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Identification of the minimum PAR4 inhibitor pharmacophore and optimization of a series of 2-methoxy-6-arylimidazo[2,1-*b*][1,3,4] thiadiazoles

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ABSTRACT

This letter describes the further deconstruction of the known PAR4 inhibitor chemotypes (MWs 490–525 and with high plasma protein binding) to identify a minimum PAR4 pharmacophore devoid of metabolic liabilities and improved properties. This exercise identified a greatly simplified 2-methoxy-6-arylimidazo [2,1-*b*][1,3,4]thiadiazole scaffold that afforded nanomolar inhibition of both activating peptide and γ-thrombin mediated PAR4 stimulation, while reducing both molecular weight and the number of hydrogen bond donors/acceptors by ~50%. This minimum PAR4 pharmacophore, with competitive inhibition, versus non-competitive of the larger chemotypes, allows an ideal starting point to incorporate desired functional groups to engender optimal DMPK properties towards a preclinical candidate.

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Protease Activated Receptor 4 (PAR4) is a G Protein-Coupled Receptor (GPCR) essential for the thrombin-induced procoagulant effect on platelets, and as such, has garnered a great deal of interest as a target for anti-platelet therapy to treat thrombosis without bleeding.[1–3] Historically dominated by antibody therapy, small molecule PAR4 antagonists are only now emerging as in vivo tool compounds and clinical candidates.[1–4] Until recently, the PAR4 antagonists in the primary literature suffered from poor DMPK properties and a lack of activity upon γ-thrombin activation.[4–7] Our lab recently divulged a new series of small PAR4 inhibitors **1** with an improved DMPK profile and weak activity upon γ-thrombin activation.[4–7] Bristol-Myers-Squibb (BMS) also disclosed a novel series of PAR4 inhibitors, represented by **2** (an analog of BMS986120), with exquisite potency against both activating peptide (AP) and γ-thrombin mediated PAR4 stimulation, but with virtually no free drug levels (rat and human f_u < 0.001).[8] Both **1** and **2** (Fig. 1) are high molecular weight compounds (490–510), with high *c*Log*P*s (>4) and the noted high plasma protein binding.[4,8]

Thus, we deconstructed **2** in an attempt to identify a minimum pharmacophore that retained potent PAR4 inhibition against both AP and γ-thrombin that could then be optimized with more favorable DMPK properties. This exercise led to the discovery that the most basic core of **2**, a 6-(benzofuran-2-yl)-2-methoxyimidazo [2,1-*b*][1,3,4]thiadiazole **3** as a potent PAR4 inhibitor (PAR4 AP IC_{50} = 1.69 nM, PAR4 γ-thrombin IC_{50} = 58.8 nM), as the minimum pharmacophore (MW = 271) of **2**.[9] However, the potential liabilities of an unsubstituted benzofuran, as in **3**, raised metabolic stability concerns. Thus, in this Letter, we describe efforts to survey alternative 6-position substituents on the 2-methoxyimidazo[2, 1-*b*][1,3,4]thiadiazole core to identify a minimum PAR4 pharmacophore that could then be further optimized with functional groups that would engender desirable DMPK properties.

In the initial SAR campaign, we elected to survey functionalized aryl moieties in the 6-position and assess if these simple analogs were sufficient to elicit PAR4 inhibition. Analogs **4**, or **3**, could be readily accessed in two steps from commercial materials (Scheme 1), and enabled the evaluation of multiple regions of the heterobiarylcore.[10] Here, commercial α-bromo ketones **5** were condensed with 5-bromo-1,3,4-thidiazol-2-amine **6** to provide 2-bromo-6-arylimidazo[2,1-*b*][1,3,4]thiadiazoles **7** in 65–86% yield. An S_NAr reaction with methoxide under mild conditions delivered analogs **4** in yields ranging from 72–80%.

SAR for select analogs **4** are highlighted in Table 1, all with molecular weights less than 325. The most basic analog, **4f** with an unsubstituted phenyl ring, was a PAR4 antagonist with a

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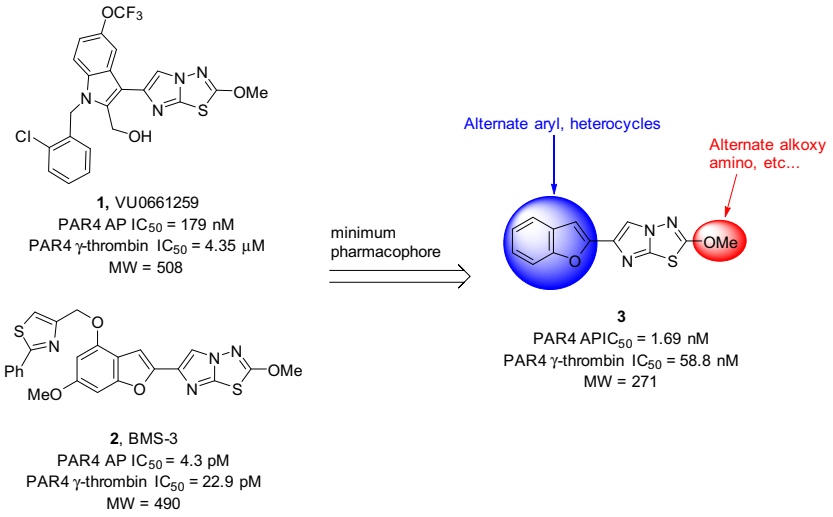
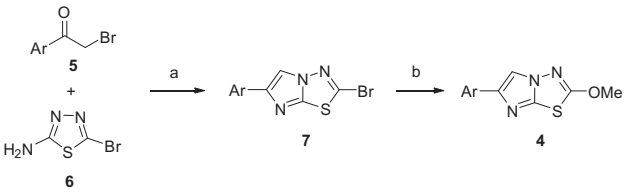
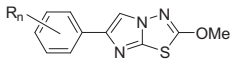


Figure 1. Structures of reported PAR4 antagonists **1** and **2**, and the minimum pharmacophore of **2**, fragment **3**, and plans to further optimize **3** into a more desirable fragment core.



Scheme 1. Synthesis of aryl analogs **4**. Reagents and conditions: (a) CH_3CN/IPA, 80 °C, 18 h, 65–86%; (b) NaOMe, DCM/MeOH (3:1), 1 h, rt 72–80%.

Table 1
Structures and activities of analogs **4**



Compd	R	PAR4-AP PAC-1 IC_{50} (nM)[a] (pIC_{50} ± SEM)	PAR4 γ-thrombin PAC-1 IC_{50} (nM)[a] (pIC_{50} ± SEM)
4a	2-CF_3	1330 (5.88 ± 0.06)	ND
4b	3-CF_3	36.3 (7.44 ± 0.10)	1310 (5.88 ± 0.24)
4c	4-CF_3	15.6 (7.81 ± 0.07)	348 (6.46 ± 0.06)
4d	2,6-diF	1300 (5.89 ± 0.05)	ND
4e	2,4-diF	175 (6.76 ± 0.03)	ND
4f	H	757 (6.12 ± 0.07)	ND
4g	4-OCH_3	592 (6.23 ± 0.11)	ND
4h	4-CH_3	57.2 (7.24 ± 0.04)	ND
4i	2-Cl	437 (6.36 ± 0.02)	ND
4j	3-Cl	72.8 (7.14 ± 0.07)	>10,000 (>5)
4k	4-Cl	39.5 (7.40 ± 0.04)	621 (6.21 ± 0.15)
4l	4-OH	>10,000 (>5)	ND
4m	4-CN	937 (6.03 ± 0.06)	ND
4n	4-OCF_3	27.7 (7.57 ± 0.09)	246 (6.64 ± 0.19)
4o	4-SO_2CH_3	1,200 (5.92 ± 0.27)	ND
4p	3,5-diCF_3	52.3 (7.28 ± 0.04)	5620 (5.25 ± 0.08)
4q	2-F	526 (6.28 ± 0.04)	ND
4r	3-F	675 (6.17 ± 0.14)	ND
4s	4-F	406 (6.39 ± 0.03)	ND
4t	3,4-diCl	28.5 (7.55 ± 0.06)	1460 (5.84 ± 0.39)
4u	4-NO_2	207 (6.68 ± 0.04)	ND
4v	4-$OCHF_2$	83.4 (7.08 ± 0.05)	2540 (5.60 ± 0.21)
4w	3-CF_3,4-F	68.8 (7.16 ± 0.05)	1090 (5.96 ± 0.15)
4x	2-NO_2	4840 (5.32 ± 0.24)	ND
4y	3-NO_2	>10,000 (>5)	ND

[a] Average of three independent determinations upon PAR4 activation with either AP or γ-thrombin.[4,9]

PAR4 IC_{50} against the AP of 757 nM. Simple substitution of the phenyl ring afforded robust SAR in terms of PAR4 inhibition against the AP, with a significant right-shift in PAR4 potency upon activation with γ-thrombin. In general, substitutions at the 4-postion of the phenyl ring afforded the most potent analogs. Moreover, lipophilic, electron-withdrawing moieties proved optimal. For example, the 4-CF_3 derivative (**4c**) was a 15.6 nM PAR4 inhibitor against the AP, and displayed an IC_{50} of 348 nM against γ-thrombin activation. This result is quite significant considering the reduced, minimum pharmacophore and activities relative to **1** and **2**. Likewise, the 4-OCF_3 congener (**4n**) was a 27.7 nM PAR4 inhibitor against the AP, and displayed an IC_{50} of 246 nM against γ-thrombin activation. The 4-Cl analog (**4k**) proved also to inhibit PAR4 upon AP activation (IC_{50} = 39.5 nM) as well as γ-thrombin (IC_{50} = 621 nM). Interestingly, the difluoromethoxy congener **4v**, of **4n**, remained potent against the AP (IC_{50} = 83.4 nM), but loss of a single fluorine atom led to no activity against γ-thrombin activation. Finally, substituents in the 2-position were generally detrimental to potency, and electron donating moieties, such as 4-OCH_3 (**4g**) and 4-OH (**4l**), led to significant diminution in potency.

Concentration response curves (CRCs) for **4c**, **4n** and **4k** against AP and γ-thrombin mediated activation are shown in Figure 2A–F, and all three proved to be competitive inhibitors of PAR4 (Fig. 2G–I). This is in sharp contrast to **1**, which is a non-competitive inhibitor of PAR4[9], and **2** which has a mixed competitive/non-competitive profile.[4] These results are exciting as until now small molecule tools with diverse modes of pharmacology and PAR4 antagonism to potentially assess in vivo effects of PAR4 inhibition have not existed.

While PAR4 activity against AP and γ-thrombin, coupled with a novel competitive mode of inhibition generated enthusiasm for these low molecular weight PAR4 antagonists **4c**, **4n** and **4k**, their in vitro DMPK profiles were suboptimal, and reminiscent of **1**.[4,8,9] While *c*Log*P*s were acceptable (3.2–3.7), experimental Log*P*s were >4 and PSAs were <40. These physiochemical properties correlated with high plasma protein binding (human and rat f_us between 0.006 and 0.014) and moderate to high intrinsic clearance in hepatic microsomes (human CL_{hep}s 13.6 to 19.9 mL/min/kg and rat CL_{hep}s 46.3 to 58.3 mL/min/kg).

These data led to second and third generation libraries aimed at replacing the 6-aryl moiety with heterocycles and surveying alternative ethers and amine substituents for the 2-methoxy group in **4c**, **4n** and **4k**. Overall, heterocyclic replacements for the 6-aryl

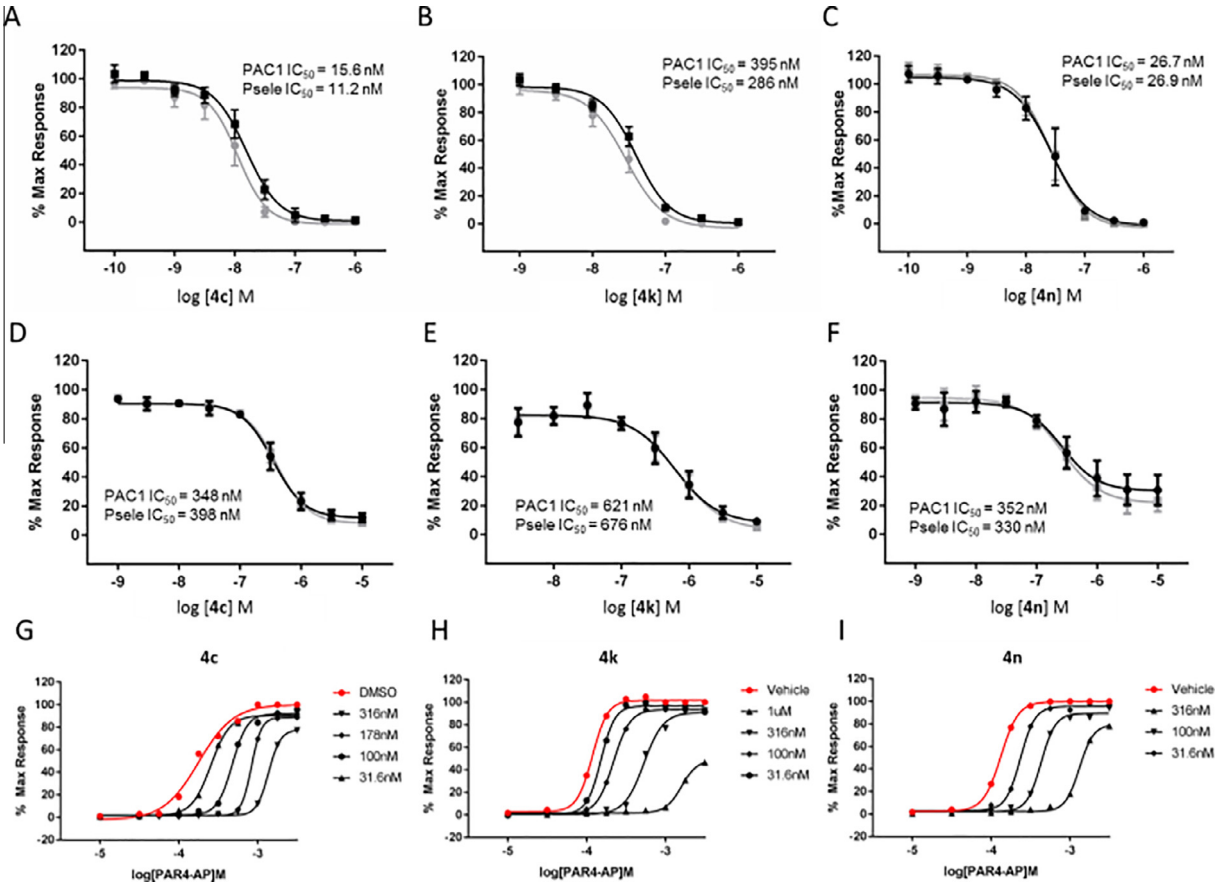


Figure 2. Molecular pharmacology profile of **4c**, **4n** and **4k**. (A–C) PAR4 antagonist CRCs ($n = 3$) against 200 μM PAR4-AP showing equivalent inhibition of both PAC-1 and P-selectin. $n = 3$, mean ± SEM; **D**–**F**) PAR4 antagonist CRCs ($n = 3$) against 100 nM γ-thrombin showing equivalent inhibition of both PAC-1 and P-selectin. $n = 3$, mean ± SEM; (G–I) Progressive fold-shift experiments showing a parallel right-ward shift of the CRC (competitive mode of PAR4 inhibition) with **4c**, **4n** and **4k.** Schild EC_{50} log DR-1 versus log [antagonist] plot slopes: **4c** (0.95 ± 0.09), **4k** (1.09 ± 1.3), **4n** (0.94 ± 0.08).[4,9]

moiety (e.g., 2-, 3- and 4-pyridyl, thienylthiazolyl) lost 10- to 50-fold activity relative to the unsubstituted phenyl comparator **4f**. Alternatives for the 2-methoxy group were equally steep. 2-Ethoxy congeners displayed activity (IC_{50}s 600–900 nM), but lost ~20-fold activity relative to **4c**, **4n** and **4k**. Larger, branched and cyclic ethers lost all PAR4 inhibitory activity. In an attempt to improve the physiochemical properties of this series, we performed S_NAr reactions on cores **7** with various primary and secondary amines. SAR was steep, with the vast majority of 2-amino congeners studied possessing no PAR4 inhibitory activity. As shown in Figure 3, only a single $N(CH_3)_2$ analog **8** was active (PAR4-AP IC_{50} = 3.45 μM), whereas the $NHCH_3$ derivative was inactive. None of the non-2-OCH_3 derivatives displayed any PAR4 activity against γ-thrombin mediated activation. Clearly, the 2-methoxy moiety is an essential element of the PAR4 pharmacophore, at least with the context of the imidazo[2,1-*b*][1,3,4]thiadiazole bicyclic scaffold.

We envisioned that deletion of the 2-hydroxy methyl moiety and transposition of the imidazo[2,1-*b*][1,3,4]thiadiazole bicycle from the 3- to the 2-position, while simultaneously truncating

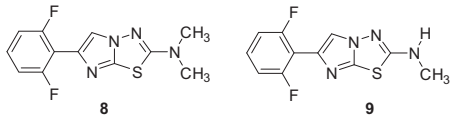


Figure 3. Structures and PAR4 activity of 2-amino congeners **8** and **9**.

the *N*-benzyl moiety to a simple *N*-Me, would afford a small molecule that aligns with fragment **3** (Fig. 4A). Indeed, this proved successful, generating indole **5**, a 20 nM PAR4 inhibitor against AP (~9-fold more potent than **1**) with a 25% reduction in molecular weight. While activity against γ-thrombin with **5** was more potent and efficacious (IC_{50} = 1.0 μM) than that of **1**, an ~50-fold difference in potency was noted between inhibition of PAR4-AP and γ-thrombin mediated stimulation (Fig. 4B), yet **5** retained selectivity versus PAR1 (IC_{50} > 10 μM) and off-target effects on the collagen receptor were eliminated (Fig. 5). Although **5** maintains γ-thrombin stimulated antagonism within four-fold of **4n**, fragments **4** represented the best path forward towards improved PAR4 inhibitors based on physiochemical properties and γ-thrombin ligand efficiency metrics (**5** LE = 0.33 vs. **4n** LE = 0.43) with competitive inhibition.

We previously reported on the potency and selectivity of example **1** noting >100 fold difference in IC_{50} values comparing PAR1-AP and PAR4-AP.[4] However, when used at micromolar concentrations that are effective against the tethered ligand (γ-thrombin mediated activation) significant off-targets against collagen I induced aggregation were noted (Fig. 5). This was surprising considering the weak effects on the primary anti-target PAR1. This was also an alarming revelation since collagen I-mediated platelet activation is intimately involved in hemostasis and thrombosis. Therefore, before focusing solely on the simplified pharmacophores **4**, we elected to re-evaluate **1**[4] in an effort to further minimize the key components required for PAR4 inhibition and address off-target effects. Fragment **3** is devoid of collagen receptor

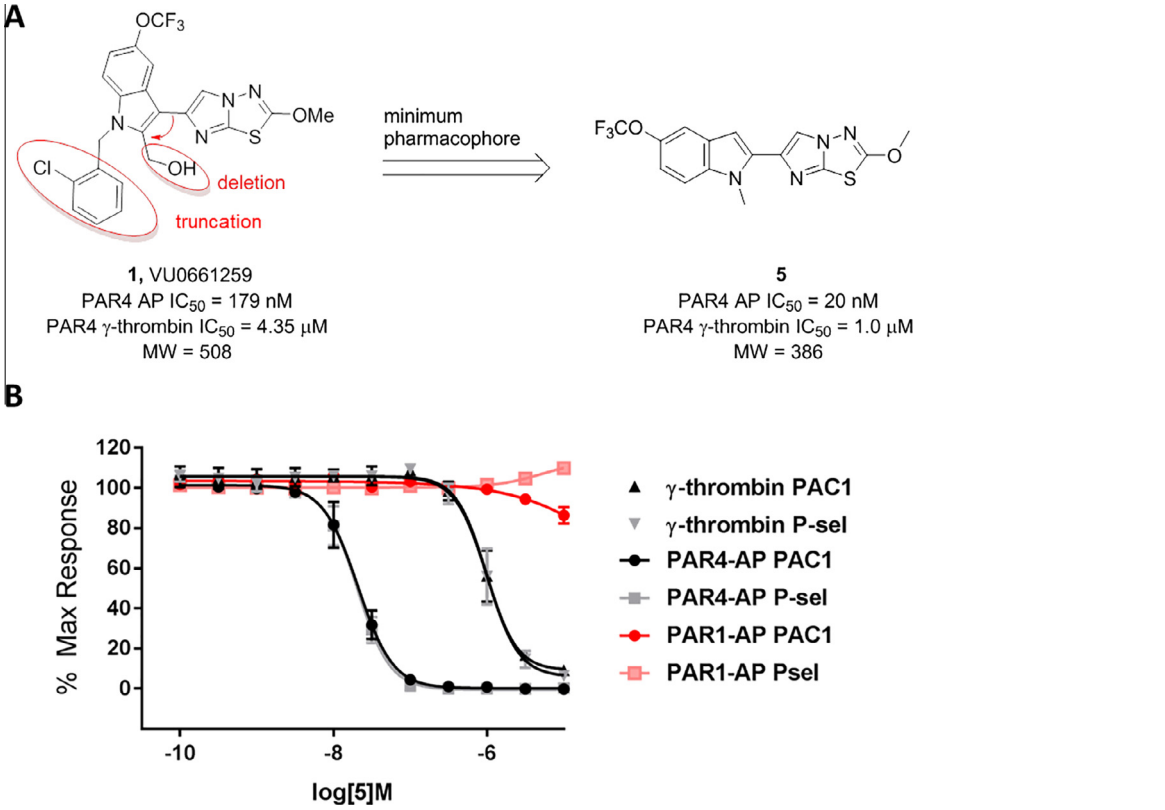


Figure 4. Identification and pharmacological profile of a minimum pharmacophore of **1**. (**A**) Strategy to mimick the minimum PAR4 pharmacophore **3** of **2** within the indole series, and identification of **5**. (B) Pharmacological profile of **5** against PAR4 γ-thrombin (100 nM) PAC-1 (IC_{50} = 1.0 μM, pIC_{50} = 6.01 ± 0.05), PAR4 γ-thrombin P-selectin (IC_{50} = 1.0 μM, pIC_{50} = 6.00 ± 0.04), PAR4-AP PAC-1(IC_{50} = 21 nM, pIC_{50} = 7.68 ± 0.04), PAR4-AP (200 μM) P-selectin (IC_{50} = 20 nM, pIC_{50} = 7.70 ± 0.03) and PAC-AP (PAC1 and P-selectin, IC_{50}s > 10 μM). *n* = 3, mean ± SEM.

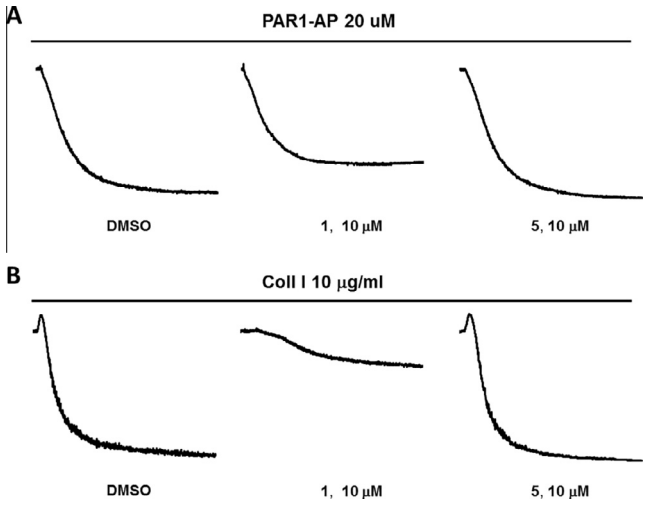


Figure 5. Identification of a minimum pharmacophore of 1 devoid of off-target effects. Washed human platelets were incubated with the indicated concentration of antagonist (**1**, **5**) for 20 min prior to activation with either (A) 20 μM PAR1-AP or (B) 10 μg/mL collagen I (Coll I). Platelets were allowed to aggregate for 10 min. Shown are representative tracings of three independent experiments.

off-target effects as are fragment inhibitors **4**, suggesting the hydroxymethyl moiety of **1** might be suspect in this series.

The PAR4 IC_{50}s from the flow cytometry assays were encouraging, but would these novel, competitive PAR4 antagonists (**4c**, **4k**, and **4n**) inhibit human platelet aggregation? As shown in Figure 6, a standard platelet aggregation assay[11] demonstrated excellent correlation and consistent data with the three competitive PAR4 inhibitors. Here, platelet aggregation was significantly inhibited at doses (316 nM to 1 μM against AP and 1 μM to 3.16 μM against γ-thrombin) of the PAR4 antagonists that abolished PAC1 binding. This was a pivotal finding, as this is the first demonstration that competitive PAR4 inhibitors are efficacious in this pharmacodynamics assay.

Furthermore, we wanted to assess the ability to inhibit platelet aggregation with **5** (Fig. 7), as there was a large disconnect between γ-thrombin and AP. Here, platelet aggregation was significantly inhibited at doses (316 nM to 1 μM against AP and only at 10 μM against γ-thrombin) of the PAR4 antagonist **5** that abolished PAC1 binding. Thus, excellent correlation once again between the FACS and aggregation assays, but poor correlation between activity against PAR4-AP and γ-thrombin induced PAR4 activation. γ-Thrombin cleavage of PAR4 generates an intramolecular tethered ligand which is covalently attached to the receptor, while PAR4-AP is a soluble surrogate for the tethered ligand. It is impossible to measure the concentration of the tethered ligand proximal to the binding pocket because it is covalently attached to the receptor. Theoretically, an infinite concentration of ligand is available due to its inability to diffuse away from the receptor. Thus, one would predict that it would take higher concentrations of an orthosteric antagonist to compete with the tethered ligand as compared to the soluble AP. Indeed, we have extensively documented this phenomenon. If this was the sole distinguishing factor between γ-thrombin and PAR4-AP, IC_{50}s should correlate. It should be possible to predict γ-thrombin IC_{50}s based on PAR4-AP IC_{50}s. However, this is not the case. Proportional differences between γ-thrombin and PAR4-AP IC_{50}s are highly variable, which is why we chose to track SAR with both ligands. Based on the data presented here we can't explain this lack of correlation, however

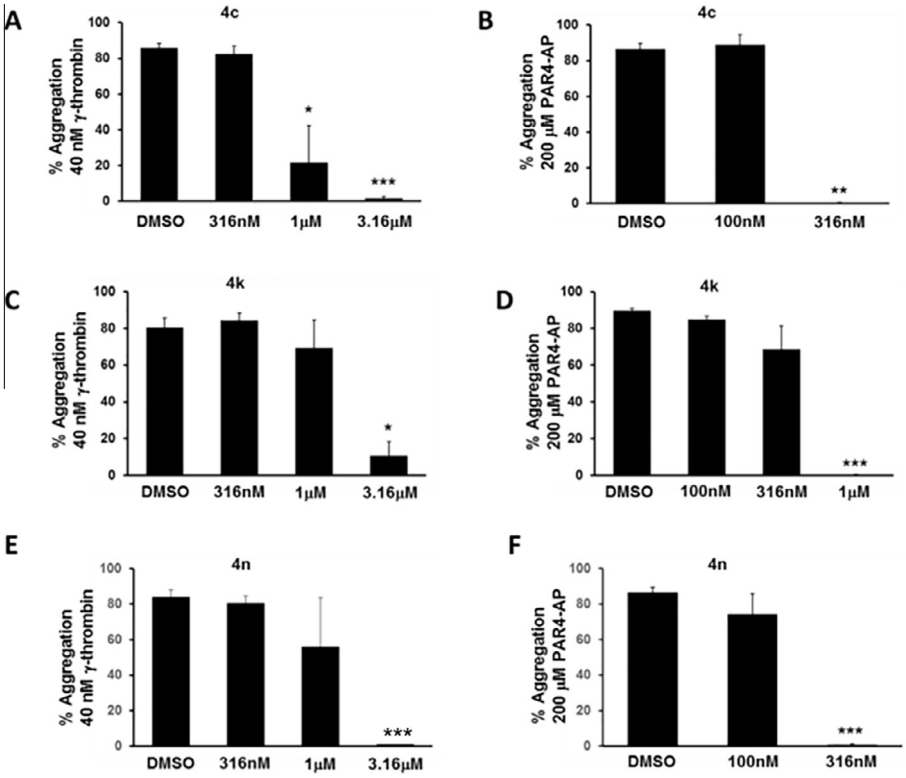


Figure 6. Activity of fragments 4 in platelet aggregation. Washed human platelets were incubated with the indicated concentration of antagonist for 20 min prior to activation with either 40 nM γ-thrombin A (**4c**), C (**4k**), E (**4n**) or 200 μM PAR4-AP B (**4c**), D (**4k**), F (**4n**). Platelet aggregation was monitored for 10 min at which point final reported % aggregation values were taken. Shown are the means ± SEM for at least three independent experiments. Values were compared to controls and significance determined by paired *t*-test. $^{*}p$-Value <0.05, $^{**}p$-value <0.005, $^{***}p$-value <0.0005.

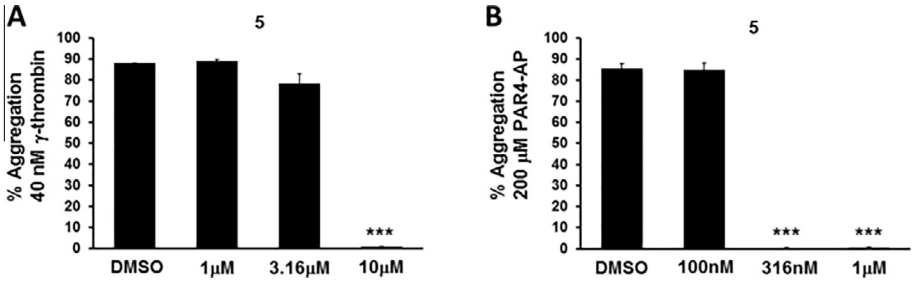


Figure 7. Activity of 5 in platelet aggregation. Washed human platelets were incubated with the indicated concentration of antagonist **5** for 20 min prior to activation with either (A) 40 nM γ-thrombin or (B) 200 μM PAR4-AP Platelet aggregation was monitored for 10 min at which point final reported % aggregation values were taken. Shown are the means ± SEM for at least three independent experiments. Values were compared to controls and significance determined by paired *t*-test. $^{***}p$-Value <0.0005.

it is important to keep in mind that one is an enzyme (γ-thrombin) and the other is a peptide (PAR4-AP). Thrombin interacts with PAR4[12,13] and its heterodimer partner PAR1[14] proximal to but outside the binding pocket. γ-Thrombin binding to PAR4 outside the binding picket, or the reorganization of the N-terminus after cleavage could induce conformational states in the receptor that the AP is incapable of inducing since it interacts only with binding pocket. This could create two distinct receptor states and therefore two distinct lines of SAR for antagonist. In support of this hypothesis our lab has previously published on functional selectivity between PAR1-AP and thrombin induced endothelial cell signaling.[15]

In summary, we identified a greatly simplified 2-methoxy-6-arylimidazo[2,1-*b*][1,3,4]thiadiazole scaffold that affords nanomolar PAR4 inhibition of AP as well as γ-thrombin, while reducing both molecular weight and the number of hydrogen bond donors/acceptors by ~50%. This simplified core represents the minimum pharmacophore for PAR4 inhibition in a competitive manner. While initial analogs did not address the DMPK liabilities of **1** and **2** (though f_u was slightly improved), the most basic core, **4h**, can serve as a sub-micromolar lead from which to build in more optimal DMPK and physiochemical properties. Similarly, **5** was identified as a minimum PAR4 pharmacophore of indole series **1**, but with an unexpected 50-fold loss in activity at γ-thrombin, yet nanomolar potency against the AP. Finally, we disclose the first example demonstrating that competitive PAR4 inhibitors significantly inhibit human platelet aggregation. Further optimization efforts around these fragments are in progress, as well as the evaluation of alternate phenyl bioisosteres beyond the imidazo[2,1-*b*][1,3,4]thiadiazole bicyclic scaffold, and these results will be reported in due course.

Acknowledgments

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References and notes

1. French, S. L.; Arthur, J. F.; Lee, H.; Nesbitt, W. S.; Andrews, R. K.; Gardiner, E. E.; Hamilton, J. R. *J. Thromb. Haemost.* **2016**, *14*, 1642–1654.
2. Gieseler, F.; Ungefroren, H.; Settmacher, U.; Hollenberg, M. D.; Kaufmann, R. *Cell. Commun. Signal.* **2013**, *11*, 86.
3. Duvernay, M.; Young, S.; Gailani, D.; Schoenecker, J.; Hamm, H. E. *Mol. Pharm.* **2013**, *83*, 781.
4. Temple, K. J.; Duvernay, M. T.; Young, S. E.; Wen, W.; Wu, W.; Maeng, J. G.; Blobaum, A. L.; Stauffer, S. R.; Hamm, H. E.; Lindsley, C. W. *J. Med. Chem.* **2016**, *59*, 7690.
5. Lee, F.-Y.; Lien, J.-C.; Huang, J.-L.; Tsai, S.-C.; Teng, C.-M.; Wu, C.-C.; Cheng, F.-C.; Kuo, S.-C. *J. Med. Chem.* **2001**, *44*, 3746.
6. Young, S. E.; Duvernay, M. T.; Lindsley, C. W.; Hamm, H. E. *PLOS One* **2013**, *8*, e65528.
7. Wen, W.; Young, S. E.; Duvernay, M. T.; Schulte, M. L.; Nance, K. D.; Melancon, B. J.; Engers, J.; Locuson, C. W.; Wood, M. R.; Daniels, J. S.; Wu, W.; Lindsley, C. W.; Hamm, H. E.; Stauffer, S. R. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4708.
8. (a) Halford, B. *C&E News* **2016**, *94*, 22; (b) Banville, J.; Remillard, R.; Ruediger, EH.; Deon, D.H.; Gagnon, M.; Dube, L.; Guy, J.; Priestly, E.S.; Posy, S.; Maxwell, B. D.; Wong, P.C. WO2013163279A1, **2013**.; (c) Priestly, E.S.; Posy, S.L.; Tremblay, F.T.; Martel, A.; Marinier, A. WO2013163241A1, **2013**.; (d) Lawrence, M.R.; Miller, M.M.; Seiffert, D.A.; Posy, S.L.; Wong, P.C.; Banville, J.; Ruediger, E.H.; Deon, D.H.; Martel, A.; Tremblay, F.; Guy, J.; Lavallee, J-F.; Gagnon, M. WO2013163244A1, **2013**.
9. Duvernay, M.T.; Temple, K.J.; Young, S.E.; Wen, W.; Wu, W.; Maeng, J.G.; Blobaum, A.L.; Stauffer, S.R.; Lindsley, C.W.; Hamm, H.E. *Mol. Pharmacol.* in press.
10. *Example experimental (**4h**).* A mixture of commercially available 2-bromo-1-(*p*-tolyl)ethan-1-one (2.35 mmol) and 5-bromo-1,3,4-thiadiazol-2-amine (3.52 mmol) were dissolved in CH_3CN/IPA (1:1; 9.4 mL) in a microwave vial that was sealed and heated to 80 °C for 18 h. Next, the vial was placed in a Microwave for 30 min at 150 °C. The solvent was evaporated and the mixture was re-suspended in DCM (20 mL), washed with saturated $NaHCO_3$ (20 mL), brine, dried over magnesium sulfate and filtered. The organic layer was concentrated under reduced pressure onto celite and loaded onto a 3 inch silica plug and purified using Teledyne ISCO Combi-Flash system (solid loading, 100% DCM, 10 min run) to afford 2-bromo-6-(*p*-tolyl)imidazo[2,1-*b*][1,3,4] thiadiazole (1.70 mmol) in 72% yield. LC–MS [M+H] = 294/296, RT = 1.174; ^1H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.68–7.70 (d, J = 8.16 Hz, 2H), 7.21–7.23 (d, J = 8.03 Hz, 2H), 2.38 (s, 3H). A solution of 2-bromo-6-(*p*-tolyl)imidazo[2,1-*b*][1,3,4]thiadiazole (1.02 mmol) in a mixture of DCM/MeOH (3:1; 20 mL) was treated at 22 °C with a 25 wt.% solution of NaOMe (2.04 mmol) in MeOH and the reaction was stirred for 1 h at room temperature. Upon completion as determined by LCMS, the reaction mixture was quenched by addition of 1% HCl (10 mL) followed by addition of saturated $NaHCO_3$ (10 mL). The aqueous layer was extracted with DCM (3 × 15 mL), dried over magnesium sulfate and filtered. The organic layer was concentrated under reduced pressure onto silica gel and purified using Teledyne ISCO Combi-Flash system (solid loading, 12G, 100% DCM, 15 min run) to afford 2-bromo-6-(*p*-tolyl)imidazo[2,1-*b*][1,3,4] thiadiazole, **4h** (0.737 mmol) in 72% yield. LC–MS [M+H] = 246, RT = 1.113; ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.66–7.68 (dt, J = 8.21 Hz, 2H), 7.19–7.21 (d, J = 8.03 Hz, 2H), 4.18 (s, 3H), 2.37 (s, 3H).
11. *Platelet aggregation* Platelets were diluted to 2.0×10^8/mL and aliquoted into glass cuvettes. PAR4 antagonists were allowed to equilibrate with platelets for 20 min prior to stimulation with the indicated agonists. Aggregations were recorded for 10 min on a model 700 Optical Lumi Aggregometer (Chrono-log, Havertown, PA).
12. Nieman, M. T.; Schmaier, A. H. *Biochemistry* **2007**, *46*, 8603.
13. Nieman, M. T. *Biochemistry* **2008**, *47*, 13279.
14. Arachiche, A.; Mumaw, M. M.; de la Fuente, M.; Nieman, M. T. *J. Biol. Chem.* **2013**, *288*, 32553.
15. McLaughlin, J. N.; Shen, L.; Holinstat, M.; Brooks, J. D.; Dibenedetto, E.; Hamm, H. E. *J. Biol. Chem.* **2005**, *280*, 25048.