# Design and Evaluation of Heterobivalent PAR1–PAR2 Ligands as Antagonists of Calcium Mobilization

Mark W. Majewski,<sup>†,§</sup> Disha M. Gandhi,<sup>†,§</sup> Ricardo Rosas, Jr.,<sup>†</sup> Revathi Kodali,<sup>‡</sup> Leggy A. Arnold,<sup>‡</sup>

<sup>†</sup>Department of Chemistry, Marquette University, P.O. Box 1881, Milwaukee, Wisconsin 53201-1881, United States <sup>‡</sup>Department of Chemistry and Biochemistry, Milwaukee Institute for Drug Discovery, University of Wisconsin, Milwaukee, Wisconsin 53211, United States

**(5)** Supporting Information

**ABSTRACT:** A novel class of bivalent ligands targeting putative proteaseactivated receptor (PAR) heteromers has been prepared based upon reported antagonists for the subtypes PAR1 and PAR2. Modified versions of the PAR1 antagonist RWJ-58259 containing alkyne adapters were connected via cycloaddition reactions to azide-capped polyethylene glycol (PEG) spacers attached to imidazopyridazine-based PAR2 antagonists. Initial studies of the PAR1–PAR2 antagonists indicated that they inhibited G alpha q-mediated calcium mobilization in endothelial and cancer cells driven by both PAR1 and PAR2 agonists. Compounds of this novel class hold promise for the prevention of restenosis, cancer cell metastasis, and other proliferative disorders.



**KEYWORDS:** Bivalent ligand, protease-activated receptor, PAR1 antagonist, PAR2 antagonist, calcium mobilization, metastasis, restenosis

Protease-activated receptors (PARs) are a unique family of class A G-protein-coupled receptors (GPCRs) that are activated by extracellular proteases, which reveal a tethered ligand at the N-terminus.<sup>1,2</sup> Self-activation of PARs by their tethered ligand agonists leads to a wide range of cellular responses, in part due to the fact that PARs are highly expressed in different tissues with varying signaling components; they can also be activated by different proteases that cleave at different locations on the Nterminus.<sup>3</sup> The unusual complexity of PAR-mediated signaling is further increased by the potential for PARs and other GPCRs to form multireceptor complexes in cell membranes.<sup>4</sup> More specifically, evidence has emerged in recent years that PARs form homomeric and heteromeric complexes among the four different PAR subtypes, and the signals mediated by these complexes are distinct from those of the monomers.<sup>5</sup> Additionally, the N-terminus of one PAR subtype can directly activate a neighboring PAR.<sup>8</sup>

The range of productive and pathological signaling for which PARs have been implicated has prompted their study as potential targets for numerous indications, including thrombosis, inflammation, stroke, kidney disease, reperfusion injury, and cancer cell metastasis (vide infra). Despite their therapeutic promise, to our knowledge only a few PAR modulators have thus far reached clinical stages, all as antithrombotic agents inhibiting platelet activation.<sup>9–12</sup>

The present lack of clinical PAR modulators for other indications may reflect in part the pleiotropic signaling of PARs, which may complicate the selective inhibition of pathological signals without adversely affecting normal signaling. For example, PAR1 antagonists may prevent thrombosis via inhibition of PAR1 on platelets, while concurrently interfering with endothelial barrier integrity by inhibiting normal PAR1 signaling in endothelium. This has inspired our recent efforts to identify biased ligands that may inhibit or activate only a subset of PAR1-mediated signaling pathways.<sup>13–15</sup>

An alternative therapeutic approach could utilize heterobivalent ligands to selectively target the differential signaling mediated by PAR heteromeric complexes (Figure 1). To our knowledge, no ligands selective for PAR heteromers have been reported. Evidence has been accumulating in recent years that GPCRs frequently form oligomers in vitro, but the in vivo



Figure 1. Proposed heterobivalent PAR ligands.

Received:	November 7, 2018
Accepted:	December 3, 2018
Published:	December 3, 2018

relevance of these oligomers has been harder to quantify, in part due to a lack of adequate chemical tools.<sup>4</sup> The concept of bivalent ligands for multimeric GPCRs was pioneered by Portoghese nearly 40 years ago,<sup>16</sup> and this strategy has been used to generate compounds primarily for the study of CNS GPCRs.<sup>17,18</sup> Recently, promising in vivo data have been obtained that suggest that bivalent ligands can modulate GPCRs in a manner distinct from that of their monovalent counterparts.<sup>19–22</sup> This approach holds particular promise for PARs, as the differential tissue expression of PAR subtypes could permit tissue-selective targeting of PAR heteromers using heterobivalent ligands. This is best exemplified by the fact that PAR2 is not expressed on human platelets; therefore, we reasoned that PAR2-directed heterobivalent ligands could permit the selective modulation of signaling by PAR2containing heteromers in vascular tissues without adversely affecting platelet activation and hemostasis.

Mueller and colleagues presented evidence that thrombinenhanced migration of melanoma cells is driven by PAR1 and PAR2, in a manner consistent with the "direct transactivation" of PAR2 by thrombin-activated PAR1.<sup>23</sup> Bar-Shavit has also reported synergistic effects of PAR1 and PAR2 activation in tumor development<sup>24,25</sup> Kuliopulos reported that signaling by PAR1–PAR2 heteromers may be implicated in restenosis, the dangerous narrowing of blood vessels which occurs in a significant fraction of patients after percutaneous interventions (PCIs).<sup>26</sup>

We hypothesize that heterobivalent antagonists may act selectively at putative PAR1-PAR2 heteromers and thus may act to inhibit cancer cell metastasis and proliferative processes such as restenosis, while minimizing impacts on normal PAR signaling. PAR1 antagonists have been investigated for restenosis in animal models,<sup>27,28</sup> and individual PAR1<sup>29,30</sup> and PAR2<sup>31</sup> antagonists have been studied in cancer cell metastasis and invasion. To our knowledge, however, none of these studies has progressed to clinical stages for these indications. One complication may arise from the fact that the tethered ligand of PAR1 can directly transactivate a neighboring PAR2 receptor, which could possibly happen even in the presence of a PAR1 antagonist.<sup>8</sup> Together, these observations make the preparation of multivalent PAR ligands an attractive strategy with benefits that may extend beyond the administration of multiple separate ligands. Herein we describe our initial efforts toward the synthesis and study of heterobivalent ligands targeting PAR1 and PAR2.

The primary considerations for the design of heterobivalent ligands are as follows: 1. What monovalent ligands should be used? 2. What spacers should be used to connect them? 3. Where should the spacer be attached to each ligand? Based on its published SAR data and potential for diverse modifications, we elected to utilize the PAR1 antagonist RWJ-58259 as the first PAR1 scaffold for our bivalent ligands<sup>27,32</sup> and an imidazopyridazine scaffold reported by Vertex as the PAR2 antagonist.<sup>33</sup> To connect the ligands, we elected to first utilize commercially available polyethylene glycol (PEG)-based spacers with a variety of lengths, particularly for their beneficial physicochemical properties, especially increased water solubility. Also beneficial is the fact that bivalent ligands with PEG spacers with excess "slack" may pay less of an entropic penalty upon binding relative to those with more hydrophobic spacers, <sup>34</sup> presumably because hydrophilic PEGs can maintain flexibility in water and do not require highly ordered solvation shells. The use of commercially available amino-PEG-azide spacers permits convenient coppercatalyzed alkyne/azide cycloadditions (CuAAC), suitable for the connection of two appropriately functionalized ligands via the PEG spacer under diverse conditions.<sup>35</sup> We recently reported the

synthesis of an alkyne-tethered version of RWJ-58259, which retained significant potency at PAR1.<sup>15</sup> Based on the SARs published for the imidazopyridazine reported by Vertex, we elected to prepare carboxyl-substituted versions of these PAR2 antagonists using the reported synthetic route (Scheme 1).<sup>33</sup> 3-





Amino-6-chloropyridazine (1) was subjected to Suzuki coupling with 4-fluorophenylboronic acid to yield the biarylamine 2. Bromination followed by alkylation with bromomethylpyruvate yielded the imidazopyridazine 4. Suzuki coupling with the propenylboronic ester A gave 5, and hydrogenation provided the ester 6. Hydrolysis followed by coupling with piperazine B yielded amide 8. Removal of the Boc group, coupling with succinic acid monomethyl ester, and hydrolysis of the resulting ester gave carboxylic acid 10a. PAR2 antagonists containing an arene substituent in place of the isopropyl group of 10a and a cyclohexyl carboxylic acid in place of the eastern ethylene group were among numerous analogs previously reported;<sup>33</sup> therefore, carboxylic acid 10b was additionally prepared (Scheme S1). Both of the carboxylic acids 10a and 10b were confirmed to be highly potent antagonists of PAR2 (vide infra), so we proceeded to couple them to a variety of PEG-based spacers (Schemes 2 and S2). Amide coupling with amino (PEG) azides, followed by CuAAC<sup>36</sup> with the previously synthesized RWJ-58259-based alkyne DG-207,<sup>15</sup> yielded heterobivalent ligands 13a-d. Control compounds 15–18 composed of the monovalent ligands with attached PEG spacers are depicted in Figure S1.

During the course of our studies, Marshall and colleagues reported several X-ray crystal structures of modified PAR2, including a cocrystal of PAR2 with the inhibitor AZ3451 at an allosteric site at the side of the receptor.<sup>37</sup> The structural similarities between **10a** and AZ3451 led us to hypothesize that they may share a binding site on PAR2, which was supported by rigid protein docking studies (Figure 2a)<sup>38</sup> and is consistent with the allosteric mode of inhibition recently reported by Fairlie for the Vertex<sup>33</sup> imidazopyridazine I-191.<sup>39</sup> Compound **10a** docks into the lipophilic pocket at the side of PAR2 in a similar pose to AZ3451, with both molecules possessing a heterocyclic nitrogen capable of accepting a hydrogen bond from Tyr210.

#### Scheme 2. Assembly of Heterobivalent PAR1-PAR2 Antagonists





**Figure 2.** (A) X-ray structure of modified PAR2 with bound allosteric ligand AZ3451 (green) and docked ligand **10a** (pink). (B) Novel bivalent ligand **20** and control compound **19** possessing a PEG-aminoalkylamide spacer.

The position of this binding site within the cell membrane suggests that the hydrophilic PEG spacer may be counterproductive and could explain the greatly decreased potency at PAR2 of our PEG-linked PAR2 antagonists. We addressed this potential issue by synthesizing modified spacers containing a terminal aminoalkylamide that could possess a higher affinity for the cell membrane above the putative PAR2 binding site (Figure 2b and Scheme S3).

The ligands were tested in our intracellular calcium mobilization assay ( $iCa^{2+}$ ) using the transformed human endothelial cell line EA.hy926, and this assay was also suitable for studying the signaling of the breast cancer cell line MDA-MB-231, which expresses both PAR1 and PAR2. Adherent cells were cultured in 96-well plates to confluence over ~48 h, then loaded with the fluorescent calcium binding dye Fluo-4/AM, according to our reported protocol.<sup>15</sup> Addition of the antagonists, followed by the relevant PAR peptide agonists TFLLRN-NH<sub>2</sub> (PAR1), SLIGKV-NH<sub>2</sub> (PAR2), or SFLLRN-NH<sub>2</sub> (PAR1/2), permitted the measurement of inhibition of Gq-driven signaling from both PAR1 and PAR2. All concentration—response curves show data

from N = 3 on a single plate, fitted using four-variable nonlinear regression with GraphPad Prism v. 5. Error bars represent standard error of the mean (SEM) for the three measurements at each concentration.

Letter

The attachment of PEG spacers to the PAR1 antagonist RWJ-58259 and the PAR2 antagonist **10a** was tolerated, albeit with 5- to 70-fold decreases in potency. Half-maximal inhibitory concentration (IC<sub>50</sub>) for the inhibition of TFLLRN-NH<sub>2</sub> (5  $\mu$ M) PAR1mediated *i*Ca<sup>2+</sup> mobilization increased from 0.02 to 0.1  $\mu$ M for the PEG spacer-linked control **17** (Figure 3a). Compound **10a** 



**Figure 3.** Inhibition of TFLLRN-NH<sub>2</sub> (5  $\mu$ M) PAR1-mediated *i*Ca<sup>2+</sup> mobilization by monovalent PAR1 ligand RWJ-58259 and spacer-linked control 17 (A) in EA.hy926 endothelial cells and (C) in MDA-MB-231 cancer cells; inhibition of SLIGKV-NH<sub>2</sub> (5  $\mu$ M) PAR2-mediated *i*Ca<sup>2+</sup> mobilization by monovalent PAR2 ligand **10a** and spacer-linked control **15** (B) in EA.hy926 endothelial cells and (D) in MDA-MB-231 cancer cells.

inhibited SLIGKV-NH<sub>2</sub> (5  $\mu$ M) PAR2-mediated *i*Ca<sup>2+</sup> mobilization with an IC<sub>50</sub> of 0.1  $\mu$ M, and the PEG spacer-linked control **15** was significantly less potent (IC<sub>50</sub> = 7  $\mu$ M, Figure 3b). This is consistent with a report from Bunnett (published during revision of this manuscript) that describes an imidazopyridazine PAR2 antagonist that suffers an approximately 1000-fold drop in potency upon attachment of a PEG tether at a similar site.<sup>40</sup> These

compounds also showed good potencies in analogous assays with MDA-MB-231 cancer cells (Figures 3c,d).

As described above, we proceeded to prepare bivalent ligands possessing spacers of different lengths, from shortest (21 atoms, **13a**) to longest (72 atoms, **13d**). To support their potential utility for future in vivo experiments, the stability of **13d** was measured in mouse plasma, and a half-life of 5.6 h was determined (see Supporting Info for details). All four ligands showed good inhibition of PAR1-driven (TFLLRN-NH<sub>2</sub>) calcium mobilization in EA.hy926 cells, with comparable IC<sub>50</sub>s ranging from 0.03 to 0.18  $\mu$ M (Figure 4a). The efficacy of the longest ligand (**13d**) was



**Figure 4.** Inhibition of TFLLRN-NH<sub>2</sub> (5  $\mu$ M) PAR1-mediated *i*Ca<sup>2+</sup> mobilization (A) and SLIGKV-NH<sub>2</sub> (5  $\mu$ M) PAR2-mediated *i*Ca<sup>2+</sup> mobilization (B) by bivalent ligands **13a**-**d** in EA.hy926 endothelial cells.

higher than the others at the highest concentrations. All four ligands had much lower potency and efficacy as PAR2 antagonists, using SLIGKV-NH<sub>2</sub> as agonist (Figure 4b). An IC<sub>50</sub> could only be estimated from the concentration—response curve fitted to the data from **13d**, with an IC<sub>50</sub> of 3.3  $\mu$ M, and with only partial inhibition at the highest concentration (31.6  $\mu$ M). The decreased potencies and efficacies of the bivalent ligands at PAR2, particularly comparing these ligands to the PEG-linked PAR2 antagonist controls, may reflect a higher affinity of the bivalent ligands for PAR1, and/or a higher receptor density for PAR1, such that ligands may be binding significantly to monomeric PAR1.

Next, the bivalent ligands 13a-d were tested for their ability to inhibit the PAR1/2 agonist SFLLRN-NH<sub>2</sub>, dosed at 3.16  $\mu$ M (Figure 5a). Again, the ligand with the longest spacer (13d) proved to be most potent, with an IC<sub>50</sub> of 0.15  $\mu$ M. It was also compared to the control compounds composed of monovalent PAR1 (17) and PAR2 (15) ligands with spacers and their combination (Figure 5b). The combination of equal concentrations of 17 and 15 gave an IC<sub>50</sub> of 0.32  $\mu$ M, resulting in an inhibition profile very similar to that of the bivalent ligand 13d.



**Figure 5.** Inhibition of SFLLRN-NH<sub>2</sub> (3.16  $\mu$ M) PAR1/2-mediated *i*Ca<sup>2+</sup> mobilization by bivalent ligands **13a–d** (A) and individual and codosed monovalent control compounds (**17** + **15**) in EA.hy926 endothelial cells (B).

The inability of the PAR2 ligand **15** to inhibit SFLLRN- $NH_2$  to any measurable degree suggests that its signaling is largely driven by PAR1 with EA.hy926 cells. Inhibition with the bivalent ligands derived from the PAR2 ligand **10b** was inferior to those derived from **10a**. These data are included in the Supporting Information (Figures S2–S4).

The bivalent ligand 13d was next studied in the breast cancer cell line with all three agonists (Figure 6). It was most effective at



**Figure 6.** Inhibition of TFLLRN-NH<sub>2</sub> (5  $\mu$ M) PAR1-mediated, SLIGKV-NH<sub>2</sub> (5  $\mu$ M) PAR2-mediated, and SFLLRN-NH<sub>2</sub> (3.16  $\mu$ M) PAR1/2-mediated *i*Ca<sup>2+</sup> mobilization of **13d** in MDA-MB-231 breast cancer cells.

the inhibition of TFLLRN-NH<sub>2</sub>-driven calcium mobilization, with an IC<sub>50</sub> of 0.14  $\mu$ M and excellent efficacy. The IC<sub>50</sub> for inhibition of SFLLRN-NH<sub>2</sub> was 3.3  $\mu$ M, with lower efficacy. The inhibition of SLIGKV-NH<sub>2</sub> by **13d** was less efficient, with an IC<sub>50</sub> estimated at 3.5  $\mu$ M, but with no inhibition observed at submicromolar concentrations. The bivalent ligand possessing a more hydrophobic spacer (**20**, Figure 2b) was also tested for its possible improved activity at PAR2; unfortunately, attachment of this spacer did not improve the inhibition of PAR2-mediated signaling (Figures S6–S8).

Finally, **13d** was tested for its ability to inhibit the natural PAR1and PAR2-activating proteases thrombin (Figure S9) and trypsin (Figure S10). Compound **13d** inhibited 1 nM thrombin with an IC<sub>50</sub> of 0.36  $\mu$ M in Ea.hy926 cells, but interestingly, the spacerlinked control **17** was ~10× more potent. The PAR2 antagonist **10a** was a potent inhibitor of 5 nM trypsin (IC<sub>50</sub> = 0.002  $\mu$ M), but attachment of a spacer (**15**) abrograted its activity, and the bivalent ligand **13d** also showed no inhibition of trypsin.

We have reported the first examples of bivalent PAR1-PAR2 antagonists, and we demonstrated that the optimal compound 13d can perform similarly to the combination of monovalent control ligands in the inhibition of intracellular calcium mobilization in the transformed endothelial cell line EA.hy926, as well as the epithelial breast cancer cell line MDA-MB-231. However, the decrease in potency observed upon attachment of PEG-based spacers to the monovalent PAR1 and PAR2 ligands means that the ligands are less potent than the combination of monovalent ligands without spacers attached, though there is always a significant disadvantage of dosing multiple drugs to obtain a desired therapeutic effect. These considerations may be less important than the ability of a heterobivalent ligand to selectively inhibit a putative heteromeric receptor complex that drives a pathological effect, such as metastasis, without interfering with healthy cell signaling. We have not yet obtained evidence that the bivalent ligands disclosed here selectively inhibit putative PAR1–PAR2 complexes; an example of such evidence would be biphasic concentration-response curves, where the ligands inhibit a population of heteromers at lower concentrations. However, the ability to detect low concentrations of such complexes is limited by the precision of the calcium mobilization assay. Receptor binding assays with labeled ligands are planned

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that would be more suitable for the detection of low populations of heteromers, and studies on PAR-driven cancer cell migration are also underway. The main weakness of the present ligands is their decrease in potency at PAR2 upon attachment of spacers to the PAR2 antagonist scaffolds, and therefore, we are presently investigating alternative PAR2 antagonists and spacers. Bivalent PAR1–PAR2 antagonists are a novel class of ligands that may hold promise for the inhibition of cancer cell metastasis, restenosis, and other proliferative diseases.

# ASSOCIATED CONTENT

#### **Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchem-lett.8b00538.

Additional abbreviations; Schemes S1–S3; Figures S1–S10; assay protocols; plasma stability assay protocol and data for **13d**; synthetic protocols; and characterization data (<sup>1</sup>H NMR, <sup>13</sup>C NMR, and LC–MS chromatograms) (PDF)

# AUTHOR INFORMATION

#### **Corresponding Author**

\*E-mail: christopher.dockendorff@mu.edu. Tel: 1-414-288-1617.

#### ORCID 0

Leggy A. Arnold: 0000-0003-1411-1572 Chris Dockendorff: 0000-0002-4092-5636

#### **Author Contributions**

<sup>§</sup>These authors contributed equally to this manuscript.

#### **Author Contributions**

Conceived project: C.D. Designed bivalent ligands: C.D. Synthesized and characterized bivalent ligands: M.W.M, D.M.G., R.R. Performed pharmacology: M.W.M., D.M.G. Analyzed data: D.M.G., M.W.M., C.D. Determined plasma stability of **13d**: R.K., L.A.A. Wrote manuscript: C.D., M.W.M., D.M.G.

## Funding

We thank Marquette University for startup funding and a Summer Faculty Fellowship/Regular Research Grant (to support R.R. and C.D.), the National Heart, Lung, and Blood Institute (NIH R15HL127636) for support of our research program, and the Clinical and Translational Science Institute of Southeastern Wisconsin (8UL1TR000055) for support of a complementary project that impacted the work disclosed here.

## Notes

An earlier version of this manuscript was submitted to the preprint server ChemRxiv: https://doi.org/10.26434/chemrxiv. 6394430.v1.

The authors declare the following competing financial interest(s): A patent application including this work has been submitted.

# ACKNOWLEDGMENTS

We thank Dr. Hartmut Weiler, Irene Hernandez, and Trudy Holyst (Blood Research Institute) for assistance with cell culture and assay troubleshooting, Dr. Michael Dwinell and Donna McAllister (Medical College of Wisconsin) for helpful advice and a sample of MDA-MB-231 cells, Prof. Nicolas Moitessier for providing access to FITTED docking software, Dr. Rachel Jones (Medical College of Wisconsin) for advice regarding the CuAAC reaction, Elliot DeMilo (Univ. Wisconsin-Milwaukee) for assistance with the stability study, and Dr. Sheng Cai (Marquette University) for assistance with LC–MS and NMR instruments. We thank ACD/Labs (NMR processing) and ChemAxon (NMR prediction and property prediction) for providing access to software.

#### ABBREVIATIONS

Boc, *tert*-butoxycarbonyl; CuAAc, copper-catalyzed alkyne/azide cycloadditions; DCE, 1,2-dichloroethane; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DPPF, 1,1'-bis(diphenylphosphino)ferrocene; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; GPCR, G-protein coupled receptor; HATU, hexafluorophosphate azabenzotriazole tetramethyl uronium; HOBt, 1-hydroxybenzotriazole; IC<sub>50</sub>, half-maximal inhibitory concentration; *i*Ca<sup>2+</sup>, intracellular calcium mobilization; MI, myocardial infarction; PAR, protease-activated receptor; PCI, percutaneous interventions; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SEM, standard error of the mean; TBTA, tris(benzyltriazolylmethyl)amine

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