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Q94 is not a selective modulator of proteinase-activated receptor 1 (PAR1) in platelets

Luc R. A. Francis, Sarah L. Millington-Burgess, Taufiq Rahman, & Matthew T. Harper

Department of Pharmacology, University of Cambridge, Cambridge, UK

Abstract
Thrombin is a potent platelet activator, acting through proteinase-activated receptors –1 and –4 (PAR1 and PAR4). Of these, PAR-1 is activated more rapidly and by lower thrombin concentrations. Consequently, PAR-1 has been extensively investigated as a target for antiplatelet drugs to prevent myocardial infarction. Q94 has been reported to act as an allosteric modulator of PAR1, potently and selectively inhibiting PAR1-Gαq coupling in multiple cell lines, but its effects on human platelet activation have not been previously studied. Platelet Ca2+ signaling, integrin αIIbβ3 activation and α-granule secretion were monitored following stimulation by a PAR1-activating peptide (PAR1-AP). Although Q94 inhibited these responses, its potency was low compared to other PAR1 antagonists. In addition, αIIbβ3 activation and α-granule secretion in response to other platelet activators were also inhibited with similar potency. Finally, in endothelial cells, Q94 did not inhibit PAR1-dependent Ca2+ signaling. Our data suggest that Q94 may have PAR1-independent off-target effects in platelets, precluding its use as a selective PAR1 allosteric modulator.

Introduction
Platelets are the key cell in arterial thrombosis, the underlying trigger of most heart attacks and ischemic strokes, and responsible for many deaths worldwide [1]. Thrombin is a potent platelet activator, acting through proteinase-activated receptor-1 and –4 (PAR1 and PAR4) [2,3]. Of these, PAR-1 is activated more rapidly and by lower thrombin concentrations. PAR1 cleavage by thrombin reveals a new N-terminal tethered ligand that triggers signaling through Gαq, Gi12/13 and, in some cells, Gq1 [3]. However, the PAR1 N-terminus can be cleaved by other proteases to reveal different tethered ligands with biased signaling compared to the ligand revealed by thrombin [5]. Activated protein C (APC) is anti-thrombotic, anti-inflammatory and pro-fibrinolytic [6]. In endothelial cells, APC cleaves PAR1 to promote barrier integrity and reduces endothelial cell death in inflammation [7], in a β-arrestin-dependent but Gαq-independent manner [8]. These opposing roles of PAR1 have important consequences for inhibition of PAR1.

Vorapaxar (SCH530348) is the only PAR1 antagonist yet approved for prevention of myocardial infarction [9,10]. Vorapaxar is a competitive, reversible non-peptide antagonist, with long receptor off-rate [11]. However, vorapaxar increases intracranial hemorrhage risk in some patients, which may be due to inhibition of the cytoprotective effects of APC in the endothelium [10,12]. An alternative strategy is to inhibit pro-thrombotic Gαq signaling downstream of PAR1 but leave Gαi-dependent cytoprotective signaling intact [4,10]. Parmodulin (ML161, JF5) are allosteric modulators of PAR1, acting at the intracellular face of PAR1 to inhibit PAR1’s Gαq-dependent Ca2+ signaling in platelets without inhibiting APC-mediated cytoprotection [12–14]. Alternatively, parmodulins may directly stimulate cytoprotective signaling from PAR1, acting as allosteric agonists [15]. The intracellular face of PAR1 therefore represents an intriguing target for drug action.

Q94 has also been reported to inhibit PAR1-Gαq coupling. It is presumed to act at the intracellular face of PAR1 as it was identified in competitive screen between a Gαq peptide mimic and the PAR1 C-terminus [16]. Q94 inhibited thrombin-induced responses in mouse lung fibroblasts, A549 cells and human microvascular endothelial cells (HMECs) [16–18]. Q94 also reduced doxorubicin-induced albuminuria in a mouse model of nephropathy [19], and activated factor X (FXa)-induced myofibroblast differentiation in lung injury [20]. We had initially planned to characterize the interaction between Q94 and PAR1 in platelets with the aim of exploring related molecular scaffolds as potential allosteric modulators. However, we were unable to repeat in platelets the inhibitory effect reported in other cells with Q94 from two sources. Our data also suggest that Q94 has PAR1-independent off-target effects in platelets, precluding its use as a selective PAR1 allosteric modulator.

Materials and Methods

Materials

Chemicals were obtained from Sigma Aldrich (UK) unless otherwise stated. Q94 was obtained from Tocris (Bristol, UK) and ChemDiv (San Diego, USA). Vorapaxar and ML161 were from Generon (Slough, UK). PAR1-AP (SFLLRN-amide) and PAR4-AP (AYPGKF-amide) were from Bachem (Weil am Rhein, Germany). U46619 was from ApexBio (Stratech, Cambridge, UK). Cal520-AM was from AAT Bioquest (Stratech, Cambridge, UK). Fluorescein...
isothiocyanate (FITC)-conjugated PAC-1 and phycoerythrin (PE)-conjugated anti-CD62P antibodies were from BD Biosciences (Wokingham, UK).

Isolation of Platelets from Human Blood

The use of blood from healthy drug-free volunteers was approved by the Human Biology Research Ethics Committee, University of Cambridge. Volunteers gave written informed consent in accordance with the Declaration of Helsinki. Blood was collected by venepuncture into vacutainers containing sodium citrate (3.2% v/v). Washed platelets were prepared as described previously [21] in HEPES-buffered saline (HBSS; pH 7.4, 135 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM MgCl₂, and 0.34 mM NaH₂PO₄ supplemented with 5 mM D-glucose) with apyrase (0.02 U/ml). Washed platelets were rested (30°C; 30 minutes) before experiments took place. CaCl₂ (2 mM) was added immediately prior to stimulation.

Platelet Ca²⁺ Signaling

Platelets were loaded with Cal520 as previously described [22]. Cal520-loaded washed platelets were incubated with antagonists or vehicle control for 30 minutes prior to stimulation, then stimulated in 96-well plates (black-walled, clear-bottomed; 180 μl/well; 5 × 10⁹ platelets/mL; 37°C) with PAR1-AP dispensed by plate reader on-board injectors (FluorStar Omega, BMD Labtech). Fluorescence (excitation, 485 nm; emission, 520 nm) was recorded every 5 seconds for 300 seconds, with 20 seconds recorded prior to stimulation.

Flow Cytometry

Platelets (1 × 10⁹/ml) were incubated with antagonists (30 minutes) prior to stimulation with agonists (5 minutes; 37°C), stained with antibodies (2 minutes; room temperature), then fixed with 1% paraformaldehyde. Samples were analyzed by flow cytometry (BD Accuri C6). Compensation was performed using OneComp eBeads (ThermoFisher). Antibodies used were FITC-conjugated PAC-1 antibody, which binds activated integrin αIIbβ₃, and PE-conjugated anti-human CD62P antibody, as a marker of α-granule secretion, at dilutions 1:20 and 1:25 respectively.

Human Umbilical Vein Endothelial Cell (HUVEC) Ca²⁺ Signaling

HUVEC were cultured as previously described [23]. For experiments, HUVEC was seeded in 96-well plates (2 × 10⁴ cells/well) and grown overnight (5% CO₂ at 37°C) to confluence. Media was removed and cells were loaded with Cal520 (2 μM in Hanks’ Balanced Salt Solution [HBSS]; 30 minutes) then washed twice with HBSS. HUVECs were incubated with antagonists (30 minutes; room temperature) prior to stimulation. PAR1-AP or control was dispensed by on-board injectors of a fluorescence plate reader (FluoStar Omega; BMG Labtech). Fluorescence was recorded every 3 seconds for 180 seconds, with 15 seconds recorded prior to stimulation.

Data Analysis

Cal520 fluorescence was normalized to the mean fluorescence prior to agonist addition to give F/F₀. Where shown, average data are mean ± standard error of mean (s.e.m.). The number of independent biological replicates is indicated in the figure legends. Inhibition curves were fitted with a variable slope model with the equation Y = 100/(1 + 10^((-Log(IC50-X)*HillSlope))), where Y is the response as % of control and X is the Log[inhibitor], using Prism (Graphpad, v9). Where this model did not give a good fit, data are presented without curve fitting. For these data, 1-way ANOVA followed by Dunnett’s multiple comparison test was used (* p < .05; ** p < .01, compared to control).

Results

Q94 Inhibits PAR1-dependent Platelet Activation with Low Potency

To investigate the effect of different PAR1 antagonists on platelet Ca²⁺ signaling, Cal520-loaded platelets were treated with different concentrations of antagonist, or DMSO (control), and stimulated with PAR1-AP (10 μM). Several orthosteric antagonists of PAR1 (vorapaxar, SCH-79797, and FR171113) inhibited the increase in Cal520 fluorescence in a concentration-dependent manner, with an IC₅₀ of 0.02 μM (vorapaxar), 0.18 μM (SCH-79797), and 0.36 μM (FR171113), respectively (Figure 1a). Similarly, ML161 and JF5, allosteric modulators of PAR1, also inhibited the increase in fluorescence in a concentration-dependent manner. The IC₅₀ of ML161 was 1.10 μM, whereas JF5 was less potent, with an IC₅₀ of 12.8 μM (Figure 1b). Although Q94 (Tocris) also inhibited fluorescence increase, its potency was low compared to most other drugs tested (IC₅₀ 16.4 μM; Q94 is shown in both Figure 1a,b to aid comparison.) Representative traces are shown in Figure 1c. To confirm the relatively low potency of Q94, we also purchased Q94 from ChemDiv, the source of Q94 in Deng et al [16]. ChemDiv Q94 showed lower potency to Q94 from Tocris (IC₅₀ of 35.9 μM; Figure 1d).

To further investigate the effect of Q94 (Tocris), PAR1-AP-induced αIIbβ₃ activation and α-granule secretion were measured. Q94 displayed some inhibitory action but again with low potency (Figure 1e,f). Vorapaxor and ML161 inhibited both platelet functions, demonstrating that this assay is sensitive to orthosteric antagonists and intracellular allosteric modulators.

Q94 Inhibits PAR1-dependent Ca²⁺ Signaling in HUVECs with Low Potency

Q94 has been previously described in human microvascular endothelial cells (HMECs) as inhibiting PAR1-dependent Ca²⁺ signaling. However, we could not see a similar inhibitory effect of Q94 (Tocris or ChemDiv) in human umbilical vein endothelial cells (HUVECs). Q94 (Tocris) partially inhibited the peak increase in F/F₀ at high concentrations, but both Q94 (Tocris) and Q94 (ChemDiv) increased AUC at concentrations greater than 1 μM (Figure 2). In contrast, PAR1-dependent Ca²⁺ signaling was inhibited by vorapaxor or ML161, demonstrating that this assay is sensitive to orthosteric antagonists and intracellular allosteric modulators.

Q94 Inhibits Platelet Activation Downstream of Several Platelet Receptors

To investigate the receptor selectivity of Q94, platelets were stimulated with PAR4-AP, the thromboxane analogue, U46619, or the P2Y₁₂ agonist, ADP. Each agonist triggered αIIbβ₃ activation and α-granule secretion. Q94 inhibited these functional responses to each agonist (Figure 3), indicating that its effect is not selective to PAR1. PAR4-AP-stimulated platelets were least affected, with inhibition of αIIbβ₃ activation but no significant inhibition of α-granule secretion. The responses to ADP were more strongly inhibited by Q94 greater than 10 μM. The effect on U46619-induced platelet activation was more complex, with Q94 enhancing αIIbβ₃ activation and α-granule secretion at 10 μM or below, an effect that reversed above 10 μM. Despite this more complex pattern, it is clear that Q94 is not selective for PAR1-dependent platelet activation.
Discussion

Platelet PAR1 has attracted extensive attention as an anti-thrombotic target [3], with vorapaxar approved for the prevention of myocardial infarction in patients without a history of stroke or bleeding [9]. However, vorapaxar blocks cytoprotective signaling downstream of APC in addition to pro-thrombotic signaling downstream of thrombin [12]. In contrast, allosteric modulators may inhibit pro-thrombotic signaling, particularly that mediated by Gαq, without blocking the cytoprotective signals, providing an alternative therapeutic approach. Unfortunately, although Q94 is reported to be a potent, selective allosteric inhibitor of PAR1-Gαq coupling, we were unable to observe potent inhibition of PAR1-dependent Ca²⁺ signaling in platelets and found off-target inhibition of other platelet activators at similar concentrations.

PAR1-dependent Ca²⁺ signaling was inhibited by a range of orthosteric antagonists and allosteric modulators. Q94 showed concentration-dependent inhibition of PAR1 signaling in this study, with an IC₅₀ of 16 μM (Q94 Tocris). We were surprised
by the high IC$_{50}$ of Q94. In a previous study, Q94 potently inhibited thrombin-induced Ca$^{2+}$ signaling in HMECs (IC$_{50}$ 0.01 μM) and was approximately 5-times more potent than SCH-79797 [18]. In contrast, in our study Q94 (Tocris) was 91-times less potent than SCH-79797. Although the IC$_{50}$ itself will depend on experimental conditions, the difference in relative potency compared to SCH-79797 suggested that Q94 (Tocris) was not acting in a potent manner in platelets. We therefore obtained Q94 from ChemDiv, the original source in Deng et al [16]. Q94 (ChemDiv) inhibition of PAR1 signaling also had a high IC$_{50}$ (36 μM). We observed similar low potency at inhibiting key platelet function responses, integrin αIIbβ3 activation and α-granule secretion. These data demonstrate that Q94 is not a potent modulator of PAR1-dependent platelet activation. Similarly, in a Doctoral thesis, Q94 (synthesized in house or purchased from ChemDiv) was reported to be ineffective on thrombin-induced Ca$^{2+}$ release in lung fibroblasts and of poor solubility [24]. It is possible that Q94 is unstable and rapidly degrades, in which case it is an unreliable modulator of PAR1. Whether lacking in potency, solubility or stability, Q94 is not a useful tool to probe PAR1-Gq signaling in platelets.

Since Q94 has been characterized in endothelial cells [18], we examined whether it has an inhibitory effect in HUVEC. Whereas vorapaxar and ML161 both inhibited PAR1-AP-induced Ca$^{2+}$
signaling. Q94 had no inhibitory effect. Surprisingly, a weak enhancement of Ca\(^{2+}\) signaling was seen at some concentrations of Q94 (both Tocris and ChemDiv). Although we do not know the mechanism that underlies this enhancement, it has also been reported in EA.hy926 cells [14]. As this enhancement was not seen with either vorapaxar or ML161, it appears to be independent of PAR1.

Most problematically, Q94 also inhibited platelet α\(_{\text{IIb}}\)β\(_{3}\) activation and α-granule secretion independently of PAR1. This suggests that the inhibition of PAR1-dependent integrin α\(_{\text{IIb}}\)β\(_{3}\) activation and α-granule secretion may be unrelated to direct inhibition of PAR1 but instead be an effect on downstream intracellular signaling. Q94 most potently inhibited ADP-induced platelet activation, with relatively less effect on PAR4-AP-induced platelet activation. This may be because activation of PAR4 results in stronger activation of platelets than does activation of P2Y\(_1\) and P2Y\(_{12}\). Q94 also had a complex effect on U46619-induced platelet activation, with enhanced platelet activation at some concentrations of Q94. The underlying mechanism of this enhancement is not clear, but it further supports the conclusion that the effects of Q94 in platelets are not specific for PAR1 signaling.

In conclusion, Q94 is not a potent, selective modulator of platelet PAR1. Rather, it appears to inhibit platelet activation downstream of several receptors with similar, low potency. Q94 is therefore not a suitable tool for studying PAR1 in platelets and is unlikely to be a useful lead in developing PAR1 allosteric modulators.

**Author Contributions**
LRAF performed and analysed experiments and edited the manuscript. SLM performed experiments, supervised experiments, and edited the manuscript. TR directed research and edited the manuscript. MTH directed research, designed experiments and wrote the manuscript.

**Disclosure Statement**
No potential conflict of interest was reported by the author(s).

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**ORCID**
Taufiq Rahman http://orcid.org/0000-0003-3830-5160

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