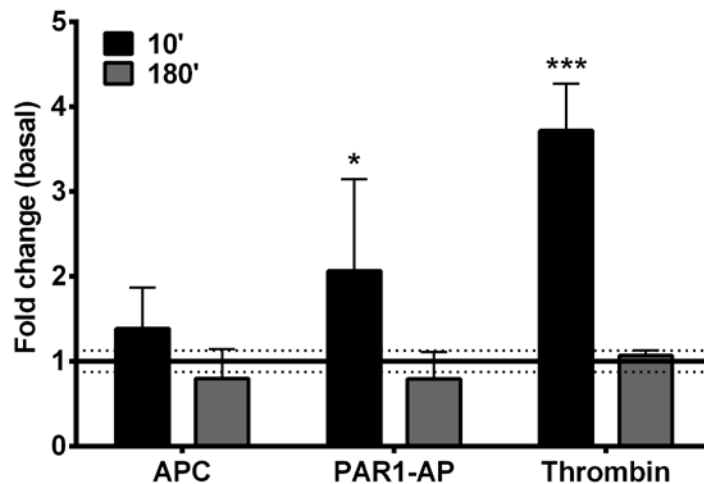
Figure 5. Barrier permeability is enhanced by APC, PAR1-AP, and thrombin. EA.hy926 cells were treated with 40 nM APC (p=0.086), 20 µM PAR1-AP (p=0.049) or 10 nM thrombin (p=0.0002) for either 10 minutes or 180 minutes prior to determination of barrier disruption via Evans Blue Albumin assay. Data presented as fold change over basal permeability, n of 2 in duplicate, mean±S.D. Solid horizontal line and dotted horizontal lines represent the mean and standard deviation respectively of basal permeability. Statistical analysis is unpaired one-tailed t-test versus basal, significant where indicated.
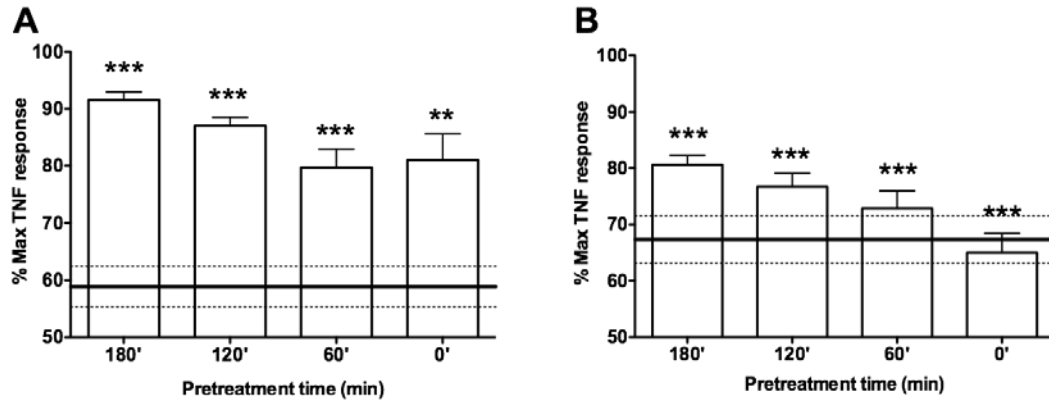
Figure 6. APC and thrombin inhibit TNFα-induced ICAM-1 surface expression. Cell surface ELISA was used to measure ICAM-1 surface expression. Cells were serum starved for 1 hour prior to pretreatment with either APC or thrombin for the indicated amount of time followed by stimulation with 10 ng/mL TNFα for 2 hours. A. Cells were treated with 20 nM APC in an n of 3 in triplicate mean±S.E.M. B. Cells treated with 20 nM thrombin n of 3 in triplicate mean±S.E.M. Data presented as percent of 2 hour TNFα treatment. Solid horizontal line and dotted horizontal lines represent the mean and standard deviation respectively of basal ICAM-1 expression. Significant where indicated. ***$p < 0.0003$, **$p < 0.005$ one sample t-test for deviation from maximal TNFα response.

PAR1-mediated inhibition of ICAM-1 is concentration dependent

The concentration dependence of PAR1-mediated ICAM-1 inhibition was also examined. Endothelial cells were pretreated with APC, thrombin or PAR1-AP prior to stimulation with TNFα. TNFα mediated ICAM-1 cell surface expression was severely inhibited by APC (Figure 7A), thrombin (Figure 7B), and PAR1-AP (Figure 7C) in EA.hy926 cells. To ensure that the observed inhibition was not due to an artifact of testing in a transformed cell line, the same experiment was performed in HUVEC. In primary cells, APC (Figure 7D) and PAR1-AP (Figure 7E) inhibited TNFα induced ICAM-1 up-regulation in a concentration dependent manner. These data, though contrary to reported phenomenon, indicate multiple PAR agonists, not only APC, can inhibit induction of a pro-inflammatory marker, ICAM-1.
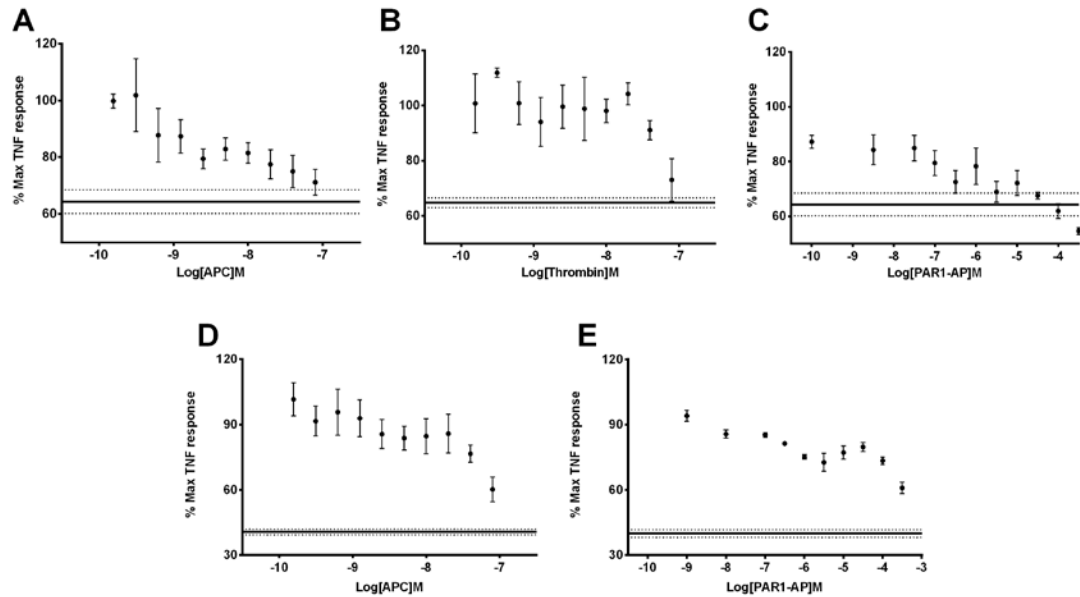
Figure 7. APC, thrombin, and PAR1-AP inhibit TNFα-mediated ICAM-1 expression in a concentration dependent manner. A. HUVECs were serum starved for 1 hour, pretreated with the indicated concentrations of APC for 3 hours followed by stimulation with 10 ng/mL TNFα for 2 hours prior to measurement of cell surface ICAM-1 via ELISA. Data presented is n of 2 in triplicate mean±S.D. B. HUVECs were serum starved for 1 hour, pretreated with the indicated concentrations of thrombin for 3 hours followed by stimulation with 10 ng/mL TNFα for 2 hours prior to assay. Data points are n of 1 in triplicate mean±S.D. C. HUVECs were serum starved for 1 hour, pretreated with the indicated concentrations of PAR1-AP for 3 hours followed by stimulation with 10 ng/mL TNFα for 2 hours prior to assay. Data points are n of 1 in triplicate mean±S.D. D. EA.hy926 cells were serum starved for 24 hours followed by pretreatment with either D. APC or E. PAR1-AP for 4 hours and subsequently stimulated with 10 ng/mL TNF for 4 hours. Data points are n of 1 in triplicate mean±S.D. Solid horizontal line and dotted horizontal lines represent the mean and standard deviation respectively of basal ICAM-1 expression.

Summary and Discussion

APC regulates calcium mobilization, cellular permeability, and ICAM-1 surface expression in a manner similar to thrombin and PAR1-AP. These findings suggest APC could be a low efficacy thrombin receptor agonist and not necessarily a modulator of PAR1 signaling. Thrombin is known to enhance calcium mobilization downstream of PAR1, however APC can also induce calcium mobilization likely through PAR1, though

that has not been definitively demonstrated. Thrombin and PAR1-AP mediated PAR1 desensitization is greater at 10 minutes of pretreatment than 180 minutes of treatment. These data indicate that re-sensitization, by enhancement of receptor reserve, takes longer than 10 minutes and even at 3 hours the potency of PAR1-AP desensitized by thrombin or PAR1-AP is lower than buffer-treated cells. Similarly, thrombin treated cells don't fully recover the maximal response at 3 hours. This may be due to the 'suicide' nature of thrombin-mediated PAR1 activation and the tendency of PAR1 to be sent to the lysosome for degradation instead of recycling. APC is reported to inhibit PAR1-mediated barrier permeability, though those results were not reproducible. However, PAR1, thrombin, and APC are capable of disrupting the barrier at 10 minutes. This 'protection' could be due to receptor desensitization and not functional selectivity. Furthermore, the anti-inflammatory nature of APC, thrombin and PAR1 on TNFα-mediated ICAM-1 cell surface expression is clear. These data suggest that PAR1 activation by multiple agonists produce anti-inflammatory responses, thus APC is not necessarily acting as an allosteric modulator but as a low potency, weakly efficacious PAR1 agonist. Alternatively, APC may not be an agonist of PAR1 but may be acting through another receptor that produces similar cellular responses as PAR1.

CHAPTER III

HIGH-THROUPUT SCREENING FOR MODULATORS OF PAR1

Introduction

As the high affinity thrombin receptor on platelets, PAR1 is an attractive target for the development of anti-platelet and anti-thrombotic therapeutics. Several peptide mimetic and small molecule antagonists have been identified and characterized on human platelets[149-152]. Antagonists of PAR1 reduce thrombin-mediated platelet aggregation, calcium mobilization, and phosphorylation of proteins *ex vivo*. However, the number of clinical candidates to date remains few. Atopaxar, a reversible PAR1 antagonist, is currently in clinical trials and a phase II study concerning its safety and efficacy in patients at risk for acute coronary syndrome has recently concluded. There appeared to be a slight increase in risk of TIMI bleeding however, the largest concern was increased liver function abnormalities and QT prolongation[102]. Vorapaxar is the sole PAR1 antagonist that completed phase III clinical trials in patients and demonstrated a good safety profile. Unfortunately for patients presenting with previous stroke, there was an increased risk for patients on Vorapaxar of developing intracranial hemorrhage noted in the TRA-2P-TIMI50 trial[105-107]. Due to the irreversible nature of PAR activation, successful antagonists of PAR1 should possess a low off-rate or act allosterically at the receptor such that competition with the tethered ligand is possible. In an effort to identify novel inhibitors of PAR1 mediated signaling, a high throughput screening assay was developed measuring intracellular calcium mobilization in a cell line. A chemically diverse compound library was screened and select compounds were identified as inhibitors for PAR1 mediated calcium mobilization in an endothelial-like cell line, EA.hy926.

Materials and Methods

Reagents

     EA.hy926 cells were a kind gift from Dr. Cora-Jean S. Edgell. Poly-Lysine treated Cellcoat Optilux, 384 well black / clear bottom were from BD biosciences (San Jose, California, USA). Fluo4-AM was purchased from Invitrogen (Grand Island, NY, USA). RWJ-56110 was synthesized in house.

High throughput calcium assay

     EA.hy926 cells are seeded at the indicated density in 40 µL of Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum 18-24 hours prior to assay. Cells were dye loaded for 1 hour with 2.5 µg/mL Fluo4-AM in calcium assay buffer (1X HBSS with calcium and magnesium without phenol red, 20 mM HEPES, 2.5 mM probenecid, 1 mM EGTA, 0.1% BSA). Cells were buffer exchanged into calcium assay buffer (without Fluo4-AM) prior to assay via the 'flick and slam' method or automated (for screening) by a BioTek ELx405 (Winooski, VT, USA). Fluorescence measurements were recorded on a Functional Drug Screening System (FDSS) 6000, Hamamatsu (Hamamatsu, Japan) at 37 ºC. 20 seconds of basal fluorescence was recorded for each experiment prior to compound or agonist addition. Compounds (3X) and agonist (10X) were injected by the FDSS and occurred simultaneously across each plate. Compounds were allowed to incubate with cells for 5 minutes prior to agonist stimulation of calcium mobilization. Experiments reported were performed in at least triplicate, on the same plate. 480:540 (ex:em) was measured each second for a total of 4 minutes 20 seconds after agonist addition. The final concentration of DMSO in the assay, if present, was 0.3%. Data was presented as % maximal agonist response or as a ratio of ex:em (480:540) max/basal fluorescence where basal is defined as ex:em at t_0.

Results

Optimization of cell density

Optimization of a high throughput calcium mobilization assay was necessary prior to screening a library of compounds. The cell line selected, EA.hy926, are primary HUVEC that have been transformed via hybridization with A549 (adenocarcinomic alveolar epithelial basal cells) cells. Although the cells are hybridomas, they retain expression of most endothelial cells markers including PAR1 and PAR2[153,154]. EA.hy926 cells were chosen as the model system not only because of the high level of endogenously expressed human PAR1, but also because it was serendipitously discovered that EA.hy926 cells displayed enhanced adherence to cell culture surfaces compared to epithelial cell lines such as HEK and CHO. Endogenous receptor expression was favored due to reduced inter-screen variability often seen in transient transfection cell assays.

To establish the optimal signal:noise ratio for screening, EA.hy926 cells were seeded at several densities and stimulated with increasing concentrations of PAR1-AP using a descriptive 8-point concentration response curve (CRC). An effective concentration for 80% of the maximal PAR1-AP response (EC_{80}) was determined from these data. PAR2-AP CRCs were also performed to determine an EC_{80} for counter screening future hits (Figure 1). The PAR1-AP maximal signal was similar at all cell densities. 8,000 cells/well allowed high PAR1 responses and little variability (Figure 1A). 10,000 cells/well allowed high PAR2-AP responses but a modest PAR1-AP response (Figure 1B). At 12,000 cells/well there was the least amount of variability of any condition, however, the maximal signal for PAR1 was not as robust as at either 8,000 or 14,000 cells/well (Figure 1C). The variability was slightly higher at 14,000 cells/well compared to 8,000 cells/well but the highest signal:noise ratio for both PAR1-AP and PAR2-AP

mediated calcium responses was produced. Thus, subsequent optimization and screening was performed at the 14,000 cellular density (Figure 1D). The estimated EC_{80} for PAR1-AP and PAR2-AP was determined as $10^{-5.5}$ M and 10^{-5} M, respectively.
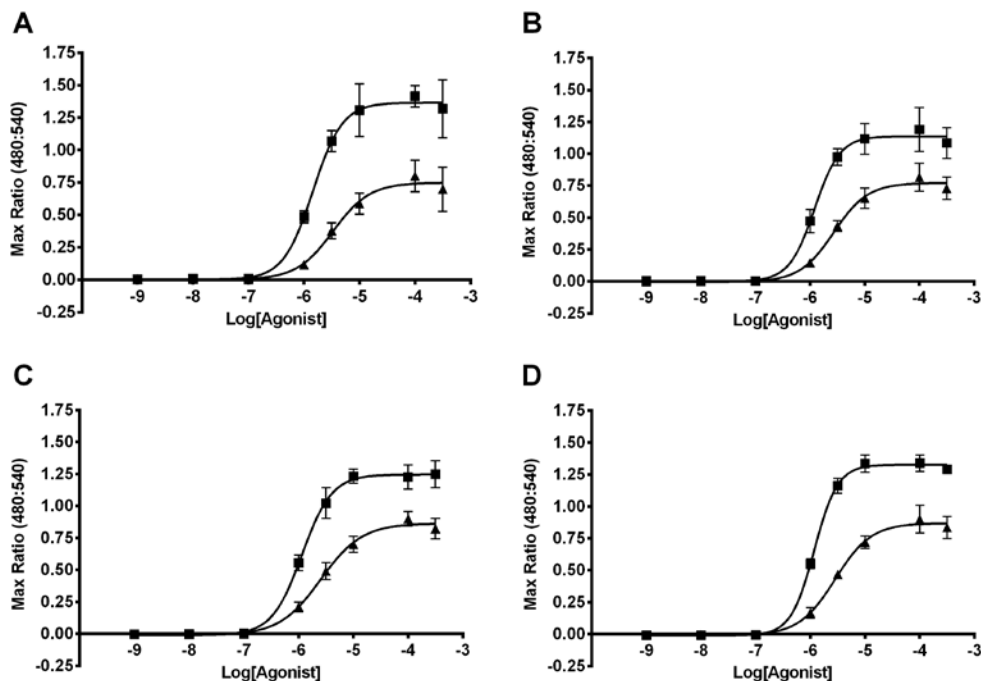
Figure 1. Optimization of EA.hy926 cell density for high-throughput screening. EA.hy926 cells were seeded at either A. 8,000 cells/well, B. 10,000 cells/well, C. 12,000 cells/well or D. 14,000 cells/well in a 384 well plate in sextuplicate. Cells were plated 24 hours prior to assay. After dye loading for 1 hour, dye loading buffer was exchanged to 45 μL calcium assay buffer. Agonists were added to the cells after 20 seconds of baseline fluorescent readings. Data presented are fluorescent max ratio, n of 1, each point performed in triplicate; mean±S.D.

Suitability of assay for high throughput screening

At the optimal cellular density of 14,000 cells/well, a Z-factor was calculated to determine if the assay was suitable for screening. Cells were treated with either assay buffer (vehicle) or the PAR1-AP EC_{80} and peak calcium was determined from the maximal fluorescence ratio as fold change versus the basal (defined as fluorescence at

t_0). A representative tracing from maximal PAR1-AP, EC_{80} PAR1-AP and vehicle treated wells is shown in Figure 2A. The Z-factor was calculated as described in Zheng et al. by performing a checkerboard assay using the EC_{80} of PAR1 and assay buffer as the signal and control respectively. The Z-factor was 0.53 which is considered an 'excellent assay' for high throughput screening[155].
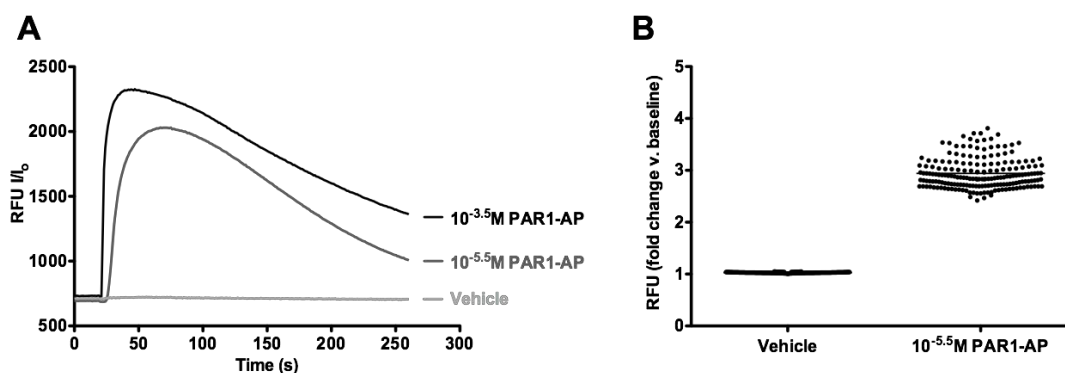
Figure 2. Suitability of Ca^{2+} assay for high throughput screening. EA.hy926 cells were seeded at a density of 14,000 cells/well, cells stimulated with PAR1-AP, and calcium mobilization was measured by fluorescence intensity for 5 minutes. A. Representative calcium tracings of raw fluorescent intensity of maximal PAR1-AP response (black), EC_{80} PAR1-AP response (grey), or vehicle (light grey). B. To determine the Z-factor, and thus the usefulness of the assay as a screening tool, cells were treated with either vehicle (calcium assay buffer) or EC_{80} PAR4-AP in checkerboard fashion in a 384 well plate. Data displayed as n of 1 with 192 replicates for each condition. Calculated Z-factor is 0.53.

Antagonist proof of concept study

A proof of concept study was conducted to ensure that the high throughput Ca^{2+} mobilization assay could identify a known PAR1 antagonist. RWJ-56110 (RWJ), a well-characterized PAR1 antagonist, was selected as the positive control. RWJ is a peptide mimetic that behaves competitively at PAR1[149]. In the assay, RWJ was able to significantly inhibit the PAR1-AP EC_{80}; therefore, the assay is suitable for identification of inhibitors of PAR1 (Figure 3).
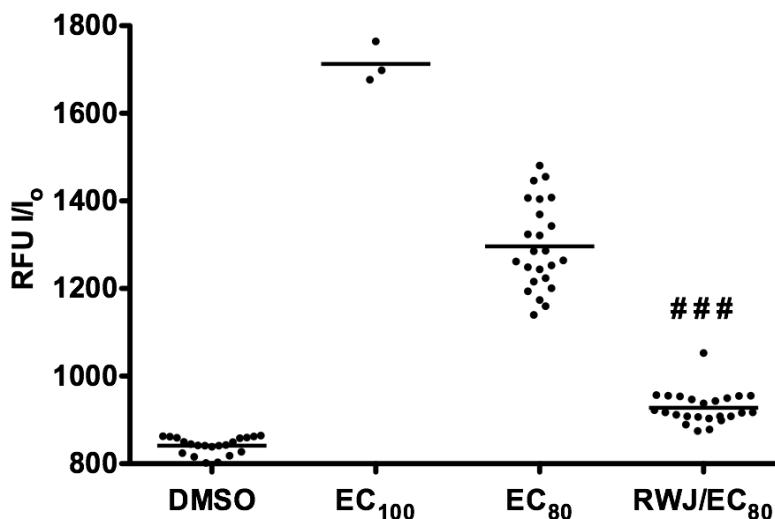
Figure 3. Antagonist proof of concept study. Cells were pretreated with either DMSO, buffer, or 10 µM RWJ-56110 for 5 minutes prior to stimulation with either an EC_{100} ($10^{-3.5}$ M) or EC_{80} ($10^{-5.5}$ M) concentration of PAR1-AP. Data represented are raw fluorescent intensity of n of 1 with at least triplicate values for each data point. Significant where indicated, unpaired two-tailed t-test versus DMSO-treated EC_{80} calcium response. ### $p<0.0001$.

Selectivity screening: agonist assessment

To incorporate non-PAR family member calcium signaling into the counter screening efforts, an appropriate ligand for induction of calcium mobilization was needed. Endothelial cells express $P2Y_2$, a purinergic G_q coupled GPCR responsible for the majority of ADP-mediated calcium mobilization in EA.hy926[156]. Additionally, EA.hy926 cells express Angiotensin type II receptor, a G_q coupled GPCR[157]. ADP and angiotensin II (ANG II) were tested in the high-throughput calcium assay. Both ADP and ANGII induced rapid intracellular calcium mobilization, indicative of G_q signaling. However, ANG II displayed weak potency and efficacy and thus was not suitable for counter screening (Figure 4A). ADP produced robust calcium responses with an estimated EC_{80} of 10^{-5}M and was subsequently used in selectivity screening (Figure 4B).

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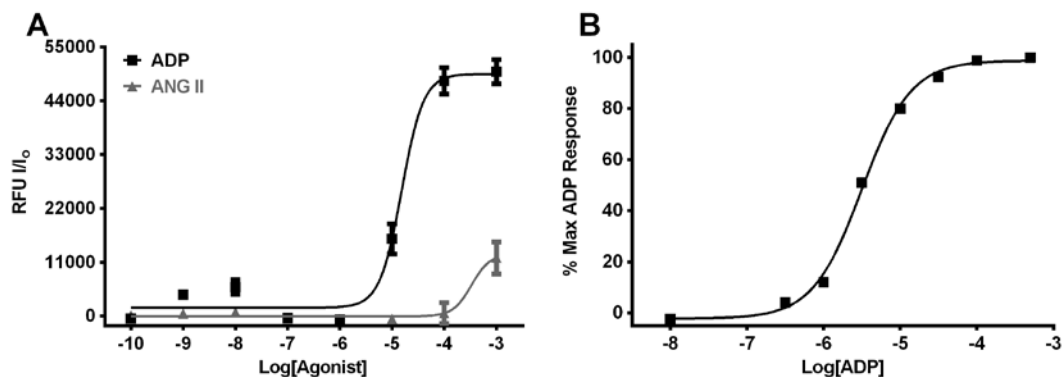
Figure 4. Counter-screen agonist selection. A. EA.hy926 cells were treated with increasing concentrations of either ADP or ANG II and calcium mobilization was measured. Data represented is an n of 1 performed in triplicate, mean±S.D. B. Concentration response curve of ADP stimulated EA.hy926 cells data presented is an n of 1 performed in triplicate mean±S.D.

Screening for novel negative modulators of PAR1 signaling

     2000 chemically diverse structures were screened as part of the Vanderbilt Spectrum Collection (Figure 5). Aside from cell plating, the assay was fully automated via the resources in the Vanderbilt Institute of Chemical Biology High-Throughput Screening Facility. The Labcyte Echo 550 was used for compound plate stamping thereby copying the spectrum collection into the compound plates used during screening. The ELx405 was used for both dye loading procedures as well as buffer exchange prior to assay. Finally, the FDSS performed all liquid handling tasks and fluorescent measurements. The tracings of all compounds that displayed even weak inhibitory activity to the PAR1-AP EC_{80} were visually inspected as well as compounds that slowed the rate of calcium mobilization initiated by PAR1-AP. Compounds displaying intrinsic fluorescence or weak agonism of calcium responses were triaged. After careful analysis, 10 compounds were suitable for validation and selectivity screening.

Fig. 5 cont.

D

Plate 4

(Scatter plot: Ratio (480:540 max-min) vs Plate 4 wells A1–P1, with labeled point E19)

E

Plate 5

(Scatter plot: Ratio (480:540 max-min) vs Plate 5 wells A1–P1, with labeled points E8 and L7)

F

Plate 6

(Scatter plot: Ratio (480:540 max-min) vs Plate 6 wells A1–P1, with labeled points E6 and K9)
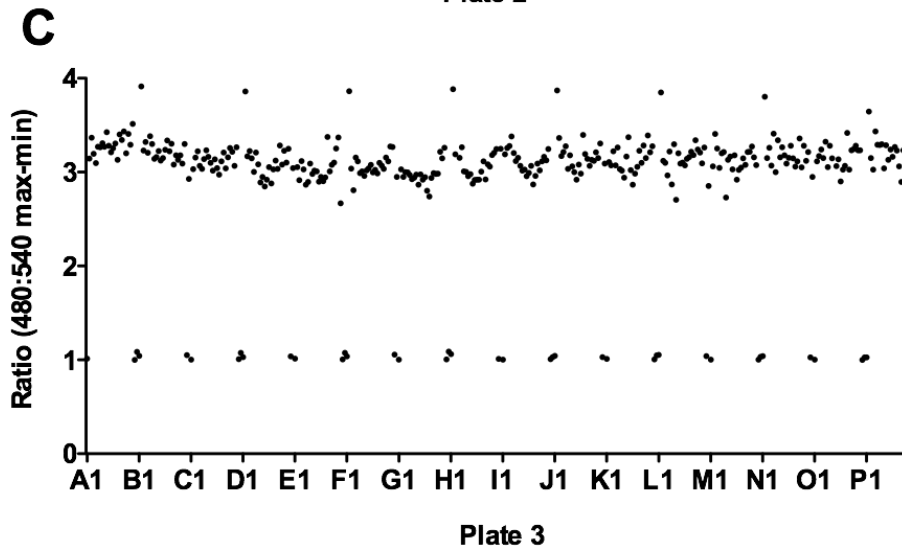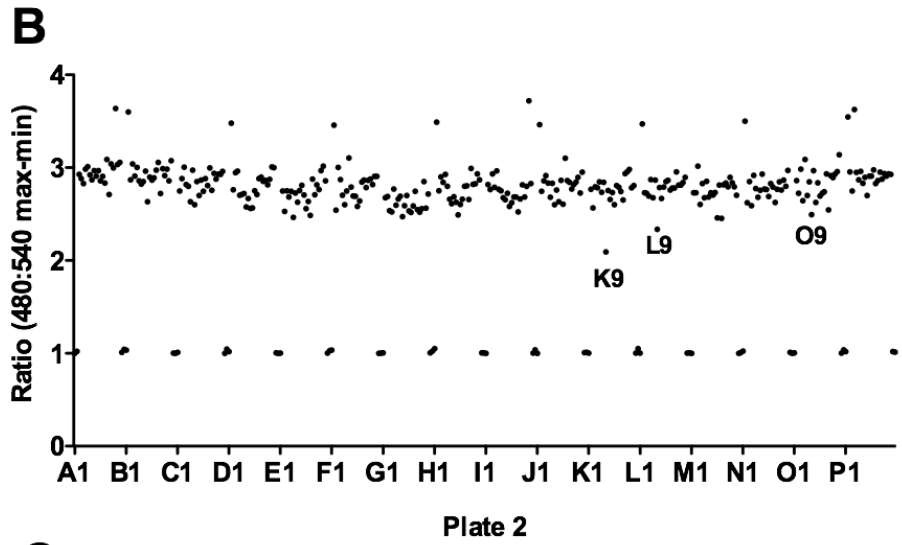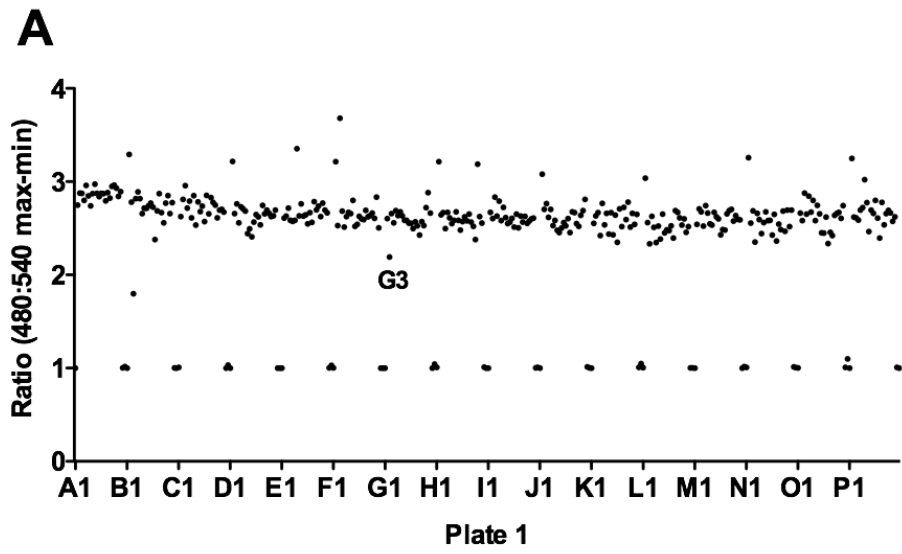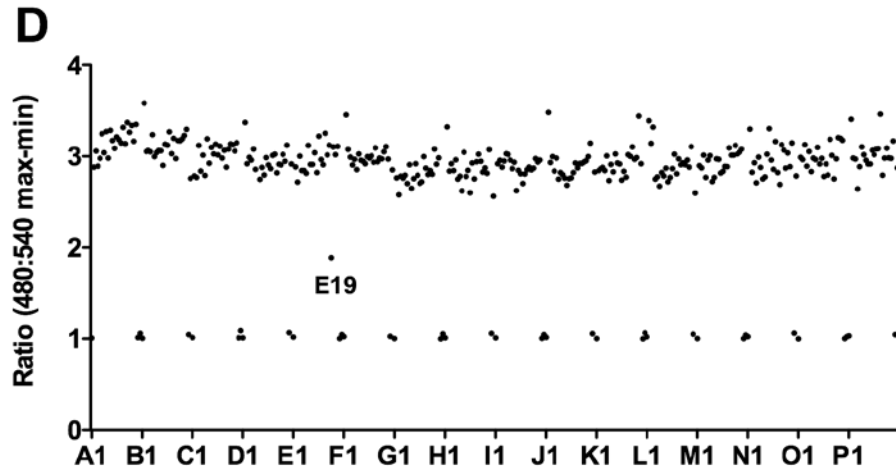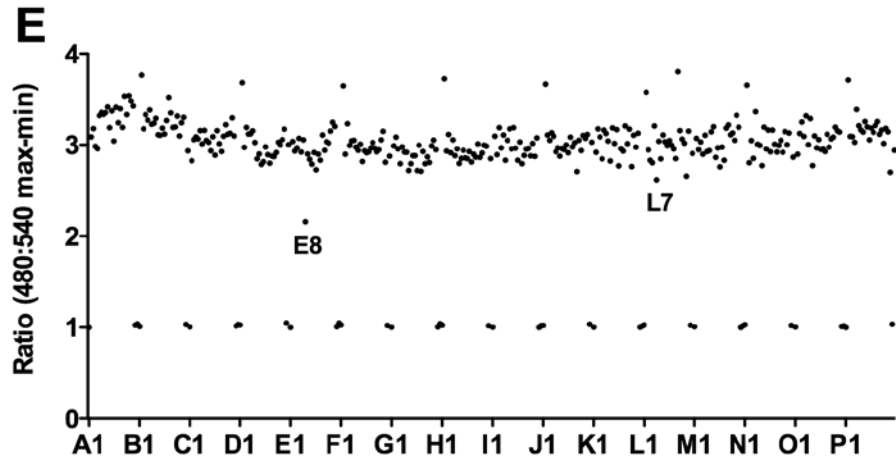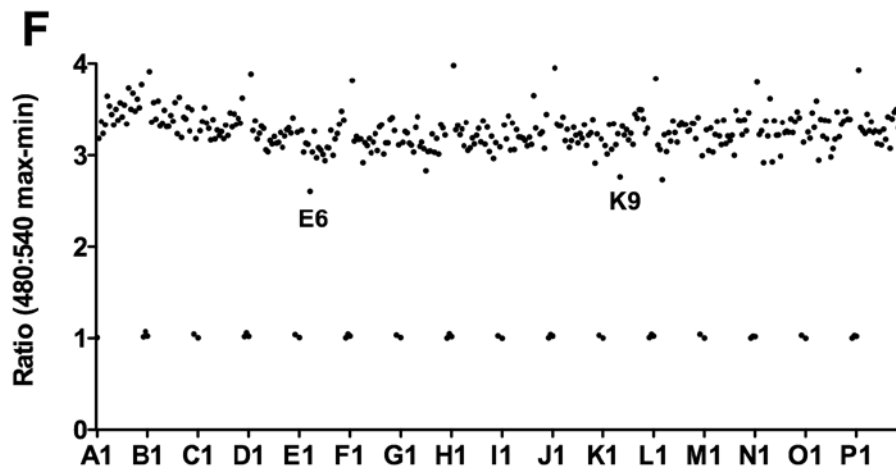
Fig. 5 cont.
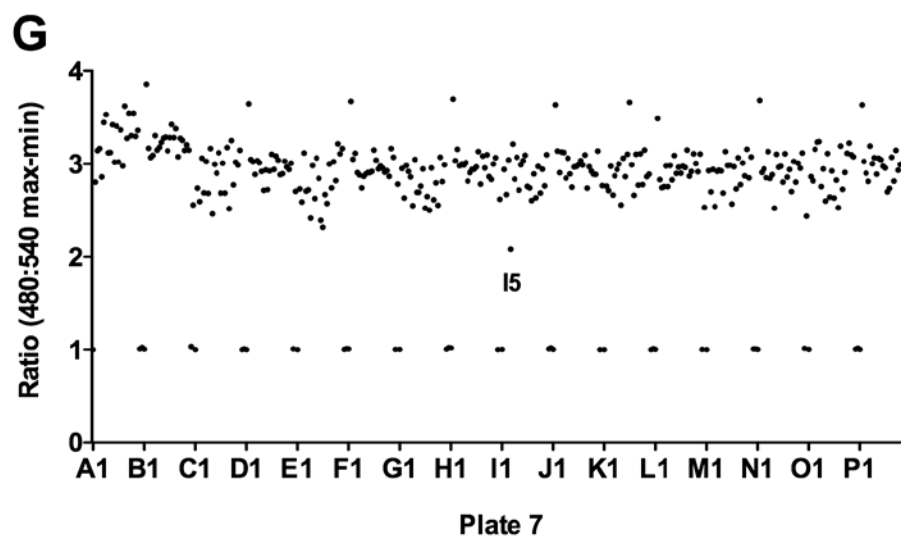
Figure 5. Spectrum collection screening results. EA.hy926 cells were screened for inhibitory action against PAR1. Cells were treated for 5 minutes with compounds from the Vanderbilt Spectrum Collection at 10 µM followed by stimulation with an EC_{80} of PAR1-AP. Data are visually inspected, all demarked compounds were considered hits by meeting the following criteria: decreased either maximum PAR1-AP mediated calcium mobilization or the rate of PAR1-AP mediated calcium mobilization and did not possess intrinsic fluorescent properties.

Validation and selectivity screening

Compounds (plate.well) 2.K9, 2.LG, 2.O9, 5.L7, 6.E6, and 6.K9 did not generate reproducible inhibitory action against PAR1-AP mediated calcium mobilization. Compound 1.G3 appeared to be a general calcium inhibitor and thus not specific or selective for any agonist tested. Compound 4.E19 weakly selectively inhibited PAR1 and not PAR2 or ADP mediated calcium signaling. 5.E8 inhibited both PAR1 and PAR2 mediated calcium signaling but not ADP and therefore may be a PAR family selective inhibitor. 7.I5 was intriguing because it weakly inhibited PAR1 mediated calcium response but potentiated ADP mediated signaling.
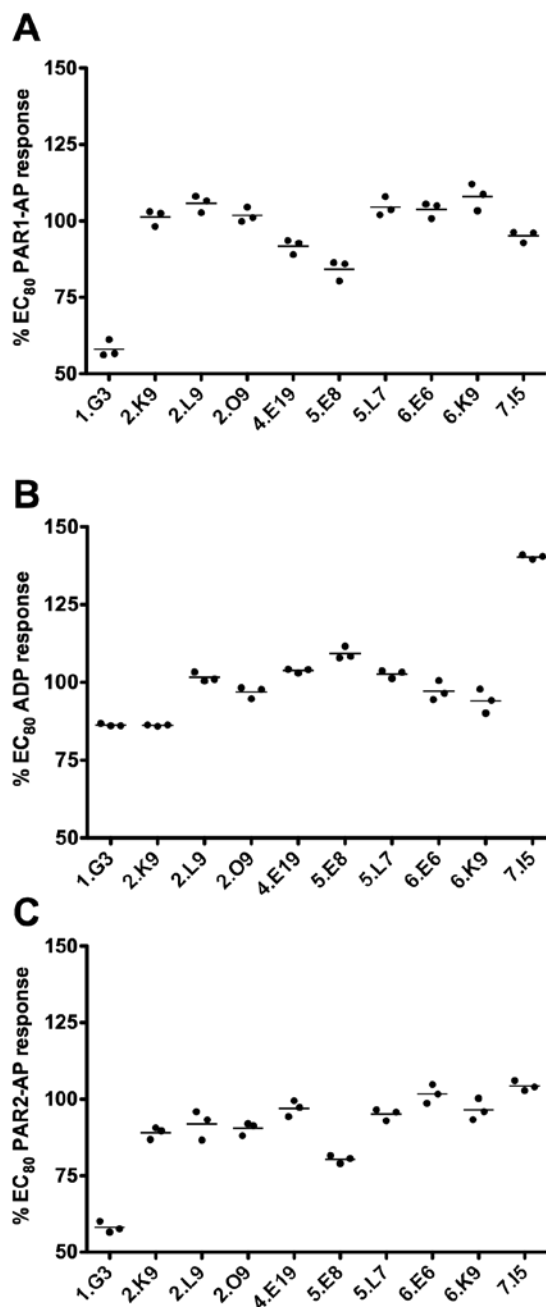
Figure 6. Hit validation and selectivity screen. Cells were treated with the hit compounds from the screen for 5 minutes prior to stimulation with an EC_{80} of either A. PAR1-AP, B. ADP, or C. PAR2-AP. Several compounds did not reproduce inhibitory activity in the validation screen. Compound 1.G3 behaved as a general calcium inhibitor. Compound 4.E19 selectively inhibited PAR1-AP. 5.E8 selectively inhibited PAR1-AP and PAR2-AP. 7.I5 inhibited PAR1-AP responses but potentiated ADP mediated calcium mobilization.

Compound identities

Compound 4.E19 (Figure 7A) is mitotane, a chemotherapeutic agent indicated for the treatment of adrenal carcinoma. Compound 5.E8 (Figure 7B) is plumbagin, a natural product possessing anti-cancer properties. Compound 7.I5 (Figure 7C) is benzyl-dimethyl-tridecyl-ammonium, a lipid-like molecule with no associated function.
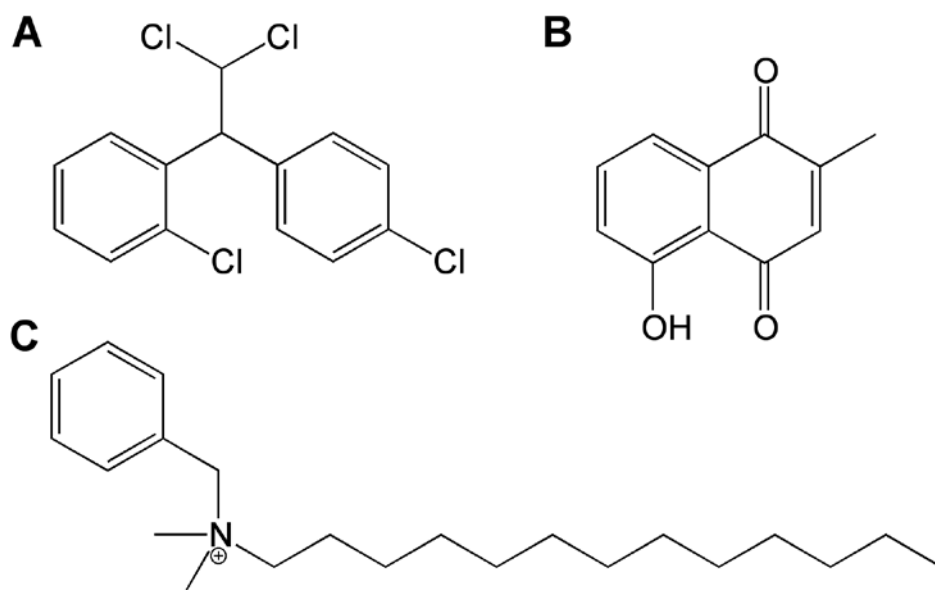
Figure 7. Identities of inhibitory compounds. A. Compound 4.E19 is mitotane. B. Compound 5.E8 is plumbagin. C. Compound 7.I15 is benzyl-dimethyl-tridecyl-ammonium.

Summary and Discussion

Novel inhibitors of PAR1-mediated calcium mobilization have been discovered as a result of using endogenously expressed PAR1 in endothelial-like Ea.hy926 cells and fluo4-AM to screen a small molecule library. EA.hy926 cells are very adherent and are an excellent choice for automated high-throughput screening assays. The high-throughput screen was designed and analyzed to capture even partial antagonists of PAR1 mediated calcium signaling. Both mitotane and plumbagin are interesting

compounds due to their selectivity profiles as well as their molecular size. The simple nature of the mitotane and plumbagin molecules favor chemical modification and structure activity relationship studies for future enhancement of potency and selectivity for PAR1. Unfortunately, the lipid-like nature of benzyl-dimethyl-tridecyl-ammonium complicates any drug discovery efforts, as lipid-like molecules tend to intercalate into the membrane non-selectively.

The current data set could be re-probed for agonist activity against PAR1. An ideal agonist candidate would manifest as a compound that alone produced a calcium response but subsequently desensitized PAR1 but not PAR2 or ADP-mediated calcium mobilization. The PAR1 agonist with rounds of SAR could produce a high affinity, low efficacy agonist deemed a functional antagonist.

CHAPTER IV

MITOTANE MODULATES PLATELET ACTIVATION

Introduction

Mitotane (o,p DDD) is a chemotherapeutic indicated for the treatment of inoperable adrenal carcinoma[158]. Mitotane possesses potent adrenotoxic activity and thus was developed as an anti-tumor agent[159]. Patients with plasma levels above 14 mg/L have greater survival rate than patients with plasma levels below 14 mg/L. Clinically, mitotane acts as an endocrine disruptor possessing estrogenic effects. Part of the estrogenic effect in humans has been attributed to increased sex hormone-binding globulin and corticosteroid-binding globulin in patients[160,161]. Mitotane is also described as a 5α reductase inhibitor, and given the role of 5α reductase in dihydroxytestosterone biosynthesis, this action likely exacerbates the estrogenic effects of mitotane[162]. Mitotane is also a potent inducer of cytochrome P450 3A4, which among many molecules, metabolizes cortisol, thus providing clinical utility for the treatment of Cushing's syndrome, a condition where patients present with excessive cortisol[162-164]. Mitotane also binds several hormone receptors including progesterone receptor, estrogen receptor-α and the androgen receptor, though no functional consequence has yet been attributed to mitotane binding [165 160].

Mitotane treatment carries a bleeding side effect in 90% of patients undergoing mitotane therapy as documented in two separate studies. In these studies a prolongation of bleeding time shortly after treatment begins was reported. These reports also suggested that the bleeding defect was likely due to a defect in platelet function based on patients presenting with normal coagulation parameters during treatment, although the mechanism of action of mitotane-mediated platelet inhibition remains unknown[166,167].

Experiments described hereafter determined mitotane inhibits platelet activation *ex vivo* in purified platelets from healthy individuals. Mitotane-mediated platelet inhibition manifested as inhibition of agonist-induced $\alpha_{IIb}\beta3$ activation and platelet aggregation. This inhibition is rapid in nature and occurs at therapeutic and even sub-therapeutic concentrations of mitotane.

Materials and Methods

Reagents

Purified compounds purchased from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mM and stored at -20 ºC until used. PAR1 activating peptide (PAR1-AP, SFLLRN) and PAR4 activating peptide (PAR4-AP, GYPGKF) were purchased from GL Biochem (Shanghai, China). NUNC 384 well plates with black optical bottoms were from Thermo (Rochester, NY, USA). Fluo4-AM was purchased from Invitrogen (Eugene, Oregon, USA). Fluorescein isothiocyanate (FITC) conjugated PAC1 and photoerythrin (PE) conjugated anti-CD62P (P-selectin) antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Platelet Preparation

Human platelets were obtained from healthy volunteers by written consent in accordance with, and approved by, the Vanderbilt University Institutional Review Board (050182). Platelets were prepared via standard washed platelet protocol as previously described[117,118]. Briefly, blood from healthy volunteers was drawn into syringes containing 3.2% sodium citrate. Platelet-rich plasma was prepared by centrifugation in a Forma 400 ML GP centrifuge at 1100 rpm for 15 minutes. 10X acid citrate dextrose was added to platelet-rich plasma and centrifuged at 2400 rpm for 10 minutes. The

supernatant was aspirated and the platelet pellet was suspended in Tyrode's buffer containing 0.1% Bovine Serum Albumin fraction V (BSA) and platelets were counted on a Beckman Z1 Coulter particle counter (Brea, CA, USA).

High-throughput platelet calcium assay

Washed platelets were prepared via standard procedure and suspended in Tyrode's buffer containing 0.1% BSA. Platelets were dye-loaded for 1 hour with Fluo4-AM in calcium assay buffer (1X HBSS without calcium or magnesium, 20 mM HEPES, 2.5 mM probenecid, 1 mM EGTA, 0.1% BSA). The calcium assay buffer-containing dye is mixed with platelets to yield a final concentration of 2.5 µg/mL Fluo4-AM and 1.0×10^8 platelets/mL. 60 µL of dye-loaded platelets were added to each well of a NUNC 384 well black optical bottom plate (Thermo, Rochester, NY, USA). Fluorescence measurements were recorded on a Functional Drug Screening System (FDSS) 6000, Hamamatsu (Hamamatsu, Japan) at 37 °C. 10 µM of each compound was added 6 minutes prior to the addition of agonist. Liquid handling was performed by the FDSS. Experiments were performed in triplicate, on the same plate, from the indicated number of donors. 480:540 (ex:em) was measured each second for a total of 12 minutes. The final concentration of DMSO in the assay was 0.5%. The difference between basal (defined as t_0) and maximal relative fluorescence unit (RFU) values (RFUs) f or ex:em 480:540 was determined for each well. Data are represented as fold over basal for each well. Data were plotted in GraphPad PRISM v.5.0.

Flow cytometry

Briefly, 60 µL of washed platelets at 1.5×10^7 platelets/mL were added to polystyrene tubes. Anti-CD62P antibody or PAC-1 antibody were diluted (per manufacturer protocol) in Tyrode's buffer containing 0.1% BSA. 40 µL of diluted antibody

was added to the platelets and incubated for 5 minutes. Platelets were pre-treated with the indicated concentrations of antagonist or DMSO control (0.5%) for 5 minutes followed by addition of agonist for 10 minutes. Platelet activity was quenched by the addition of ice-cold 1.5% paraformaldehyde followed by dilution in 1X phosphate-buffered saline. Platelets were analyzed on a BD FACS Canto II (Franklin Lakes, NJ, USA). Fluorescent intensity was determined for 100,000 events within the platelet gate. Data were collected and analyzed via FACS DiVa software. 100% max response was determined for each donor as the DMSO-treated control stimulated with either 200 µM PAR4-AP, 20 µM PAR1-AP, or 10 nM thrombin. Samples treated with inhibitor were normalized as a percentage of the DMSO-treated control. Normalized data were plotted in GraphPad PRISM v.5.0. Dose response curves and subsequent IC_{50} values were generated using the 'log(inhibitor) vs. response variable slope' parameter.   Data plotted are mean±S.E.M. or mean±S.D.

Aggregation

Briefly, washed platelets were prepared to a final concentration of $2.0x10^8$ platelets/mL in Tyrode's buffer containing 0.1% BSA. Compounds or DMSO control (0.1%) were added 10 minutes prior to stimulation with either PAR1-AP or PAR4-AP. Light transmittance was recorded by a Chrono-Log Model 700 Aggregometer. 100% light transmission signifies full platelet aggregation.

Results

Mitotane inhibits platelet activation

Platelets from healthy individuals treated *ex vivo* with 14 mg/L mitotane had reduced aggregation responses to thrombin and collagen mediated activation (Figure

1A). Only aggregation mediated by high dose thrombin was not significantly inhibited by

mitotane. In addition to aggregation, ATP secretion, indicative of platelet dense granule

release, downstream of the thrombin and collagen receptors was inhibited by therapeutic

concentrations of mitotane (Figure 1B). These data indicate that mitotane has direct

inhibitory effects on human platelet activation driven by the major platelet agonists,

thrombin and collagen.
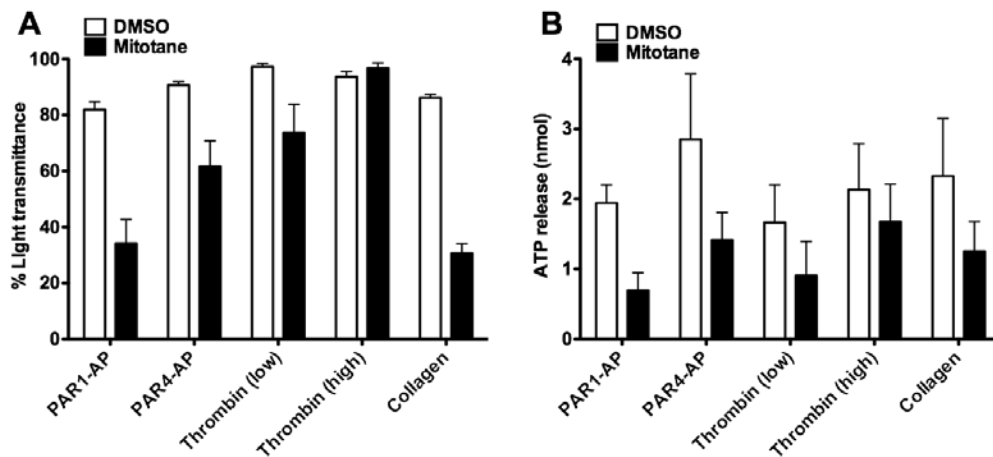
Figure 1. Mitotane inhibits thrombin and collagen mediated platelet activation.
Platelets were treated with 14 mg/L mitotane for 10 minutes prior to stimulation with 20
µM PAR1-AP, 200 µM PAR4-AP, 2 nM thrombin (low), 10 nM thrombin (high), or 10
µg/mL collagen.   A.   Platelet aggregation for agonists represented as % light
transmittance where 100% transmittance is fully aggregated platelets. Data represented
are an n of 6 performed in singlicate mean±S.E.M. B. ATP release, in nmol, in response
to platelet agonists with and without treatment with mitotane. Data presented are an n of
3 performed in singlicate mean±S.E.M.

Mitotane inhibits $\alpha_{IIb}\beta3$ activation in a concentration dependent manner

To fully characterize the inhibitory effect of mitotane on platelets, $\alpha_{IIb}\beta3$ activation

was measured. $\alpha_{IIb}\beta3$ exists on the platelet surface in an inactive conformation and

upon platelet activation, by a number of agonists, $\alpha_{IIb}\beta3$ undergoes a conformation shift

to its active state to allow fibrin binding. PAC1 binding measures only receptors in the

50

active conformation. Mitotane treatment alone induces a 4.5 fold increase in active $\alpha_{IIb}\beta3$.

However, mitotane treatment severely inhibited thrombin and convulxin, a GPVI agonist, mediated $\alpha_{IIb}\beta3$ activation (Figure 1A). Furthermore, mitotane inhibition of thrombin, PAR1-AP, and PAR4-AP induced $\alpha_{IIb}\beta3$ activation is dose dependent (Figure 1B). These data suggest that while mitotane can activate $\alpha_{IIb}\beta3$, subsequent stimulation of the platelet does not yield additional receptor activation.
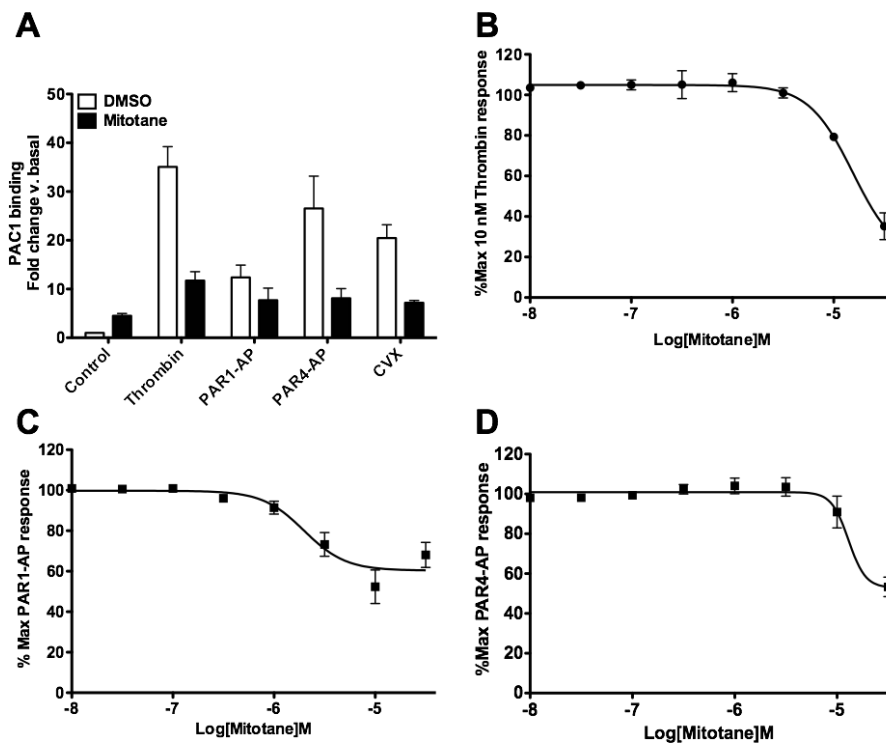
Figure 2. Mitotane inhibits $\alpha_{IIb}\beta3$ activation downstream of thrombin receptors. Platelets were treated with increasing concentrations of mitotane for 5 minutes followed by stimulation with thrombin, 20 µM PAR1-AP, 200 µM PAR4-AP, or 500 ng/mL Convulxin (CVX). A. 14 mg/mL mitotane mediated activation or inhibition of agonist induced $\alpha_{IIb}\beta3$ activation. Data represented is and n of at least 3 in singlicate mean±S.E.M. B. Concentration dependence of mitotane inhibition on thrombin mediated $\alpha_{IIb}\beta3$ activation. Data presented is an n of 2 performed in singlicate mean±S.D. C. Concentration dependence of mitotane inhibition on PAR1-AP mediated $\alpha_{IIb}\beta3$ activation. N of 3, in singlicate mean±S.E.M. D. Concentration dependence of mitotane inhibition on PAR4-AP mediated $\alpha_{IIb}\beta3$ activation. Data displayed is an n of 2 performed in singlicate mean±S.D.

Mitotane activates alpha granule secretion and calcium mobilization

To characterize the effect of mitotane on alpha granule release, P-selectin cell surface expression was measured. P-selectin antigen normally present in the lumen of alpha granules is expressed on the platelet surface upon degranulation. Mitotane alone appears to fully activate P-selectin cell surface expression such that subsequent activation by thrombin, PAR1-AP, PAR4-AP, or convulxin did not produce additional alpha granule degranulation (Figure 3A). Since calcium mobilization is tightly coupled to granule secretion, the effect of mitotane on intracellular calcium mobilization was measured. Mitoane alone produced a 10-fold increase in peak calcium mobilization (Figure 3B). Mitotane elicited only a modest potentiation of PAR1-AP and PAR4-AP peak calcium mobilization, however, the calcium tracing revealed that mitotane greatly enhanced the area under the curve for agonist-induced calcium mobilization (Figure 3C).

Similar chemical entities inhibit PAR-mediated $\alpha_{IIb}\beta3$ activation

Mitotane is a de-chlorinated metabolite of the now banned commercial insecticide DDT and shares structural similarities, nominally the dual ring structure and substituents in the ortho or para positions, with pesticides still in use. The action of the DDT family of compounds and several other chemical species were examined on agonist-induced $\alpha_{IIb}\beta3$ activation. In a concentration dependent manner, 4,4 DDT, 2,4 DDT, and 4,4 DDD inhibited both PAR1-AP (Figure 4A) and PAR4-AP (Figure 4B) mediated $\alpha_{IIb}\beta3$ activation. In addition, a few pesticides with similar chemical structures as mitotane that are also approved for commercial use, showed inhibitory action on PAR1 mediated $\alpha_{IIb}\beta3$ activation. Chloroflurenol, irgasan, and methoxychlor inhibited PAR1-AP mediated $\alpha_{IIb}\beta3$ activation in human platelets (Figure 4C). Chlorofurenol is registered for use as an herbicide for the control of broadleaf weeds. Irgasan, also known as triclosan is antibiotic and antifungal. Irgasan is present in consumer products

including no-rinse hand sanitizers. Methoxychlor was banned as of 2003, it was used as an insecticide replacement for DDT. These data indicate that organochloride insecticides particularly those with di-benzene rings may have inhibitory activity on the platelet.
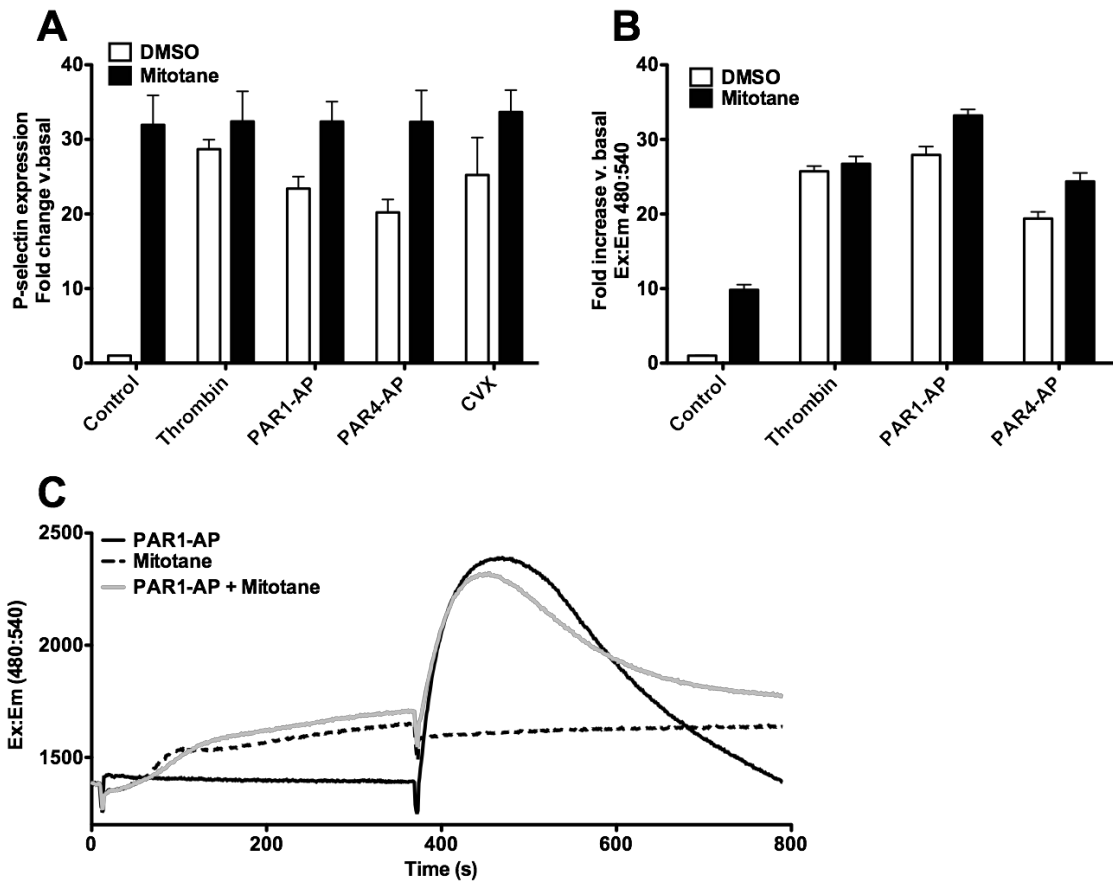
Figure 3. Mitotane activates alpha granule secretion and calcium mobilization. A. Platelets treated with mitotane or DMSO control for 5 minutes prior to challenge with thrombin, PAR1-AP, PAR4-AP, or convulxin. N of 2 in singlicate, mean±S.D. B. Peak Ca^{2+} levels of platelets treated with mitotane or DMSO control for 5 minutes prior to challenge with thrombin, PAR1-AP, or PAR4-AP. N of 3 in triplicate mean±S.E.M. C. Kinetic tracing of PAR1-AP mediated calcium mobilization (black line), 14 mg/L mitotane treated platelets (dashed line), or mitotane treated platelets subsequently stimulated with PAR1-AP (grey line). A representative well of each condition from a single donor is represented.
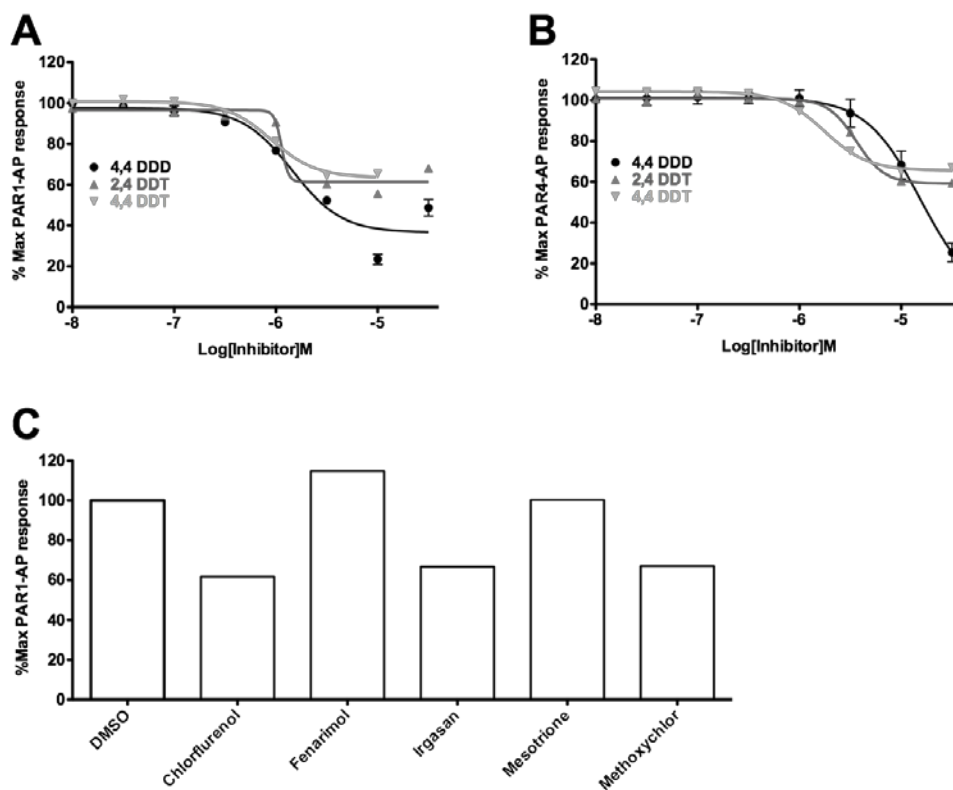
Figure 4. Similar chemical entities inhibit PAR mediated α$_{IIb}$β3 activation. Platelets were treated with increasing concentrations of 4,4, DDD, 2,4 DDT, or 4,4 DDT for 5 minutes prior to stimulation with either A. 20 µM PAR1-AP or B. 200 µM PAR4-AP n of 1 or 2 mean±S.D. where indicated. C. Platelets were treated with 10 µM of the indicated pesticides or DMSO control prior to stimulation with 20 µM PAR1-AP. α$_{IIb}$β3 activation was measured by flow cytometry. Data presented is an n of 1 in singlicate.
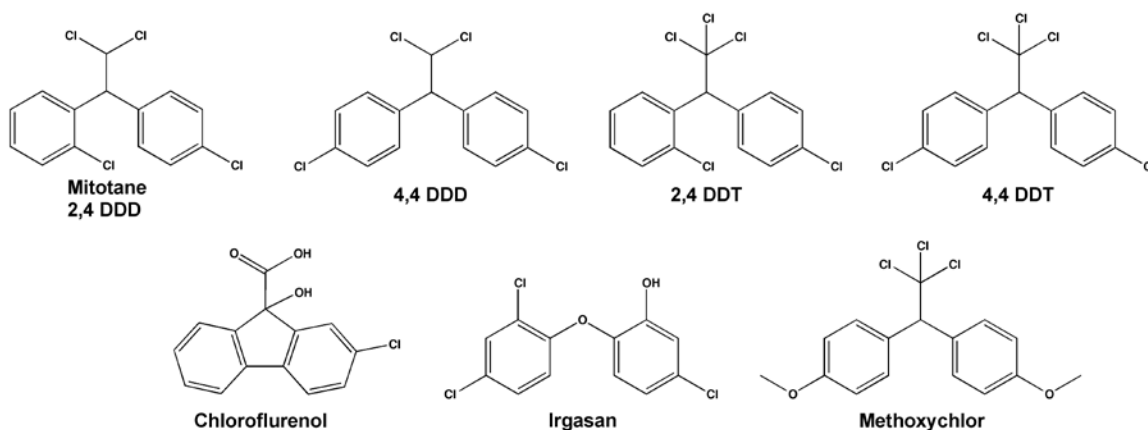
Figure 5. Chemical structures of inhibitory compounds.

Summary and Discussion

Mitotane is a modulator of thrombin and collagen receptor signaling in human platelets. Platelets isolated from healthy individuals and acutely treated with therapeutic concentrations of mitotane show reduced platelet aggregation in response to thrombin receptor agonists and collagen receptor agonists. Patients administered mitotane frequently have a bleeding side effect likely resulting from the partial inhibition of thrombin and collagen induced $\alpha_{IIb}\beta3$ activation. Furthermore, mitotane inhibits dense granule release thus nullifying feed forward purinergic receptor signaling, also a critical component for hemostasis, as patients taking clopidogrel have an increased risk of bleeding. Interestingly, mitotane alone induces Ca^{2+} signaling and potentiates peak agonist induced Ca^{2+} mobilization. Mitotane potentiates agonist-induced Ca^{2+} mobilization not by enhancing peak Ca^{2+}, but by initiating calcium release alone and an apparent impairment of calcium re-uptake. Due to the efficient coupling between calcium mobilization and degranulation, mitotane-mediated calcium enhancement is the probable cause for mitotane mediated P-selectin cell surface expression. It is important to note that although mitotane initiated full alpha granule secretion, mitotane inhibited agonist induced dense granule secretion, as measured by ATP release. These data interestingly suggest that the release of different types of platelet granules may be distinctly regulated and thus pharmacologically targeted.

The dual nature of mitotane action on platelets is likely due to mitotane acting on at least two distinct targets. Key elements of mitotane action that should be addressed include the mechanism(s) by which mitotane is causing inhibition of $\alpha_{IIb}\beta3$ activation. Structure-activity relationships may be used to separate the inhibitory actions of mitotane from the potentiating actions of mitotane to discover a novel class of anti-platelet agents. Mitotane could be acting through hormone receptors in the platelet to modulate signaling. Mitotane binds several hormone receptors and the platelet expresses several of them

including: estrogen receptor β, androgen receptor, mineralocorticoid receptor and the glucocorticoid receptor[168-170]. Initial attempts to pharmacologically ascribe a specific hormone receptor to mitotane function proved futile as the hormone receptor antagonists alone significantly modulated PAR1-AP mediated platelet activation (data not shown). Hormone receptor antagonists are often functional antagonists and can produce agonist effects if the concentration of receptor or compound is high enough. Platelet-specific knockout mice strategies may be employed to definitively exclude hormone receptors as the mechanism through which mitotane is acting.

CHAPTER V

SYNTHESIS AND CHARACTERIZATION OF INDOLE DERIVED PAR4 ANTAGONISTS

Introduction

Thrombin, a key factor in coagulation and inflammation, typically elicits cellular responses via activation of protease activated receptors (PARs). The PAR family consists of four GPCRs that are uniquely activated by proteolytic cleavage of the *N*-terminus resulting in the generation of a new *N*-terminus serving as a tethered ligand, the endogenous agonist for the receptor[75]. Human platelets express both PAR1 and PAR4. PAR1 contains a hirudin-like domain that is able to bind thrombin at sub-nanomolar concentrations however PAR4 lacks this binding domain and is considered the low affinity thrombin receptor on platelets[115,116,171].

PAR activation by thrombin in the platelet is responsible for a number of responses essential to hemostasis. Specifically, PAR-mediated platelet activation causes rapid intracellular calcium mobilization, secretion of autocrine hormones such as 5-HT and ADP, the production of eicosanoids such as thromboxane, expression of glycoproteins such as P-selectin important for platelet interaction with hematopoetic cells involved in coagulation and inflammation, and conversion of the fibrin receptor integrin $\alpha_{IIb}\beta3$ to a high affinity conformation.

PARs are attractive targets for anti-platelet and antithrombotic therapy, as both genetic and pharmacological inhibition results in blockade of arterial thrombosis in animal models[112,121]. To date many PAR1 antagonists have been synthesized and characterized[172-175] and the high affinity non-peptide PAR1 antagonist, Vorapaxar, completed phase III clinical trials. The Vorapaxar TRA-CER trial was terminated due to an association of treatment with an increased risk of bleeding including intracranial

hemorrhage however, the TRA-2P-TIMI 50 trial, excluding patients presenting with previous stroke, was completed and showed that PAR1 antagonism is effective in reducing cardiovascular death and ischemic events [105-107]. PAR4 as the low affinity thrombin receptor is consequently engaged by high concentrations of thrombin and thus subject to differential temporal engagement by thrombin. Signaling differences between PAR1 and PAR4 are well documented indicating that although activated by the same endogenous agonist and purportedly couple to the same G-proteins, platelet thrombin receptor signaling is fundamentally distinct[60,117-119,176,177]. Though the physiological role of PAR1 is becoming clear, the role of PAR4 in thrombosis and hemostasis remains unknown due to a lack of a pharmacokinetically, pharmacodynamically sufficient antagonists thus the need for potent, selective PAR4 antagonists remains.

YD-3 is the sole non-peptide, selective, PAR4 receptor antagonist[123,125,126,178,179]. The utility of YD-3 as a PAR4 antagonist has been demonstrated in *ex vivo* platelet assays[123,126] as well as an *in vivo* mouse model of angiogeneisis[124]. The published synthetic route of YD-3 is lengthy, 9 steps beginning from cyclohexanone[123]. The inactive isomer (N^2 instead of N^1 of indazole becomes benzylated) comprises at least 20% of the final yield prohibiting an efficacious synthesis. A primary goal of a novel synthetic route was deletion of the N^2 indazole nitrogen and replacement with an indole or azaindole, effectively eliminating the formation of the inactive regioisomer. In parallel, efforts were made to survey substituted aryl/heteroaryl moieties in multiple regions of YD-3, while also exploring replacements for, and the necessity of, the ethyl ester, a potential labile moiety. In order to rapidly determine structure activity relationships for larger libraries of analogs, we also developed a high throughput purified platelet Ca^{2+} assay to measure PAR4 mediated activation of platelets. There remains improvement not only in the synthesis of YD-3 but also in the physiochemical properties of the molecule.

Materials and Methods

Reagents

Purified compounds were dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mM and stored at -20 ºC until used. PAR1 activating peptide (PAR1-AP, SFLLRN) and PAR4 activating peptide (PAR4-AP, GYPGKF) were purchased from GL Biochem (Shanghai, China). NUNC 384 well plate black optical bottom was from Thermo (Rochester, NY, USA). Fluo4-AM was purchased from Invitrogen (Eugene, Oregon, USA). Fluorescein isothiocyanate (FITC) conjugated PAC1 and photoerythrin (PE) conjugated anti-CD62P (P-selectin) antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Platelet Preparation

Human platelets were obtained from healthy volunteers in accordance with and approved by the Vanderbilt University Institutional Review Board (050182). Written informed consent was obtained from all individuals. Platelets were prepared via standard washed platelet protocol as previously described[117,118]. Briefly, blood from healthy volunteers (averaging 30 ± 6.6 years of age and comprised of 53% males and 47% females) was drawn into syringes containing 3.2% sodium citrate. Platelet rich plasma was prepared by centrifugation in a Forma 400 ML GP centrifuge at 1100 rpm for 15 minutes. 10X acid citrate dextrose was added to platelet rich plasma and centrifuged at 2400 rpm for 10 minutes. The supernatant was aspirated and the platelet pellet was suspended in Tyrode's buffer containing 0.1% Bovine Serum Albumin fraction V (BSA) and counted on a Beckman Z1 Coulter particle counter (Brea, CA). Platelets were used at either 2.0×10^8 or 0.15×10^8 cells/mL for the two assays.

High-throughput platelet calcium assay

Washed human platelets were prepared via standard procedure and suspended in Tyrode's buffer containing 0.1% BSA. Platelets were dye loaded for 1 hour with Fluo4-AM in calcium assay buffer (1X HBSS without calcium or magnesium, 20 mM HEPES, 2.5 mM probenecid, 1 mM EGTA, 0.1% BSA). The calcium assay buffer containing dye is mixed with platelets to yield a final concentration of 2.5 µg/mL Fluo4-AM and $1.0x10^8$ platelets/mL. 60 µL of dye loaded platelets were added to each well of a NUNC 384 well plate black optical bottom plate (Thermo, Rochester, NY, USA). Fluorescence measurements were recorded on a Functional Drug Screening System (FDSS) 6000, Hamamatsu (Hamamatsu, Japan) at 37 °C. 10 µM of each compound was added 6 minutes prior to the addition of 80 µM PAR4-AP. Compounds were injected by the FDSS and occurred simultaneously across each plate. Experiments reported were performed in triplicate, on the same plate, from the indicated number of donors. 480:540 (ex:em) was measured each second for a total of 12 minutes. The final concentration of DMSO in the assay was 0.5%. The difference between basal (defined as t_0) and maximal relative fluorescence unit (RFU) values (ΔRFUs) for ex:em 480:540 was determined for each well. The triplicate ΔRFU for DMSO treated control wells stimulated with 80 µM PAR4-AP was averaged and the average valueΔ_c was set to 100% max response for each plate. Each well was subsequently normalized as a percent of theΔ_C control (value/Δ_C x 100). Normalized data was plotted in GraphPad PRISM v.5.0.

Flow cytometry

Briefly, 60 µL of washed platelets at a concentration of $1.5x10^7$ platelets/mL were added to polystyrene tubes. Anti-CD62P antibody or PAC-1 antibody were diluted (per manufacturer protocol) in Tyrode's buffer containing 0.1% BSA. 40 µL of diluted antibody was added to the platelets and allowed to bind for 5 minutes. Platelets were pre-treated

with indicated concentrations of antagonist or DMSO control for 5 minutes followed by addition of PAR1-AP or PAR4-AP for 10 minutes. Platelet activity was quenched by the addition ice cold 1.5% paraformaldehyde followed by dilution in 1X phosphate buffered saline. The final DMSO concentration was 0.5%. Platelets were stored up to 18 hours at 4 °C before flow cytometric analysis. Analysis was carried out on a BD FACS Canto II (Franklin Lakes, NJ, USA). Fluorescent intensity was determined for 100,000 events within the platelet gate. Data was collected and analyzed via FACS DiVa software. 100% max response was determined for each individual as the DMSO treated control stimulated with either 200 μM PAR4-AP, 20 μM PAR1-AP, or 10 nM thrombin. Samples treated with antagonist were normalized as a percentage of the DMSO treated control. Normalized data was plotted in GraphPad PRISM v.5.0. Dose response curves and subsequent IC_{50} values were generated using the 'log(inhibitor) vs. response variable slope' parameter. Data plotted is mean±S.E.M. or mean±S.D.

Aggregation

Briefly, washed human platelets were prepared to a final concentration of 2.0×10^8 platelets/mL in Tyrode's buffer containing 0.1% BSA. Compounds or DMSO control were added 10 minutes prior to stimulation with either PAR1-AP or PAR4-AP. The final DMSO concentration was 0.1%. Light transmittance was recorded by a Chrono-Log Model 700 Aggregometer. 100% light transmission signifies full platelet aggregation.

Results

Synthetic scheme for YD-3 and novel compounds

Although the novel synthetic route allowed for rapid generation of YD-3, the route was again subject to formation of an inactive isomer of YD-3 previously described

(compound 31 in Chen et al)[123]. Initially, planning to employ commercially available 3-bromoindoles and azaindoles, for a rapid two-step route to access analogs of YD-3, it was discovered that the 3-bromo analogs proved to be unstable to the reaction conditions. Thus, a multi-dimensional iterative parallel synthesis approach was employed and an expedient three-step approach was developed (Figure 1), beginning with indoles and azaindoles, which were *N*-alklyated with various commercially available benzyl and heteroarylmethyl bromides[180,181]. A selective bromination in the 3-position and a subsequent microwave-assisted Suzuki coupling between brominated indoles and heteroaryl boronic acids (Figure 1) delivered YD-3 analogs (APPENDIX B)[182 183].
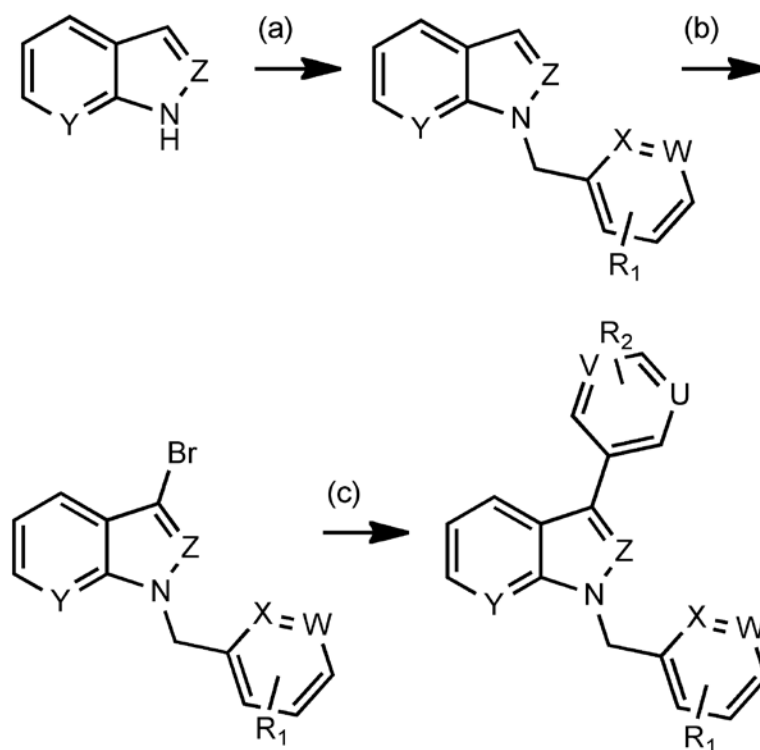
Figure 1. Synthetic scheme for YD-3 and novel compounds. Reagents and conditions. (a) 1.2 equiv. Ar (Het)CH$_2$Br, 1.2 equiv. NaH, rt, 40 min, 39-65%; (b) 1.1 equiv. NBS, 4 °C, 16h, 76-90%; (c) 10 mol% Pd$_2$(dba)$_3$, 20 mol% PCy$_3$, 1.1 equiv. Ar(Het)B(OH)$_2$ or Ar(Het)B(OR)$_2$, 1.7 equiv. K$_3$PO$_4$, dioxane/H$_2$O, mw, 120 °C, 30 min, 17-56%. U,V,W,X,Y,Z = CH or N. YD-3: U,V,W,X,Y= CH, Z=N, R$_2$=CH$_2$COOCH$_2$CH$_3$

High throughput screen identifies novel indole derived PAR4 antagonists

Protease activated receptor-4 is coupled to G_q and stimulates intracellular calcium mobilization in platelets via the dense tubular system as well as lysosome-related acidic calcium stores[35,36]. Measurement of intracellular calcium mobilization is a common, cost effective practice used to screen for agonists and antagonists of G_q coupled GPCRs. Extracellular calcium was withheld in the calcium assay for consistency with aggregation and flow cytometry assays, which are also carried out in calcium free buffers. The use of a low concentration of PAR4-AP made it possible to identify low affinity competitive inhibitors to better inform future structure activity relationship (SAR) studies. The assay was validated as an acceptable high throughput screening assay using the methods of Zhang et al[155]. The average Z-factor from two individuals is 0.33 ± 0.3 (mean±S.D.) demonstrating the assay is sufficient for determining a 'hit' (Figure 2A). An inhibition time course was performed where samples were treated for increasing amounts of time with 10 μM YD-3 and subsequently stimulated with 80 μM PAR4-AP. YD-3 significantly inhibited PAR4-AP as early as 1 minute and longer treatment times yielded more inhibition (Figure 2B). However, 6 and 12 minutes of YD-3 treatment did not give significantly different levels of inhibition, thus the 6 minute treatment time was selected in the interest of shorter total assay time (Figure 2B). Figure 2C illustrates the kinetics of calcium mobilization for platelets stimulated with 80 μM PAR4-AP and how YD-3 can slow the mobilization of calcium as well as diminish the maximum change in fluorescence (ΔRFU). Using the high-throughput calcium mobilization assay and YD-3 as a control the analogues that were effective at inhibiting a submaximal concentration of PAR4 agonist peptide (PAR4-AP) at 10 μM (Figure 2D) was determined. Similar to the SAR reported previously that led to the discovery of YD-3, SAR was very shallow, with few analogs displaying activity comparable to YD-3. From 3 rounds of synthesis we produced 38 compounds of which 1, 3, 5, VU0469152, VU046154, VU0469155,

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VU0469907, VU0476680, VU0476683, VU0476684, VU0476686, and VU0476689 were capable of inhibiting PAR4 mediated platelet calcium mobilization to less than 50% of the maximum response (Figure 2D), indicating that indole was a suitable replacement for the indazole core. Of note, the corresponding carboxylic acid of the terminal ethyl ester, 2, was inactive as were other ester replacements including 6, and VU0469133-912 (APPENDIX B). Of the compounds tested only 1, 3, and 5 were able to fully antagonize a maximal PAR4-AP response (Figure 3). Other 'hits' from the screening process did not fully antagonize PAR4 mediated $\alpha_{IIb}\beta3$ activation even at 10 µM concentration. The addition of chlorine in the 3 position of YD-3 was shown to give modest improvement in the potency of YD-3 for PAR4. Because of the lack of a terminal ester, attempts were made to improve upon the most potent partial antagonist VU0469155, a methoxy ester derivative, by adding a chlorine in the meta position of the benzyl group (VU047689). There was a modest improvement in the potency against PAR4 but not enough to warrant further investigation.

Potency and selectivity of lead compounds

In human platelets, in addition to receptor mediated calcium mobilization via coupling to G_q, PAR stimulation converts the fibrin receptor $\alpha_{IIb}\beta3$ to its high affinity fibrin binding conformation, and triggers granule secretion containing P-selectin molecules (CD62P). The PAC-1 antibody binds only the high affinity conformation of $\alpha_{IIb}\beta3$ and is an accurate readout for $\alpha_{IIb}\beta3$ activation. Potency of full antagonists was measured against both PAR1 and PAR4 mediated $\alpha_{IIb}\beta3$ activation (via PAC-1 binding) and P-selectin expression (anti-CD62p binding) using flow cytometry.
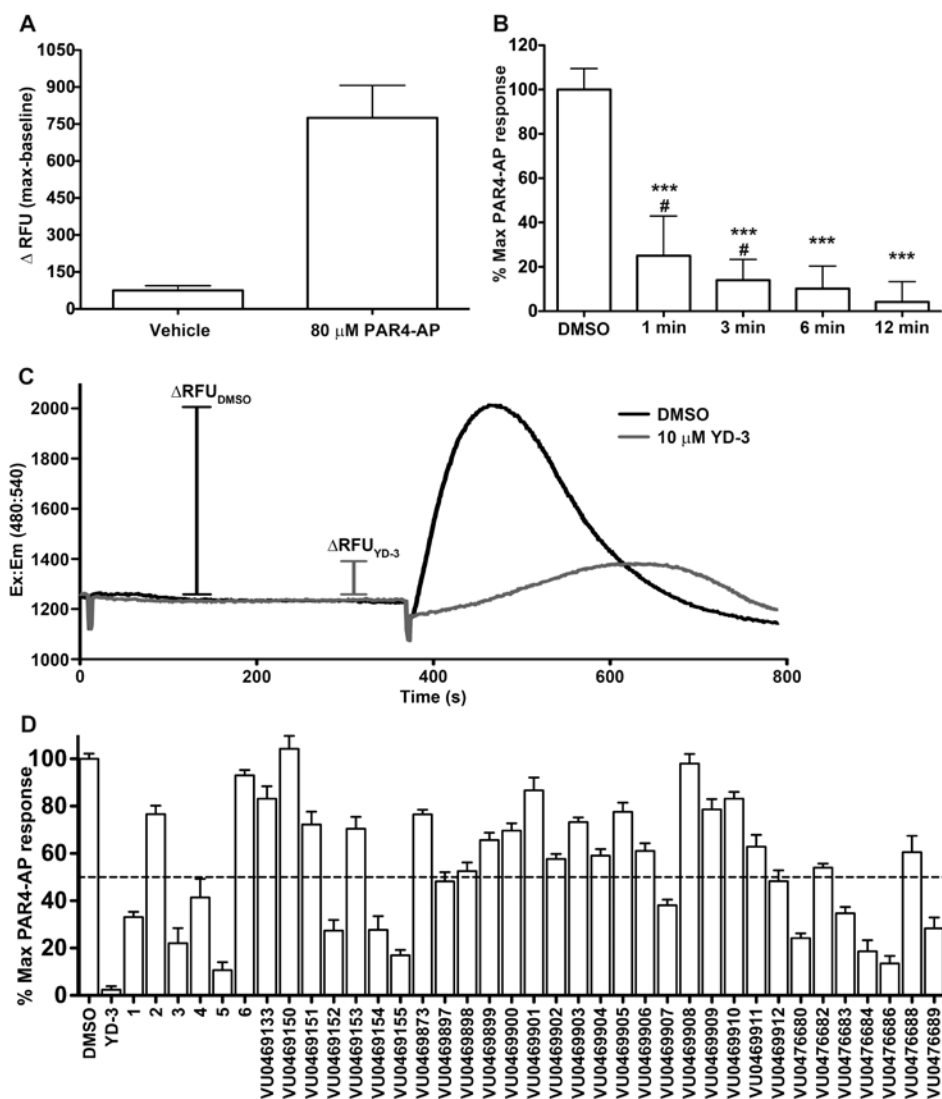
Figure 2. High throughput platelet calcium mobilization identifies novel indole derived PAR4 antagonists. A. To determine the Z-factor, and thus the usefulness of the assay as a screening tool, platelets were treated with either vehicle (calcium assay buffer) or 80 µM PAR4-AP in checkerboard fashion in a 384 well plate. Representative data from 1 donor is displayed as mean ± S.D. using 192 replicates of each condition in a single plate. Z-factor of the represented sample is 0.35 and a second volunteer (data not shown) was 0.31. B. Platelets were treated with 10 µM YD-3 for the indicated period of time followed by stimulation with 80 µM PAR4-AP. Data represented is an n of 2 volunteers, mean ± S.D. performed in triplicate. Means are significantly different where indicated. Unpaired one-tailed t-test: time points versus DMSO treated control ***p< 0.0001. Unpaired one-tailed t-test: time points versus 12 minute time point #p<0.05. C. Representative tracings of platelets treated with DMSO or 10 µM YD-3 for 6 minutes followed by stimulation with 80 µM PAR4-AP. D. Compounds were screened by treating platelets with 10 µM compound or DMSO control followed by challenge with 80 µM PAR4-AP. Data represented as percent 80 µM PAR4 response n of 2 volunteers mean±S.D. Compounds that displayed greater than 50% inhibitory activity (below dashed line) were subject to further investigation.
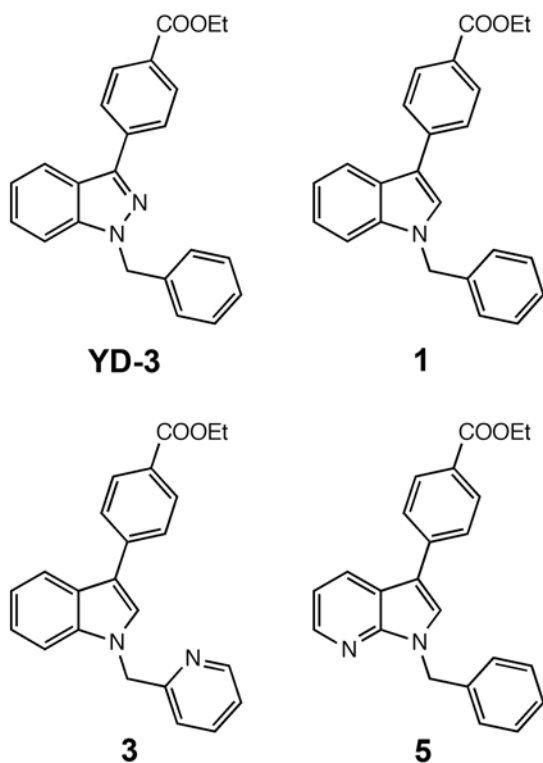
Figure 3. Structures of full antagonists for PAR4: YD-3, 1, 3, and 5.

There was not any notable functional selectivity between the molecules ability to inhibit αIIbβ3 activation (Figure 3, black) and P-selectin expression (Figure 3, grey). 1 (Figure 4D) was less potent than YD-3 (Figure 4B, Figure S4) at inhibiting PAR4 mediated responses demonstrating IC_{50} values for PAR4 mediated $\alpha_{IIb}\beta3$ activation of 66 ± 1 nM versus 26 ± 1 nM respectively. However, YD-3 inhibited PAR1-AP mediated $\alpha_{IIb}\beta3$ activation by 55% at 10 μM, a previously undocumented observation (Figure 4A). Interestingly, 1 reduced maximum PAR1-AP $\alpha_{IIb}\beta3$ response by 38% (Figure 4C) and 3 and 5 inhibited PAR1 by 31% (Figure 4E) 56% (Figure 4F) respectively. Together these data suggest that addition of nitrogen into the indole core may contribute to off target effects against PAR1. Both 3 and 5 were significantly less potent then either YD-3 or 1 with IC_{50}'s for PAR4 mediated $\alpha_{IIb}\beta3$ activation of 1.0 ± 1.1 μM and 170 ± 1 nM

respectively (Figure 4F, 4H). These data suggest that indole can serve as a highly

selective PAR4 inhibitor with only modest loss of potency compared to YD-3.
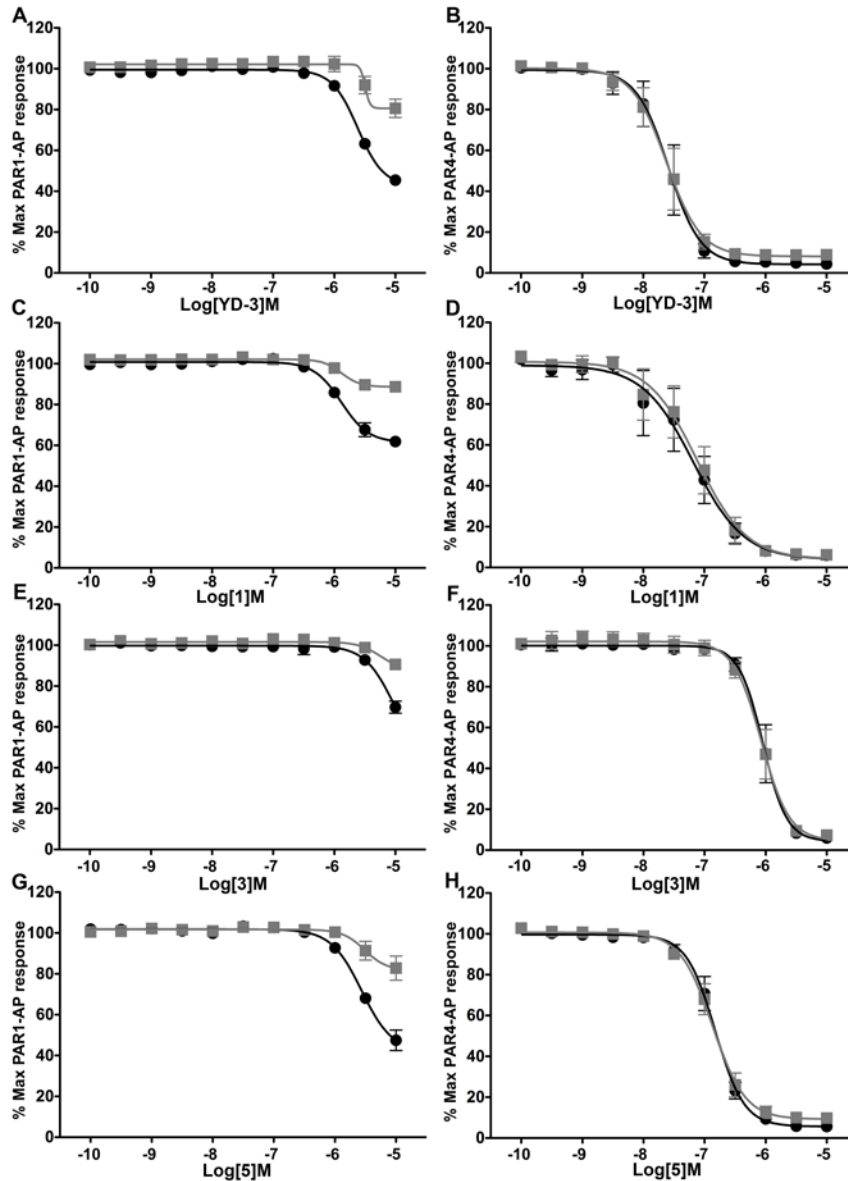
Figure 4. 1, 3, and 5 are full PAR4 antagonists. 1, 3, and 5 were compared with YD-3 potency against PAR1 and PAR4 mediated $\alpha_{IIb}\beta3$ activation and P-selectin expression. Platelets were treated with compound or DMSO control for 5 minutes prior to stimulation with 20 µM PAR1-AP (left panels) or 200 µM PAR4-AP (right panels) for 10 minutes. $\alpha_{IIb}\beta3$ activation (black) and P-selectin expression (grey) in PAR activated platelets by flow cytometry. The order of potency for PAR4 antagonism is YD-3>1>5>3. Values are displayed as mean±S.E.M. n of 3 volunteers performed in singlicate.

The PAR4 partial antagonists: VU0469152, VU046154, VU0469155, VU0469907, VU0476680, VU0476683, VU0476684, VU0476686, and VU0476689, displayed a similar magnitude of inhibition against PAR1 mediated platelet activation as YD-3, 1, 3, and 5 (Figure 5) however, because these compounds did not fully antagonize PAR4 mediated platelet responses even at 10 µM, the additional loss of selectivity indicates poor lead compounds.
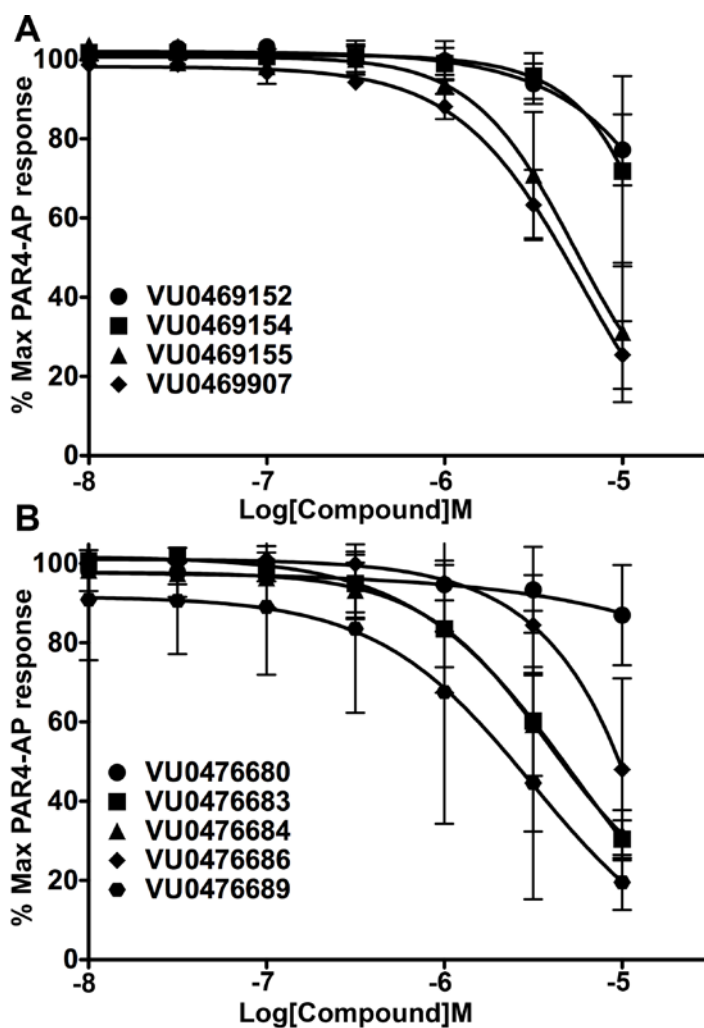
Figure 5. Several compounds act as partial antagonists of PAR4. Platelets were treated with indicated concentrations of compound for 5 minutes prior to stimulation with 200 µM PAR4-AP. $\alpha_{IIb}\beta 3$ activation was measured via flow cytometric analysis of PAC1 binding. Hits from the second round (A) and third round (B) of optimization are shown. Data is presented as mean±S.D. n of 2 volunteers.

Potency of compounds against thrombin mediated platelet activation

Inhibition of the PAR4-AP does not completely describe the potency of any PAR4 antagonist. Compounds should elicit an inhibitory effect against thrombin either alone or in combination with a PAR1 antagonist, such as RWJ-56110[173]. Against a high dose of thrombin where both PAR1 and PAR4 are engaged, PAR4 antagonists alone cannot significantly inhibit thrombin mediated $\alpha_{IIb}\beta3$ activation [171]. The ability of 1 µM YD-3, 1µM 1, 3.16 µM 3, and 1 µM 5 to inhibit thrombin mediated $\alpha_{IIb}\beta3$ activation at concentrations where each compound previously demonstrated complete inhibition of PAR4 mediated $\alpha_{IIb}\beta3$ activation and low inhibition for PAR1 mediated $\alpha_{IIb}\beta3$ activation was subsequently tested. YD-3, 1, 3, and 5 were able to reduce PAR1 mediated $\alpha_{IIb}\beta3$ activation by 8.4 ± 2.4%, 14.1 ± 2.8%, 7.2 ± 1.3%, and 7.2 ± 1.4% respectively when used at these lower concentrations (Figure 4A, C, E, G). 10 µM RWJ-56110 alone was able to significantly inhibit thrombin mediated $\alpha_{IIb}\beta3$ activation by 18.4 ± 5.4% and when used in combination with either YD-3 or 1, was able to reduce thrombin mediated signaling by 42.9 ± 9.7% and 37.1 ± 6.4% respectively (Figure 6). RWJ and YD-3 demonstrated dual inhibition of PAR1 and PAR4 against thrombin since the experimentally determined reduction in thrombin mediated $\alpha_{IIb}\beta3$ activation by the combination of RWJ and YD-3 (42.9 ± 9.7%) was greater than the addition of the effect of RWJ-56110 on thrombin and the effect of YD-3 on PAR1-AP (26.8 ± 6.0%). Compound 1 in conjunction with RWJ-56110 also slightly inhibited thrombin mediated $\alpha_{IIb}\beta3$ activation to a greater extent than addition of the inhibitory effects of each alone (37.1 ± 6.4% compared to 32.5 ± 6.1%). Neither 3 (14 ± 2% reduction) nor 5 (17 ± 2% reduction) when added in conjunction with RWJ-56110 were able to inhibit thrombin mediated platelet $\alpha_{IIb}\beta3$ activation to a greater magnitude than RWJ-56110 alone. These data suggest that even slight losses of antagonist potency against PAR4-AP responses translates into dramatic reductions in potency against thrombin. Additionally, these data suggest that for platelets stimulated

with high concentrations of thrombin, the contribution of PAR1 signaling to αIIbβ3 activation is greater than PAR4.
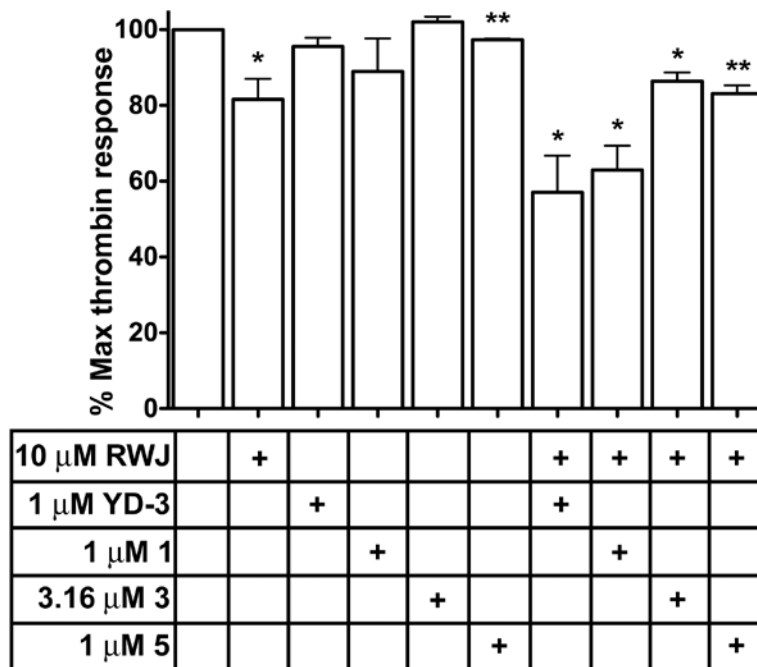
Figure 6. Dual PAR1/PAR4 inhibition significantly inhibits thrombin mediated platelet activation. Platelets were treated with PAR1 antagonist RWJ-56110 or PAR4 full antagonists either alone or in combination prior to stimulation with 10 nM thrombin for 10 minutes. $\alpha_{IIb}\beta3$ activation was measured, data reported is mean±S.E.M. n of 3 volunteers. One sample t-test for significant deviation from DMSO control p-values *$p<0.05$, **$p<0.005$.

Condition	1	2	3	4	5	6	7	8	9	10
10 μM RWJ		+					+	+	+	+
1 μM YD-3			+				+			
1 μM 1				+				+		
3.16 μM 3					+				+	
1 μM 5						+				+

Lead compounds inhibit PAR-AP mediated aggregation

The activities of the full antagonists YD-3, 1, 3, and 5, were assessed via classical measurement of platelet activity, platelet aggregation. We demonstrate using YD-3 as a control, 1, and 5 are able to significantly inhibit PAR4 but not PAR1 mediated platelet aggregation in healthy subjects (Figure 7). Compound 3 also inhibits PAR4 mediated platelet aggregation versus PAR1 mediated platelet aggregation though non-significantly (p=0.054).
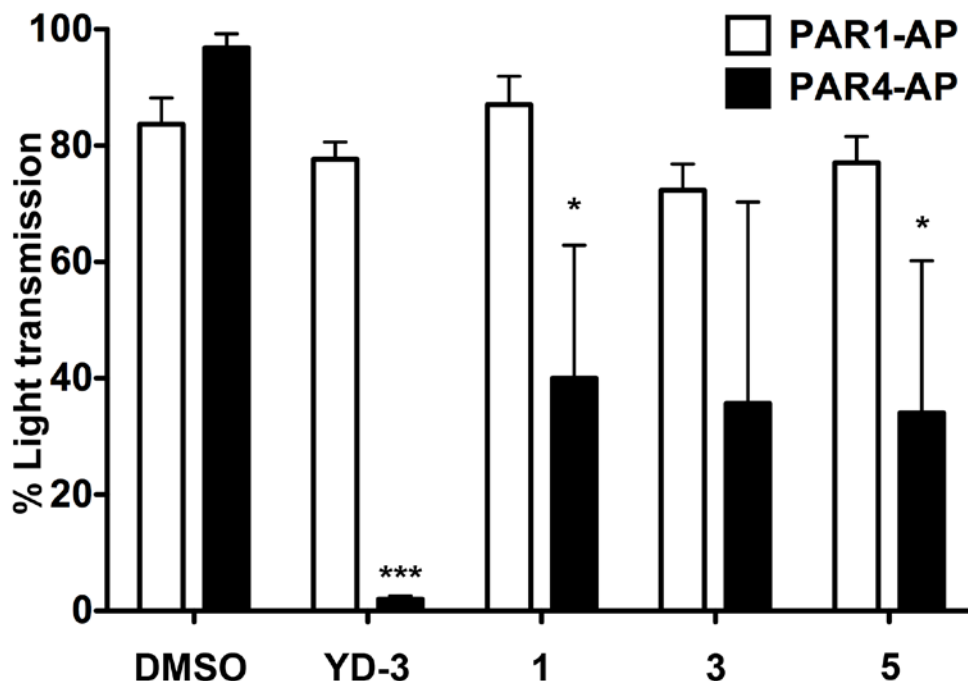
Figure 7. Full PAR4 antagonists are selective for PAR4 mediated platelet aggregation. Platelets were treated with YD-3, 1, 3 or 5 for 10 minutes prior to stimulation with either 20 µM PAR1-AP (white bars) or 200 µM PAR4-AP (black bars). Data reported as % light transmittance where 100% represents fully aggregated platelets. Unpaired two-tailed t-test versus DMSO treated agonist control. Data displayed as mean±S.E.M n of 3 or more. Means are significantly different where indicated. *** $p<0.0001$, * $p<0.05$.

Mechanism of action of YD-3 and compound 1

To examine the putative mechanism of action of the most potent antagonists, YD-3 and 1, PAR4-AP mediated P-selectin cell surface expression was measured to generate highly descriptive twelve point CRCs in the presence of increasing concentrations of antagonist. YD-3 is previously described as competitive in nature by platelet aggregation[126]. This finding was confirmed by P-selectin expression. Figure 8 shows parallel rightward shifts in the PAR4 CRC in response to increasing concentrations of compound, indicative of a competitive mechanism of action for both YD-3 (Figure 8A) and 1 (Figure 8B).

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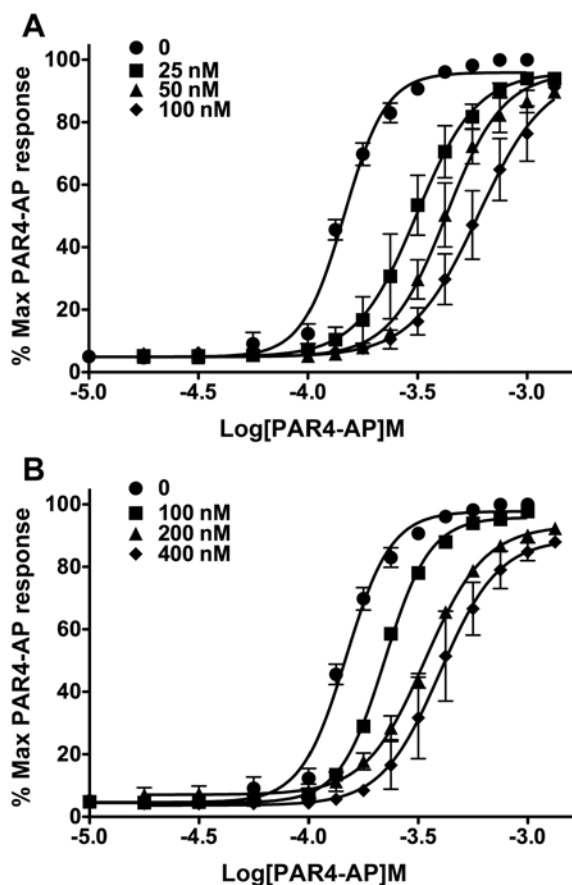
Figure 8. YD-3 and 1 act as competitive antagonists against PAR4. Platelets were treated with indicated concentrations of A) YD-3 or B) 1 for 5 minutes prior to stimulation with a full PAR4-AP concentration response curve. P-selectin was measured via flow cytometry. Data represented as mean±S.E.M n of 3.

Summary and Discussion

These data indicate that indole is a suitable scaffold for selective inhibitory activity against PAR4 mediated platelet activation and thus serve as a novel scaffold with which to build future PAR4 antagonists. Given the large number of commercially available derivatives of indole, bromides, aryl boronic acids and boronic esters, the synthetic routes presented are capable of rapidly generating numerous compounds. The use of a high throughput purified platelet calcium mobilization assay allows for rapid identification of molecules with inhibitory activity towards PAR4. Compound 1 retains

antagonist activity against both the PAR4 peptide as well as thrombin and as such is the most potent novel compound presented here. The other scaffolds 3 (2-pyridine) and 5 (7-azaindole), retained selectivity for PAR4 versus PAR1 but sacrificed potency against PAR4 activating peptide and most importantly thrombin. Both YD-3 and, to a lesser extent, 1 display weak off-target effects against PAR1. The inability of compounds 1, 3, and 5 to fully inhibit PAR4 mediated platelet aggregation is reflective not of partial inhibition of the response but rather that a couple individuals fully aggregated (though slowly) in the presence of 1, 3, or 5. This phenomenon is likely reflective of the loss of affinity of these compounds for PAR4. The apparent discrepancy of YD-3 selectivity for PAR4 versus PAR1 between platelet aggregation assays and flow cytometric analysis ($\alpha_{IIb}\beta3$ activation) can be at least partially explained by signaling convergence. PAR mediated aggregation is dependent on $\alpha_{IIb}\beta3$ activation; however, ADP signaling, calcium mobilization, and granule secretion also play a significant role[117]. Additionally, there is evidence that thrombin mediated platelet aggregation, particularly PAR1-AP stimulated platelet aggregation, is resistant to $\alpha_{IIb}\beta3$ blockade [184,185]. Gawaz et al. demonstrate using direct $\alpha_{IIb}\beta3$ blockade that approximately 50% of total $\alpha_{IIb}\beta3$ receptor is sufficient for TRAP (PAR1-AP) to elicit 80% of maximum TRAP mediated aggregation. Thus, inhibition of 55% of PAR1-AP mediated $\alpha_{IIb}\beta3$ activation may not be enough to inhibit PAR1 mediated platelet aggregation[184].

Thrombin mediated $\alpha_{IIb}\beta3$ activation was not abolished even when both antagonists were added prior to stimulation. One possible explanation is that thrombin binds GPIb receptors, which may contribute to signaling[186]. Of note, other than YD-3, PAR4 antagonists did not have a large effect against thrombin when putative PAR1 off target effects were accounted for. A probable rationale for this observation is that thrombin activated protease activated receptors create tethered ligands which are essentially irreversible agonists such that any orthosteric molecule must be either

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irreversible, or have such high affinity that the displacement of the tethered ligand is possible. Alternatively, one could envision that negative allosteric modulators may prove to be a better approach to inhibit thrombin activated PARs. Though we have identified indole as a favorable scaffold for future production of PAR4 antagonists, there is considerable room for improvement for antagonists of PAR4 and as such, efforts are underway to discover higher potency compounds.

CHAPTER VI

SYNOPSIS

Targeting thrombin receptors

The targeting of thrombin receptors, PAR1 and PAR4, for therapeutic intervention truly represents interesting challenges for drug discovery and design. First, the irreversible activation by thrombin will require antagonists be very high affinity (likely in the form of low dissociation) to be efficacious, or like Vorapaxar, bind irreversibly to the receptor. An alternative strategy, particularly for PAR1 antagonism, may be functional antagonism. Since PAR1 when activated is rapidly sorted to lysosomes for degradation and is not recycled to the cell surface, a high affinity, low efficacy partial agonist would serve as a functional antagonist, as long as the agonist shifted the receptor into a state that induced phosphorylation. For either PAR1 or PAR4 an ideal strategy for receptor inhibition is allosteric modulation such that instead of attempting to compete for tethered ligand binding, the negative allosteric modulator induces a receptor conformation which precludes tethered ligand binding, enhances the tethered ligand off rate, reduces the tethered ligand on-rate or reduces the receptor- G-protein coupling efficiency of tethered agonist induced activation. Furthermore, since irreversible PAR1 inhibition has been shown to be relatively safe in humans, antibody based therapeutics may be considered. Antibodies directed towards the hirudin-like binding domain on PAR1 or spanning the cleavage site of either receptor would provide the most efficacious antibodies. Off-target issues with prolonged PAR1 antagonism are likely to arise since PAR1 expression is ubiquitous, for instance murine models demonstrate the necessity of functional PAR1 for embryonic development. The restricted expression

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profile of PAR4 however, is amenable to irreversible or long-term antagonist interventions.

The feasibility for discovering each class of inhibitors presents a separate challenge and in the case of PAR4 is presently impossible due to lack of sufficient tool compounds. High-throughput functional screens can identify antagonists, negative allosteric modulators, and partial agonists. Partial agonist leads can be counter screened to ensure selectivity and that they induce receptor phosphorylation. Subsequent medicinal chemistry efforts could improve the affinity of inhibitors or partial agonist compounds and, in the case of PAR1, the mechanism can be described as competitive or non-competitive for ligand binding, and affinity could be determined. PAR1 drug discovery efforts are aided by the availability of a radiolabeled high affinity agonist peptide, whereas PAR4 lacks this tool. At present YD-3 and compound 1 are described as competitive only by function, not ligand binding, therefore these compounds may not be acting at the level of the receptor which may manifest as off target effects once administered *in vivo*. Furthermore, the lack of a radioligand probe, particularly antagonist probe, for PAR4 reduces the amount of pharmacological information that can be gathered. With a suitable probe, PAR4 inhibitors could be described as competitive or non-competitive at the receptor. Furthermore, PAR4 expression levels on platelets, cancer cells, and in murine models could be accurately described. While the understanding of PAR1 in human physiology and disease is burgeoning, it is imperative that an acceptable PAR4 antagonist probe be developed so that understanding of the receptor and its contribution to thrombosis and hemostasis is better understood.

Progress towards the specific aims

We made considerable progress on the specific aims as outlined in Chapter I. We successfully synthesized YD-3 by two distinct routes having either two or three

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steps. By converting to the indole scaffold thus eliminating the nitrogen in the 2 position of the indole ring, we effectively eliminated the formation of the inactive isomer of YD-3. We synthesized a library of over 30 compounds, screened them in a high-throughput calcium assay, and characterized potency by measurement of both integrin $\alpha_{IIb}\beta3$ activation and P-selectin secretion. Although none of the compounds were more potent than YD-3, valuable SAR data about the pharmacophore was gathered. The indole ring tolerates nitrogen substitutions, while the ethyl ester seems required for low nanomolar potency. Although we could not use radioligands to determine the mechanism of action of our novel compounds, we performed Schild analysis in an effort to classify molecules as competitive or non-competitive. Both YD-3 and lead Compound 1 displayed competitive behavior against the PAR4-AP. However, allosteric compounds or compounds which work downstream of the receptor may also appear competitive in functional assays, thus the mechanism of action of PAR4 antagonists remains undefined. Due to the compounds lack of favorable pharmacokinetic profiles, synthesized compounds were not subject to testing in *ex vivo* models of thrombosis.

APPENDIX A

NMR SPECTROSCOPY
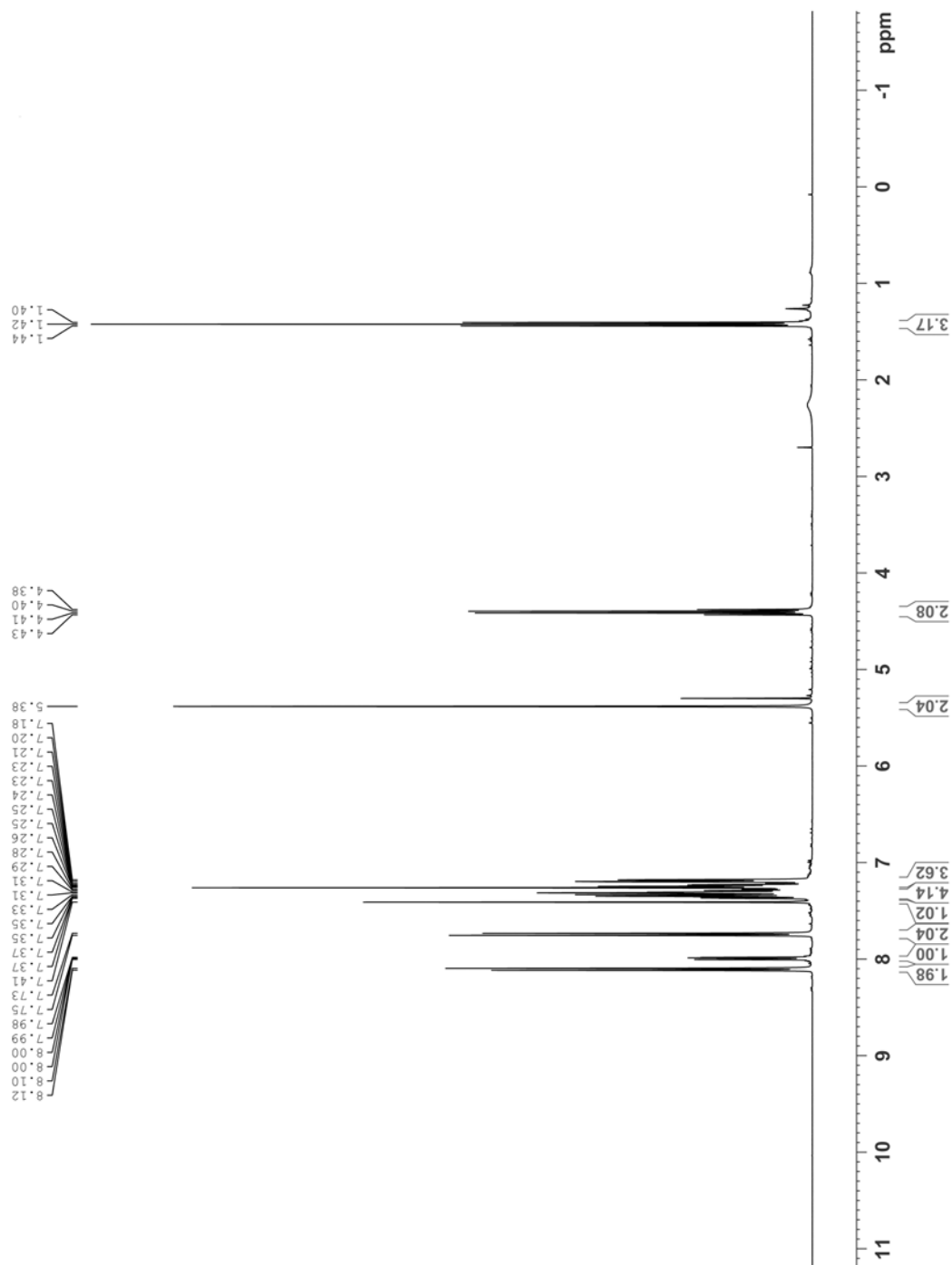
APPENDIX A.1 ^{1}H NMR spectra for Compound 1 400 MHz

1.40
1.42
1.44

4.38
4.40
4.41
4.43

5.38
7.18
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8.00
8.10
8.12

3.17

2.08

2.04

3.62
4.14
1.02
2.04
1.00
1.98
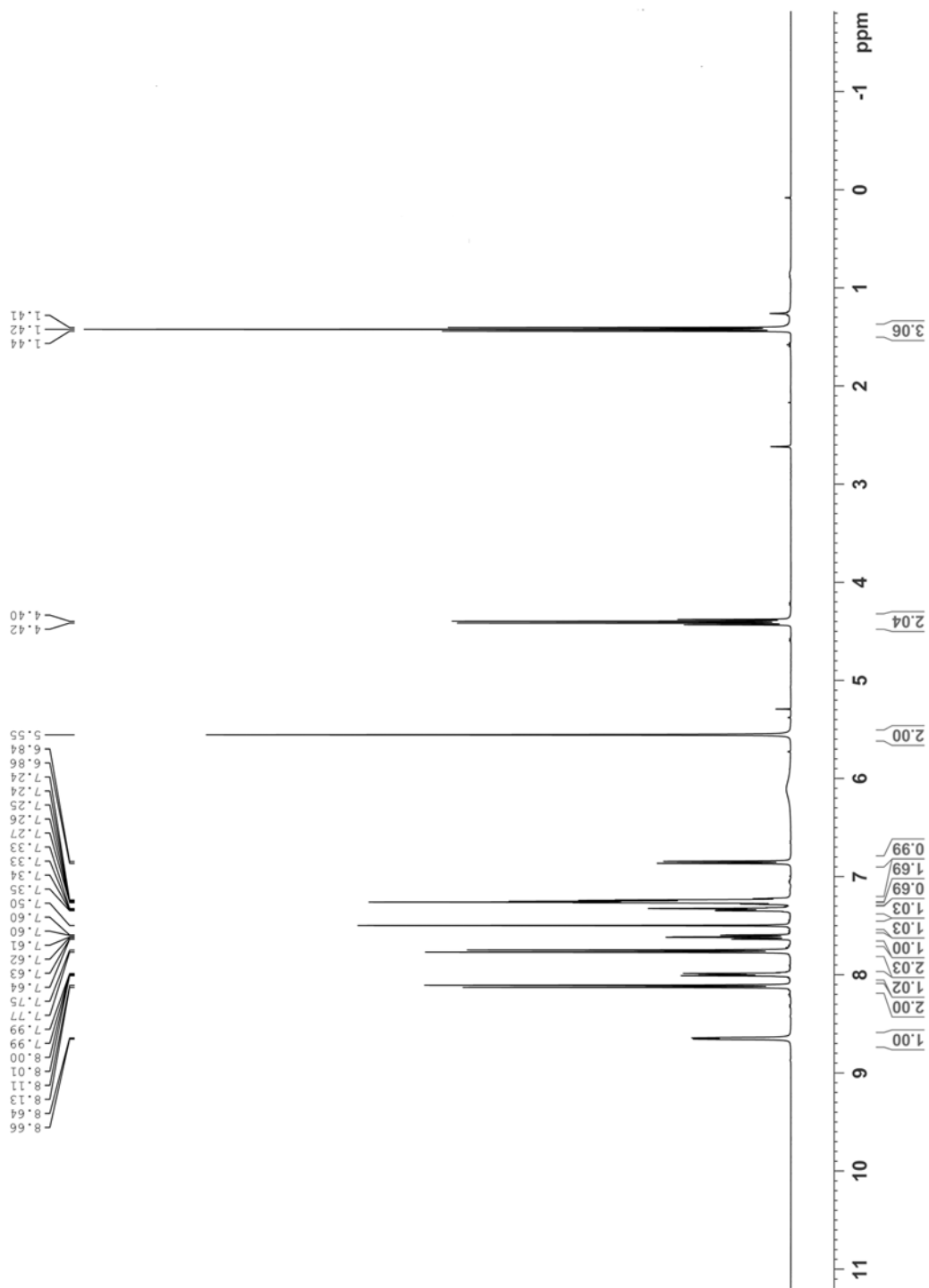
APPENDIX A.2 ¹H NMR spectra of Compound 3 400 MHz
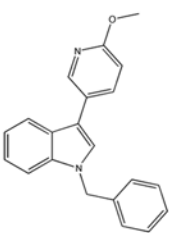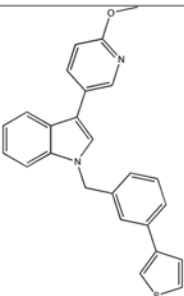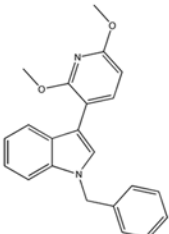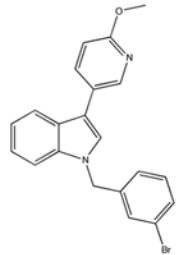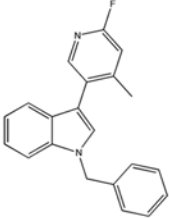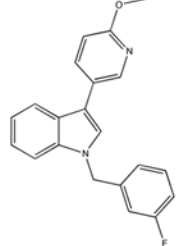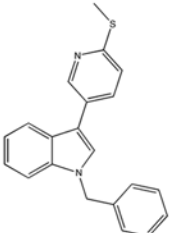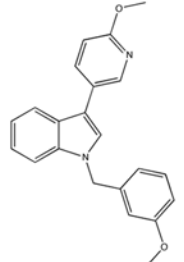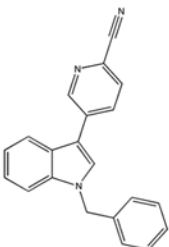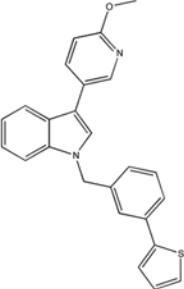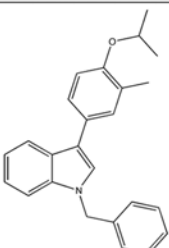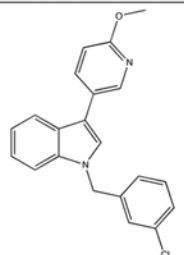
APPENDIX A.3 ^{1}H NMR spectra of Compound 5 400 MHz
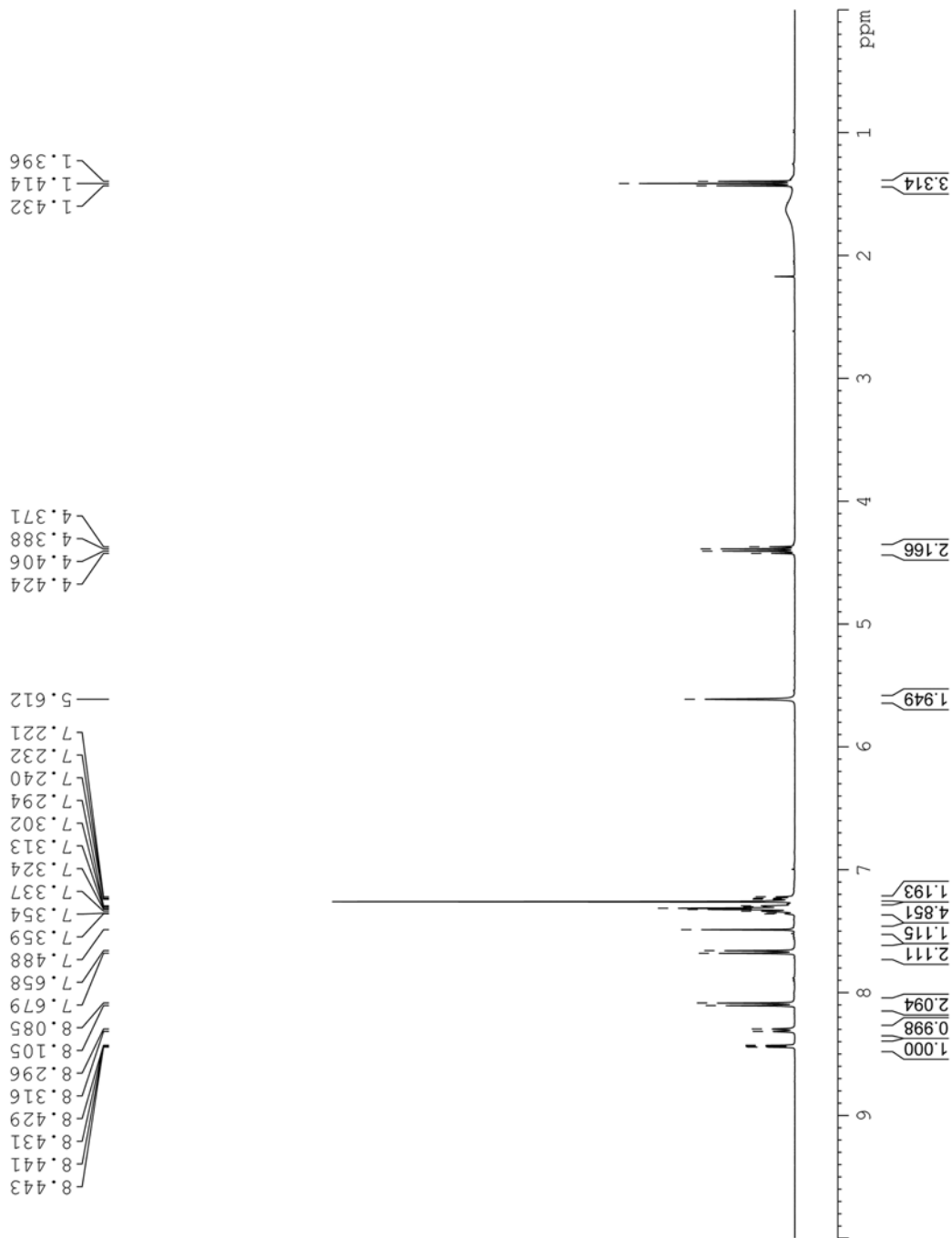
APPENDIX B

NAMES AND STRUCTURES OF COMPOUNDS

Compound	Structure	Compound	Structure
YD-3		VU0469901	
1		VU0469902	
2		VU0469903	
3		VU0469904	
4		VU0469905	
5		VU0469906	
6		VU0469907	

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Compound	Structure	Compound	Structure
VU0469133		VU0469908	
VU0469150		VU0469909	
VU0469151		VU0469910	
VU0469152		VU0469911	
VU0469153		VU0469912	
VU0469154		VU0476680	

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Compound	Structure	Compound	Structure
VU0469155		VU0476682	
VU0469873		VU0476683	
VU0469897		VU0476684	
VU0469898		VU0476686	
VU0469899		VU0476688	
VU0469900		VU0476689	

BIBLIOGRAPHY

1   Esmon, C. & Jackson, C. The conversion of prothrombin to thrombin. IV. The function of the fragment 2 region during activation in the presence of factor V. *The Journal of biological chemistry* **249**, 7791-7797 (1974).

2   Esmon, C. & Jackson, C. The conversion of prothrombin to thrombin. III. The factor Xa-catalyzed activation of prothrombin. *The Journal of biological chemistry* **249**, 7782-7790 (1974).

3   Versteeg, H. H., Heemskerk, J. W., Levi, M. & Reitsma, P. H. New fundamentals in hemostasis. *Physiological reviews* **93**, 327-358, doi:10.1152/physrev.00016.2011 (2013).

4   Butkowski, R., Elion, J., Downing, M. & Mann, K. Primary structure of human prethrombin 2 and alpha-thrombin. *The Journal of biological chemistry* **252**, 4942-4957 (1977).

5   Mann, K., Elion, J., Butkowski, R., Downing, M. & Nesheim, M. Prothrombin. *Methods in enzymology* **80 Pt C**, 286-302, doi:10.1016/s0076-6879(81)80025-0 (1981).

6   Van Der Meijden, P. E. *et al.* Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. *J Thromb Haemost* **10**, 1355-1362, doi:10.1111/j.1538-7836.2012.04758.x (2012).

7   Odell, T. T., Jr. & Jackson, C. W. Polyploidy and maturation of rat megakaryocytes. *Blood* **32**, 102-110 (1968).

8   Zimmet, J. & Ravid, K. Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Experimental hematology* **28**, 3-16 (2000).

9   Schulze, H. *et al.* Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood* **107**, 3868-3875, doi:10.1182/blood-2005-07-2755 (2006).

10   Italiano, J. E., Jr., Lecine, P., Shivdasani, R. A. & Hartwig, J. H. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* **147**, 1299-1312 (1999).

11   Gerrard, J. M. *et al.* Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules. *J Clin Invest* **66**, 102-109, doi:10.1172/JCI109823 (1980).

12   Nurden, A. T. & Nurden, P. The gray platelet syndrome: clinical spectrum of the disease. *Blood reviews* **21**, 21-36, doi:10.1016/j.blre.2005.12.003 (2007).

13   Berneis, K. H., Da Prada, M. & Pletscher, A. Micelle formation between 5-hydroxytryptamine and adenosine triphosphate in platelet storage organelles. *Science* **165**, 913-914 (1969).

14   Holmsen, H. & Weiss, H. J. Secretable storage pools in platelets. *Annual review of medicine* **30**, 119-134, doi:10.1146/annurev.me.30.020179.001003 (1979).

15   Ruiz, F. A., Lea, C. R., Oldfield, E. & Docampo, R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J Biol Chem* **279**, 44250-44257, doi:10.1074/jbc.M406261200 (2004).

16   Offermanns, S., Toombs, C. F., Hu, Y. H. & Simon, M. I. Defective platelet activation in G alpha(q)-deficient mice. *Nature* **389**, 183-186, doi:10.1038/38284 (1997).

17   Murugappan, S., Tuluc, F., Dorsam, R. T., Shankar, H. & Kunapuli, S. P. Differential role of protein kinase C delta isoform in agonist-induced dense granule secretion in human platelets. *J Biol Chem* **279**, 2360-2367, doi:10.1074/jbc.M306960200 (2004).

18   Wang, D. *et al.* Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity* **13**, 25-35 (2000).

19   Walker, T. R. & Watson, S. P. Synergy between Ca2+ and protein kinase C is the major factor in determining the level of secretion from human platelets. *The Biochemical journal* **289 (Pt 1)**, 277-282 (1993).

20   Werner, M. H., Senzel, L., Bielawska, A., Khan, W. & Hannun, Y. A. Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Molecular pharmacology* **39**, 547-556 (1991).

21   Gerrard, J. M. *et al.* A role for protein kinase C in the membrane fusion necessary for platelet granule secretion. *Blood* **74**, 2405-2413 (1989).

22   Brass, L. F. & Joseph, S. K. A role for inositol triphosphate in intracellular Ca2+ mobilization and granule secretion in platelets. *J Biol Chem* **260**, 15172-15179 (1985).

23   O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. Inositol 1,4,5-trisphosphate releases Ca2+ from a Ca2+-transporting membrane vesicle fraction derived from human platelets. *J Biol Chem* **260**, 956-962 (1985).

24   White, J. G. Interaction of membrane systems in blood platelets. *The American journal of pathology* **66**, 295-312 (1972).

25   Gerrard, J. M., White, J. G., Rao, G. H. & Townsend, D. Localization of platelet prostaglandin production in the platelet dense tubular system. *The American journal of pathology* **83**, 283-298 (1976).

26   Cutler, L., Rodan, G. & Feinstein, M. B. Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. *Biochimica et biophysica acta* **542**, 357-371 (1978).

27   van Nispen Tot Pannerden, H. E., van Dijk, S. M., Du, V. & Heijnen, H. F. Platelet protein disulfide isomerase is localized in the dense tubular system and does not become surface expressed after activation. *Blood* **114**, 4738-4740, doi:10.1182/blood-2009-03-210450 (2009).

28   Chen, H. & Kahn, M. L. Reciprocal signaling by integrin and nonintegrin receptors during collagen activation of platelets. *Molecular and cellular biology* **23**, 4764-4777 (2003).

29   Inoue, O., Suzuki-Inoue, K., Dean, W. L., Frampton, J. & Watson, S. P. Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. *J Cell Biol* **160**, 769-780, doi:10.1083/jcb.200208043 (2003).

30   Briddon, S. J., Melford, S. K., Turner, M., Tybulewicz, V. & Watson, S. P. Collagen mediates changes in intracellular calcium in primary mouse megakaryocytes through syk-dependent and -independent pathways. *Blood* **93**, 3847-3855 (1999).

31   Achison, M. *et al.* Integrin-independent tyrosine phosphorylation of p125(fak) in human platelets stimulated by collagen. *J Biol Chem* **276**, 3167-3174, doi:10.1074/jbc.M007186200 (2001).

32   Polanowska-Grabowska, R., Geanacopoulos, M. & Gear, A. R. Platelet adhesion to collagen via the alpha 2 beta 1 integrin under arterial flow conditions causes rapid tyrosine phosphorylation of pp125FAK. *The Biochemical journal* **296 (Pt 3)**, 543-547 (1993).

33   Clements, J. L. *et al.* Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. *J Clin Invest* **103**, 19-25, doi:10.1172/JCI5317 (1999).

34   Hallam, T. J. & Rink, T. J. Responses to adenosine diphosphate in human platelets loaded with the fluorescent calcium indicator quin2. *The Journal of physiology* **368**, 131-146 (1985).

35   Cavallini, L., Coassin, M. & Alexandre, A. Two classes of agonist-sensitive Ca2+ stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. *The Biochemical journal* **310 (Pt 2)**, 449-452 (1995).

36   Jardin, I. *et al.* Differential involvement of thrombin receptors in Ca2+ release from two different intracellular stores in human platelets. *The Biochemical journal* **401**, 167-174, doi:10.1042/BJ20060888 (2007).

37   Braun, A. *et al.* Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood* **113**, 2056-2063, doi:10.1182/blood-2008-07-171611 (2009).

38   Macfarlane, D. E. & Mills, D. C. Inhibition by ADP of prostaglandin induced accumulation of cyclic AMP in intact human platelets. *Journal of cyclic nucleotide research* **7**, 1-11 (1981).

39   Waldo, G. L. & Harden, T. K. Agonist binding and Gq-stimulating activities of the purified human P2Y1 receptor. *Molecular pharmacology* **65**, 426-436, doi:10.1124/mol.65.2.426 (2004).

40   Hoffmeister, M. *et al.* Cyclic nucleotide-dependent protein kinases inhibit binding of 14-3-3 to the GTPase-activating protein Rap1GAP2 in platelets. *J Biol Chem* **283**, 2297-2306, doi:10.1074/jbc.M706825200 (2008).

41   McBride, P. A., Mann, J. J., Polley, M. J., Wiley, A. J. & Sweeney, J. A. Assessment of binding indices and physiological responsiveness of the 5-HT2 receptor on human platelets. *Life sciences* **40**, 1799-1809 (1987).

42   Shenker, A., Goldsmith, P., Unson, C. G. & Spiegel, A. M. The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem* **266**, 9309-9313 (1991).

43   Offermanns, S., Laugwitz, K., Spicher, K. & Schultz, G. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 504-508, doi:10.1073/pnas.91.2.504 (1994).

44   Klages, B., Brandt, U., Simon, M. I., Schultz, G. & Offermanns, S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* **144**, 745-754 (1999).

45   Dorsam, R. T., Kim, S., Jin, J. & Kunapuli, S. P. Coordinated signaling through both G12/13 and G(i) pathways is sufficient to activate GPIIb/IIIa in human platelets. *J Biol Chem* **277**, 47588-47595, doi:10.1074/jbc.M208778200 (2002).

46   de Bruyn, K. M., Zwartkruis, F. J., de Rooij, J., Akkerman, J. W. & Bos, J. L. The small GTPase Rap1 is activated by turbulence and is involved in integrin [alpha]IIb[beta]3-mediated cell adhesion in human megakaryocytes. *J Biol Chem* **278**, 22412-22417, doi:10.1074/jbc.M212036200 (2003).

47   Brandt, J. T. *et al.* A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. *American heart journal* **153**, 66 e69-16, doi:10.1016/j.ahj.2006.10.010 (2007).

48   Wiviott, S. D. *et al.* Randomized comparison of prasugrel (CS-747, LY640315), a novel thienopyridine P2Y12 antagonist, with clopidogrel in percutaneous coronary intervention: results of the Joint Utilization of Medications to Block Platelets Optimally (JUMBO)-TIMI 26 trial. *Circulation* **111**, 3366-3373, doi:10.1161/CIRCULATIONAHA.104.502815 (2005).

49   Scarborough, R. M. *et al.* Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of Sistrurus m. barbouri. *J Biol Chem* **266**, 9359-9362 (1991).

50   Zeymer, U. *et al.* Randomized comparison of eptifibatide versus abciximab in primary percutaneous coronary intervention in patients with acute ST-segment elevation myocardial infarction: results of the EVA-AMI Trial. *Journal of the American College of Cardiology* **56**, 463-469, doi:10.1016/j.jacc.2009.08.093 (2010).

51   Hartman, G. D. *et al.* Non-peptide fibrinogen receptor antagonists. 1. Discovery and design of exosite inhibitors. *Journal of medicinal chemistry* **35**, 4640-4642 (1992).

52   Topol, E. J. *et al.* Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. *N Engl J Med* **344**, 1888-1894, doi:10.1056/NEJM200106213442502 (2001).

53   Valgimigli, M. *et al.* Comparison of angioplasty with infusion of tirofiban or abciximab and with implantation of sirolimus-eluting or uncoated stents for acute myocardial infarction: the MULTISTRATEGY randomized trial. *JAMA : the journal of the American Medical Association* **299**, 1788-1799, doi:10.1001/jama.299.15.joc80026 (2008).

54   Gallwitz, M., Enoksson, M., Thorpe, M. & Hellman, L. The extended cleavage specificity of human thrombin. *PLoS One* **7**, e31756, doi:10.1371/journal.pone.0031756 (2012).

55   Stenberg, P., McEver, R., Shuman, M., Jacques, Y. & Bainton, D. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *The Journal of cell biology* **101**, 880-886, doi:10.1083/jcb.101.3.880 (1985).

56   Sultan, C. *et al.* Involvement of platelet glycoprotein IIb-IIIa (alpha IIb-beta 3 integrin) in thrombin-induced synthesis of phosphatidylinositol 3',4'-bisphosphate. *The Journal of biological chemistry* **266**, 23554-23557 (1991).

57   Curtis, J. E., Helgerson, S. L., Parker, E. T. & Lollar, P. Isolation and characterization of thrombin-activated human factor VIII. *J Biol Chem* **269**, 6246-6251 (1994).

58   Esmon, C. T. The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity. *J Biol Chem* **254**, 964-973 (1979).

59   Magdeleyns, E. J., Keuren, J. F. & Curvers, J. Factor Va, bound to microparticles released during platelet storage, is resistant to inactivation by activated protein C. *Transfusion* **47**, 1880-1888, doi:10.1111/j.1537-2995.2007.01411.x (2007).

60   Duvernay, M. T., Young, S., Gailani, D., Schoenecker, J. & Hamm, H. E. Protease-activated Receptor (PAR) 1 and PAR4 Differentially Regulate Factor V

Expression from Human Platelets. *Molecular pharmacology*, doi:10.1124/mol.112.083477 (2013).

61   Monkovic, D. D. & Tracy, P. B. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. *J Biol Chem* **265**, 17132-17140 (1990).

62   Esmon, C. T., Esmon, N. L. & Harris, K. W. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J Biol Chem* **257**, 7944-7947 (1982).

63   Suzuki, K. *et al.* Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. *The EMBO journal* **6**, 1891-1897 (1987).

64   Weitz, J. I., Hudoba, M., Massel, D., Maraganore, J. & Hirsh, J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. *J Clin Invest* **86**, 385-391, doi:10.1172/JCI114723 (1990).

65   Granger, C. B. *et al.* Apixaban versus warfarin in patients with atrial fibrillation. *N Engl J Med* **365**, 981-992, doi:10.1056/NEJMoa1107039 (2011).

66   Markwardt, F. in *Methods in enzymology* Vol. Volume 19 (ed Laszlo Lorand Gertrude E. Perlmann) 924-932 (Academic Press, 1970).

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

68   Connolly, S. J. *et al.* Dabigatran versus warfarin in patients with atrial fibrillation. *N Engl J Med* **361**, 1139-1151, doi:10.1056/NEJMoa0905561 (2009).

69   Suh, T. T. *et al.* Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes & development* **9**, 2020-2033 (1995).

70   Davey, M. & Lüscher, E. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature* **216**, 857-858, doi:10.1038/216857a0 (1967).

71   Detwiler, T. & Feinman, R. Kinetics of the thrombin-induced release of adenosine triphosphate by platelets. Comparison with release of calcium. *Biochemistry* **12**, 2462-2468, doi:10.1021/bi00737a015 (1973).

72   Detwiler, T. & Feinman, R. Kinetics of the thrombin-induced release of calcium (II) by platelets. *Biochemistry* **12**, 282-289, doi:10.1021/bi00726a017 (1973).

73   Martin, B., Feinman, R. & Detwiler, T. Platelet stimulation by thrombin and other proteases. *Biochemistry* **14**, 1308-1314, doi:10.1021/bi00677a032 (1975).

74   Tam, S., Fenton, J. & Detwiler, T. Dissociation of thrombin from platelets by hirudin. Evidence for receptor processing. *The Journal of biological chemistry* **254**, 8723-8725 (1979).

75   Vu, T., Hung, D., Wheaton, V. & Coughlin, S. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068, doi:10.1016/0092-8674(91)90261-v (1991).

76   Regard, J., Sato, I. & Coughlin, S. Anatomical profiling of G protein-coupled receptor expression. *Cell* **135**, 561-571, doi:10.1016/j.cell.2008.08.040 (2008).

77   Camerer, E., Huang, W. & Coughlin, S. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5255-5260, doi:10.1073/pnas.97.10.5255 (2000).

78   Cooper, D., Pechkovsky, D., Hackett, T., Knight, D. & Granville, D. Granzyme K activates protease-activated receptor-1. *PLoS One* **6**, doi:10.1371/journal.pone.0021484 (2011).

79   Knecht, W. *et al.* Trypsin IV or mesotrypsin and p23 cleave protease-activated receptors 1 and 2 to induce inflammation and hyperalgesia. *The Journal of biological chemistry* **282**, 26089-26100, doi:10.1074/jbc.M703840200 (2007).

80   Kuliopulos, A. *et al.* Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. *Biochemistry* **38**, 4572-4585, doi:10.1021/bi9824792 (1999).

81   Trivedi, V. *et al.* Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell* **137**, 332-343, doi:10.1016/j.cell.2009.02.018 (2009).

82   Wang, T. *et al.* Granzyme B-induced neurotoxicity is mediated via activation of PAR-1 receptor and Kv1.3 channel. *PLoS One* **7**, doi:10.1371/journal.pone.0043950 (2012).

83   Gerszten, R. *et al.* Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface. *Nature* **368**, 648-651, doi:10.1038/368648a0 (1994).

84   Scarborough, R. *et al.* Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *The Journal of biological chemistry* **267**, 13146-13149 (1992).

85   Barr, A., Brass, L. & Manning, D. Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor.G protein coupling. *The Journal of biological chemistry* **272**, 2223-2229, doi:10.1074/jbc.272.4.2223 (1997).

86   Kim, S. *et al.* Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. *Blood* **99**, 3629-3636 (2002).

87   Moers, A. *et al.* G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat Med* **9**, 1418-1422, doi:10.1038/nm943 (2003).

88   Nieswandt, B., Schulte, V., Zywietz, A., Gratacap, M. P. & Offermanns, S. Costimulation of Gi- and G12/G13-mediated signaling pathways induces integrin alpha IIbbeta 3 activation in platelets. *J Biol Chem* **277**, 39493-39498, doi:10.1074/jbc.M207256200 (2002).

89   Covic, L., Gresser, A. L. & Kuliopulos, A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* **39**, 5458-5467 (2000).

90   Tiruppathi, C. *et al.* G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. *Proc Natl Acad Sci U S A* **97**, 7440-7445 (2000).

91   Ishii, K. *et al.* Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. *J Biol Chem* **269**, 1125-1130 (1994).

92   Trejo, J. & Coughlin, S. R. The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. *J Biol Chem* **274**, 2216-2224 (1999).

93   Trejo, J., Hammes, S. R. & Coughlin, S. R. Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting. *Proc Natl Acad Sci U S A* **95**, 13698-13702 (1998).

94   Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J. & Trejo, J. beta - Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *J Biol Chem* **277**, 1292-1300, doi:10.1074/jbc.M109160200 (2002).

95   Chen, C. H., Paing, M. M. & Trejo, J. Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. *J Biol Chem* **279**, 10020-10031, doi:10.1074/jbc.M310590200 (2004).

96   Connolly, A., Ishihara, H., Kahn, M., Farese, R. & Coughlin, S. Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**, 516-519, doi:10.1038/381516a0 (1996).

97   Griffin, C., Srinivasan, Y., Zheng, Y., Huang, W. & Coughlin, S. A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science (New York, N.Y.)* **293**, 1666-1670, doi:10.1126/science.1061259 (2001).

98   Khoufache, K. *et al.* PAR1 contributes to influenza A virus pathogenicity in mice. *J Clin Invest* **123**, 206-214, doi:10.1172/JCI61667 (2013).

99   Kogushi, M. *et al.* The novel and orally active thrombin receptor antagonist E5555 (Atopaxar) inhibits arterial thrombosis without affecting bleeding time in guinea pigs. *European journal of pharmacology* **657**, 131-137, doi:10.1016/j.ejphar.2011.01.058 (2011).

100   Takeuchi, M. *et al.* in *European heart journal.* 14-14 (OXFORD UNIV PRESS GREAT CLARENDON ST, OXFORD OX2 6DP, ENGLAND).

101   Serebruany, V. L., Kogushi, M., Dastros-Pitei, D., Flather, M. & Bhatt, D. L. The in-vitro effects of E5555, a protease-activated receptor (PAR)-1 antagonist, on platelet biomarkers in healthy volunteers and patients with coronary artery disease. *Thromb Haemost* **102**, 111-119, doi:10.1160/TH08-12-0805 (2009).

102   Goto, S., Ogawa, H., Takeuchi, M., Flather, M. D. & Bhatt, D. L. Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. *European heart journal* **31**, 2601-2613, doi:10.1093/eurheartj/ehq320 (2010).

103   Chackalamannil, S. *et al.* Discovery of a novel, orally active himbacine-based thrombin receptor antagonist (SCH 530348) with potent antiplatelet activity. *Journal of medicinal chemistry* **51**, 3061-3064, doi:10.1021/jm800180e (2008).

104   Kosoglou, T. *et al.* Pharmacodynamics and pharmacokinetics of the novel PAR-1 antagonist vorapaxar (formerly SCH 530348) in healthy subjects. *European journal of clinical pharmacology* **68**, 249-258, doi:10.1007/s00228-011-1120-6 (2012).

105   Morrow, D. A. *et al.* Vorapaxar in the secondary prevention of atherothrombotic events. *New England Journal of Medicine* **366**, 1404-2817 (2012).

106   Tricoci, P. *et al.* Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *The New England journal of medicine* **366**, 20-53, doi:10.1056/NEJMoa1109719 (2012).

107   Scirica, B. M. *et al.* Vorapaxar for secondary prevention of thrombotic events for patients with previous myocardial infarction: a prespecified subgroup analysis of the TRA 2 degrees P-TIMI 50 trial. *Lancet* **380**, 1317-1324, doi:10.1016/S0140-6736(12)61269-0 (2012).

108   Seminario-Vidal, L. *et al.* Thrombin promotes release of ATP from lung epithelial cells through coordinated activation of rho- and Ca2+-dependent signaling pathways. *J Biol Chem* **284**, 20638-20648, doi:10.1074/jbc.M109.004762 (2009).

109   Hauck, R. W., Schulz, C., Schomig, A., Hoffman, R. K. & Panettieri, R. A., Jr. alpha-Thrombin stimulates contraction of human bronchial rings by activation of protease-activated receptors. *The American journal of physiology* **277**, L22-29 (1999).

110   Weiss, E. J., Hamilton, J. R., Lease, K. E. & Coughlin, S. R. Protection against thrombosis in mice lacking PAR3. *Blood* **100**, 3240-3244, doi:10.1182/blood-2002-05-1470 (2002).

111   Vandendries, E. R., Hamilton, J. R., Coughlin, S. R., Furie, B. & Furie, B. C. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse

model of thrombosis. *Proc Natl Acad Sci U S A* **104**, 288-292, doi:10.1073/pnas.0610188104 (2007).

112   Hamilton, J. R., Cornelissen, I. & Coughlin, S. R. Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. *J Thromb Haemost* **2**, 1429-1435, doi:10.1111/j.1538-7836.2004.00783.x (2004).

113   Kaufmann, R. *et al.* The two-receptor system PAR-1/PAR-4 mediates alpha-thrombin-induced [Ca(2+)](i) mobilization in human astrocytoma cells. *Journal of cancer research and clinical oncology* **126**, 91-94 (2000).

114   Hamilton, J. R., Frauman, A. G. & Cocks, T. M. Increased expression of protease-activated receptor-2 (PAR2) and PAR4 in human coronary artery by inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. *Circulation research* **89**, 92-98 (2001).

115   Kahn, M. *et al.* A dual thrombin receptor system for platelet activation. *Nature* **394**, 690-694, doi:10.1038/29325 (1998).

116   Xu, W. F. *et al.* Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6642-6646 (1998).

117   Holinstat, M. *et al.* PAR4, but not PAR1, signals human platelet aggregation via Ca2+ mobilization and synergistic P2Y12 receptor activation. *The Journal of biological chemistry* **281**, 26665-26739, doi:10.1074/jbc.M602174200 (2006).

118   Voss, B., McLaughlin, J., Holinstat, M., Zent, R. & Hamm, H. PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. *Molecular pharmacology* **71**, 1399-1805, doi:10.1124/mol.106.033365 (2007).

119   Shapiro, M. J., Weiss, E. J., Faruqi, T. R. & Coughlin, S. R. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *The Journal of biological chemistry* **275**, 25216-25221, doi:10.1074/jbc.M004589200 (2000).

120   Cornelissen, I. *et al.* Roles and interactions among protease-activated receptors and P2ry12 in hemostasis and thrombosis. *Proc Natl Acad Sci U S A* **107**, 18605-18610, doi:10.1073/pnas.1013309107 (2010).

121   Sambrano, G. R., Weiss, E. J., Zheng, Y. W., Huang, W. & Coughlin, S. R. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature* **413**, 74-78, doi:10.1038/35092573 (2001).

122   Young, S. E., Duvernay, M. T., Schulte, M. L., Lindsley, C. W. & Hamm, H. E. Synthesis of indole derived protease-activated receptor 4 antagonists and characterization in human platelets. *PLoS One* **8**, e65528, doi:10.1371/journal.pone.0065528 (2013).

123   Chen, H.-S. *et al.* Synthesis and antiplatelet activity of ethyl 4-(1-benzyl-1H-indazol-3-yl)benzoate (YD-3) derivatives. *Bioorganic & medicinal chemistry* **16**, 1262-1340, doi:10.1016/j.bmc.2007.10.070 (2008).

124   Peng, C. Y. *et al.* The indazole derivative YD-3 specifically inhibits thrombin-induced angiogenesis in vitro and in vivo. *Shock* **34**, 580-585, doi:10.1097/SHK.0b013e3181df00a3 (2010).

125   Wu, C. *et al.* YD-3, a novel inhibitor of protease-induced platelet activation. *British journal of pharmacology* **130**, 1289-1385, doi:10.1038/sj.bjp.0703437 (2000).

126   Wu, C.-C. *et al.* Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. *Thrombosis and haemostasis* **87**, 1026-1059 (2002).

127   Kataoka, H. *et al.* Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* **102**, 3224-3231, doi:10.1182/blood-2003-04-1130 (2003).

128   Stearns-Kurosawa, D., Kurosawa, S., Mollica, J., Ferrell, G. & Esmon, C. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 10212-10216, doi:10.1073/pnas.93.19.10212 (1996).

129   Walker, F. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *The Journal of biological chemistry* **255**, 5521-5524 (1980).

130   Walker, F. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. *The Journal of biological chemistry* **256**, 11128-11131 (1981).

131   Griffin, J., Evatt, B., Zimmerman, T., Kleiss, A. & Wideman, C. Deficiency of protein C in congenital thrombotic disease. *The Journal of clinical investigation* **68**, 1370-1373, doi:10.1172/jci110385 (1981).

132   Cheng, T. *et al.* Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. *Nature medicine* **9**, 338-342, doi:10.1038/nm826 (2003).

133   Oganesyan, V. *et al.* The crystal structure of the endothelial protein C receptor and a bound phospholipid. *The Journal of biological chemistry* **277**, 24851-24854, doi:10.1074/jbc.C200163200 (2002).

134   Gu, J.-M. *et al.* Disruption of the endothelial cell protein C receptor gene in mice causes placental thrombosis and early embryonic lethality. *The Journal of biological chemistry* **277**, 43335-43343, doi:10.1074/jbc.M207538200 (2002).

135   Li, W. *et al.* Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin. *Journal of thrombosis and haemostasis : JTH* **3**, 1351-1359, doi:10.1111/j.1538-7836.2005.01385.x (2005).

136   Essler, M. *et al.* Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. *The Journal of biological chemistry* **273**, 21867-21874, doi:10.1074/jbc.273.34.21867 (1998).

137   Singleton, P. A., Dudek, S. M., Chiang, E. T. & Garcia, J. G. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1646-1656, doi:10.1096/fj.05-3928com (2005).

138   Russo, A., Soh, U., Paing, M., Arora, P. & Trejo, J. Caveolae are required for protease-selective signaling by protease-activated receptor-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6393-6397, doi:10.1073/pnas.0810687106 (2009).

139   Joyce, D., Gelbert, L., Ciaccia, A., DeHoff, B. & Grinnell, B. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *The Journal of biological chemistry* **276**, 11199-11203, doi:10.1074/jbc.C100017200 (2001).

140	Riewald, M., Petrovan, R., Donner, A., Mueller, B. & Ruf, W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science (New York, N.Y.)* **296**, 1880-1882, doi:10.1126/science.1071699 (2002).

141	Riewald, M. & Ruf, W. Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. *The Journal of biological chemistry* **280**, 19808-19814, doi:10.1074/jbc.M500747200 (2005).

142	Bae, J.-S., Yang, L., Manithody, C. & Rezaie, A. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood* **110**, 3909-3916, doi:10.1182/blood-2007-06-096651 (2007).

143	Ludeman, M. *et al.* PAR1 cleavage and signaling in response to activated protein C and thrombin. *The Journal of biological chemistry* **280**, 13122-13128, doi:10.1074/jbc.M410381200 (2005).

144	Bae, J.-S., Yang, L. & Rezaie, A. Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 2867-2872, doi:10.1073/pnas.0611493104 (2007).

145	Feistritzer, C. & Riewald, M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood* **105**, 3178-3184, doi:10.1182/blood-2004-10-3985 (2005).

146	Guo, H. *et al.* Neuroprotective activities of activated protein C mutant with reduced anticoagulant activity. *The European journal of neuroscience* **29**, 1119-1130, doi:10.1111/j.1460-9568.2009.06664.x (2009).

147	Hirano, K. *et al.* Distinct Ca2+ requirement for NO production between proteinase-activated receptor 1 and 4 (PAR1 and PAR4) in vascular endothelial cells. *The Journal of pharmacology and experimental therapeutics* **322**, 668-677, doi:10.1124/jpet.107.121038 (2007).

148	Bae, J.-S., Yang, L. & Rezaie, A. Factor X/Xa elicits protective signaling responses in endothelial cells directly via PAR-2 and indirectly via endothelial protein C receptor-dependent recruitment of PAR-1. *The Journal of biological chemistry* **285**, 34803-34812, doi:10.1074/jbc.M110.163642 (2010).

149   Andrade-Gordon, P. *et al.* Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci U S A* **96**, 12257-12262 (1999).

150   Damiano, B. P., Derian, C. K., Maryanoff, B. E., Zhang, H. C. & Gordon, P. A. RWJ-58259: a selective antagonist of protease activated receptor-1. *Cardiovascular drug reviews* **21**, 313-326 (2003).

151   Rilatt, I., Mirabel, E., Le Grand, B. & Perez, M. Discovery and SAR of small molecule PAR1 antagonists. *Bioorganic & medicinal chemistry letters* **20**, 903-906, doi:10.1016/j.bmcl.2009.12.072 (2010).

152   Bocquet, A. *et al.* Effects of a new PAR1 antagonist, F 16618, on smooth muscle cell contraction. *European journal of pharmacology* **611**, 60-63, doi:10.1016/j.ejphar.2009.03.056 (2009).

153   Edgell, C. J., McDonald, C. C. & Graham, J. B. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A* **80**, 3734-3737 (1983).

154   Edgell, C. J. *et al.* Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. *In vitro cellular & developmental biology : journal of the Tissue Culture Association* **26**, 1167-1172 (1990).

155   Zhang, Chung & Oldenburg. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of biomolecular screening* **4**, 67-73 (1999).

156   Raqeeb, A., Sheng, J., Ao, N. & Braun, A. P. Purinergic P2Y2 receptors mediate rapid Ca(2+) mobilization, membrane hyperpolarization and nitric oxide production in human vascular endothelial cells. *Cell calcium* **49**, 240-248, doi:10.1016/j.ceca.2011.02.008 (2011).

157   Benndorf, R., Boger, R. H., Ergun, S., Steenpass, A. & Wieland, T. Angiotensin II type 2 receptor inhibits vascular endothelial growth factor-induced migration and in vitro tube formation of human endothelial cells. *Circulation research* **93**, 438-447, doi:10.1161/01.RES.0000088358.99466.04 (2003).

158   Lubitz, J. A., Freeman, L. & Okun, R. Mitotane use in inoperable adrenal cortical carcinoma. *JAMA : the journal of the American Medical Association* **223**, 1109-1112 (1973).

159   Kaminsky, N., Luse, S. & Hartroft, P. Ultrastructure of adrenal cortex of the dog during treatment with DDD. *Journal of the National Cancer Institute* **29**, 127-159 (1962).

160   Nader, N. *et al.* Mitotane has an estrogenic effect on sex hormone-binding globulin and corticosteroid-binding globulin in humans. *The Journal of clinical endocrinology and metabolism* **91**, 2165-2170, doi:10.1210/jc.2005-2157 (2006).

161   van Seters, A. P. & Moolenaar, A. J. Mitotane increases the blood levels of hormone-binding proteins. *Acta endocrinologica* **124**, 526-533 (1991).

162   Chortis, V. *et al.* Mitotane therapy in adrenocortical cancer induces CYP3A4 and inhibits 5alpha-reductase, explaining the need for personalized glucocorticoid and androgen replacement. *The Journal of clinical endocrinology and metabolism* **98**, 161-171, doi:10.1210/jc.2012-2851 (2013).

163   Bledsoe, T., Island, D. P., Ney, R. L. & Liddle, G. W. An Effect of O,P'-Ddd on the Extra-Adrenal Metabolism of Cortisol in Man. *The Journal of clinical endocrinology and metabolism* **24**, 1303-1311 (1964).

164   Luton, J. P. *et al.* Treatment of Cushing's disease by O,p'DDD. Survey of 62 cases. *N Engl J Med* **300**, 459-464, doi:10.1056/NEJM197903013000903 (1979).

165   Scippo, M. L. *et al.* Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Analytical and bioanalytical chemistry* **378**, 664-669, doi:10.1007/s00216-003-2251-0 (2004).

166   Haak, H. R. *et al.* Optimal treatment of adrenocortical carcinoma with mitotane: results in a consecutive series of 96 patients. *British journal of cancer* **69**, 947-951 (1994).

167   Haak, H. R., Caekebeke-Peerlinck, K. M., van Seters, A. P. & Briet, E. Prolonged bleeding time due to mitotane therapy. *Eur J Cancer* **27**, 638-641 (1991).

168   Khetawat, G. *et al.* Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood* **95**, 2289-2296 (2000).

169   Moro, L. *et al.* Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase. *Blood* **105**, 115-121, doi:10.1182/blood-2003-11-3840 (2005).

170   Moraes, L. A. *et al.* Ligand-specific glucocorticoid receptor activation in human platelets. *Blood* **106**, 4167-4175, doi:10.1182/blood-2005-04-1723 (2005).

171   Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H. & Coughlin, S. R. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation* **103**, 879-887, doi:10.1172/JCI6042 (1999).

172   Bernatowicz, M. S. *et al.* Development of potent thrombin receptor antagonist peptides. *Journal of medicinal chemistry* **39**, 4879-4887, doi:10.1021/jm960455s (1996).

173   Andrade-Gordon, P. *et al.* Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci U S A* **96**, 12257-12262 (1999).

174   Ahn, H. S. *et al.* Inhibition of cellular action of thrombin by N3-cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7H-pyrrolo[3, 2-f]quinazoline-1,3-diamine (SCH 79797), a nonpeptide thrombin receptor antagonist. *Biochemical pharmacology* **60**, 1425-1434 (2000).

175   Clasby, M. C. *et al.* Discovery and synthesis of a novel series of quinoline-based thrombin receptor (PAR-1) antagonists. *Bioorganic & medicinal chemistry letters* **16**, 1544-1548, doi:10.1016/j.bmcl.2005.12.042 (2006).

176   Cleator, J., Zhu, W., Vaughan, D. & Hamm, H. Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. *Blood* **107**, 2736-2780, doi:10.1182/blood-2004-07-2698 (2006).

177   Holinstat, M. *et al.* Protease-activated receptor signaling in platelets activates cytosolic phospholipase A2α differently for cyclooxygenase-1 and 12-lipoxygenase catalysis. *Arteriosclerosis, thrombosis, and vascular biology* **31**, 435-477, doi:10.1161/atvbaha.110.219527 (2011).

178   Peng, C.-Y. *et al.* The indazole derivative YD-3 specifically inhibits thrombin-induced angiogenesis in vitro and in vivo. *Shock (Augusta, Ga.)* **34**, 580-585, doi:10.1097/SHK.0b013e3181df00a3 (2010).

179   Lee, F. *et al.* Synthesis of 1-benzyl-3-(5'-hydroxymethyl-2'-furyl)indazole analogues as novel antiplatelet agents. *Journal of medicinal chemistry* **44**, 3746-3755 (2001).

180   Kennedy, J. P. *et al.* Application of combinatorial chemistry science on modern drug discovery. *Journal of combinatorial chemistry* **10**, 345-354, doi:10.1021/cc700187t (2008).

181   Lindsley, C. W. *et al.* Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorganic & medicinal chemistry letters* **15**, 761-764, doi:10.1016/j.bmcl.2004.11.011 (2005).

182   Mistry, A. G., Smith, K. & Bye, M. R. A superior synthethic method for the bromination of indoles and benzimidazoles. *Tetrahedron letters* **27**, 1051-2105 (1986).

183   Kudo, N., Perseghini, M. & Fu, G. A versatile method for Suzuki cross-coupling reactions of nitrogen heterocycles. *Angewandte Chemie (International ed. in English)* **45**, 1282-1286, doi:10.1002/ange.200503479 (2006).

184   Gawaz, M. *et al.* Incomplete inhibition of platelet aggregation and glycoprotein IIb-IIIa receptor blockade by abciximab: importance of internal pool of glycoprotein IIb-IIIa receptors. *Thromb Haemost* **83**, 915-922 (2000).

185   Kleiman, N. S. *et al.* Differential inhibition of platelet aggregation induced by adenosine diphosphate or a thrombin receptor-activating peptide in patients treated with bolus chimeric 7E3 Fab: implications for inhibition of the internal pool of GPIIb/IIIa receptors. *Journal of the American College of Cardiology* **26**, 1665-1671, doi:10.1016/0735-1097(95)00391-6 (1995).

186   Dormann, D., Clemetson, K. J. & Kehrel, B. E. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* **96**, 2469-2478 (2000).