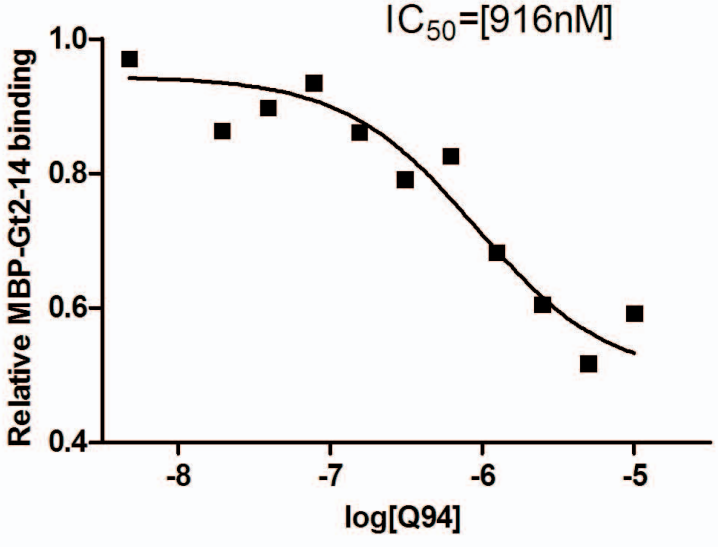
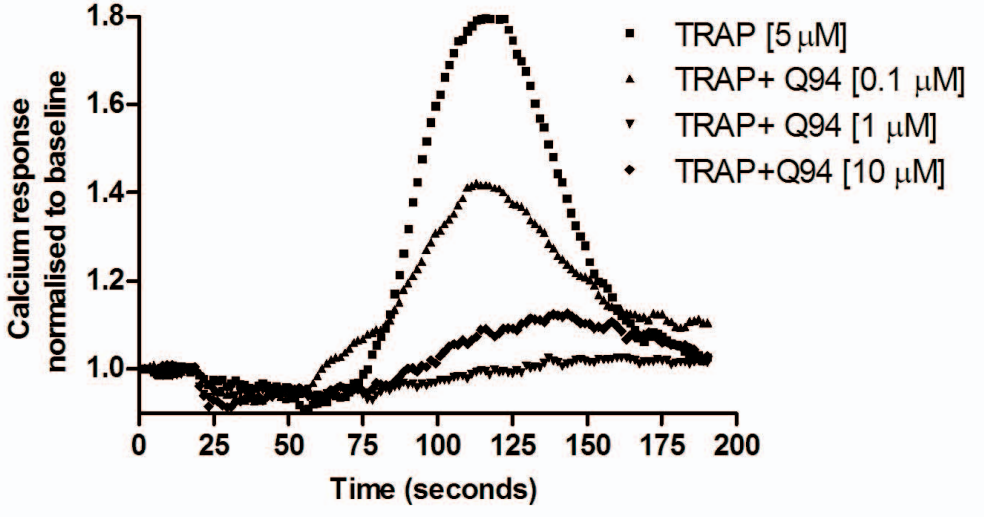
E07-07-0720 Chambers

Supplementary Data Figure 1. Q94 displaces the high affinity binding peptide MBP-T2-14 from activated PAR_1 and blocks thrombin induced Ca^{2+} transients in HEK293 cells. For the binding studies (left panel) PAR_1 receptors expressed in membranes of SF9 cells were treated with [100 nM] TRAP. Purified T2-14 fused with *E. coli* maltose binding protein (MBP-T2-14)[100 nM] was added to wells and allowed to bind to the receptor. Increasing amounts of Q94 were added to wells in triplicate, binding of MBP-T2-14 was measured using an ELISA for MBP. Data presented are the mean of individual wells from 3 separate experiments. A non-linear regression analysis was generated using GraphPad Prism, version 5.0. For the Ca^{2+} transient studies (right panel), assays were performed using Molecular Devices Calcium Plus Assay Kit according to the manufacturer's protocol. HEK cells were seeded overnight at 50,000 cells/100 μl/well in 96-well plates coated with poly-D-lysine. Probenecid was made fresh and added to the loading buffer (2.5 mM final). Compound dilutions were added to the wells and allowed to incubate at 37°C, with 5% CO_2 for 1 hr, after which 100 μl of loading dye was added and the plate incubated at 37°C, with 5% CO2 for 1 hour. The parameters were an excitation wavelength of 485nm, and an emission wavelength of 525 nm. Basal conditions were established and basal fluorescence (F_B) recorded before addition of agonist (TRAP; 5μM). Subsequently, fluorescence readings (F_S) were taken every 2 seconds for 190 seconds. Data presented are the mean (F_S-F_B) of 3 individually recorded wells. Results were quantified and averaged using GraphPad Prism, Version 5.0.

Supplementary Figure 1





Supplementary data

Selection screen criteria for Q94

The novel PAR_1 antagonist, Q94, is a small molecule (MW<500) that meets the Lipinski rule of five. The compound was chosen on the basis of its ability to compete for binding at the carboxyl-terminus of PAR_1, with a high affinity receptor binding peptide probe (HABP) designed around the 11 amino acids found at the carboxyl-terminus of Gαq. The HABP probe (T2-14) was selected from a proprietary library of Gαq related peptides, constructed by sequential substitution of the 11 native C-terminal amino acids with each of the 20 known amino acids. This was fused with *E. coli* maltose binding protein (MBP-T2-14), to enable recognition by MBP ELISA. IC_{50} values for binding of the HABPs to the receptor carboxyl-terminus showed that MBP-T2-14 actually had a higher binding affinity for PAR_1 than native Gαq C-terminal peptide. The selective nature of the MBP-T2-14 for PAR_1 was supported by the fact that both a β2AR-x probe (which contains a sequence obtained from screening the activated β2-adrenergic receptor with a Gαs library), and RHO8 probe (identified from a Gαt library for its ability to bind activated Rhodopsin) showed little or no binding affinity for PAR_1. Q94 was found to compete off the highly selective MBP-T2-14, from activated PAR_1 receptors with an IC_{50} of 916nM (Supplementary Figure 1, left panel). Additionally, this compound was found to effectively inhibit thrombin receptor activating peptide (TRAP) induced calcium transients (Supplementary data, Figure 1, right panel).

HABP	C-terminal sequence	IC50 [nM]
MBP-Gq	L Q L N L K E Y N L V	320
MBP-T2-14	L Q L N L K x Y N x V	106
MBP-B2AR-x	Q R x H L R x Y E x L	8277
MBP-RHO-8	L L E N L R D C G M F	8411

Table I. IC_{50} Values for high affinity C-terminal PAR_1 receptor binding proteins. Using competitive ELISA the affinity of selected purified MBP-C-terminal fusion proteins for activated PAR_1 were determined. β2AR-x contains a sequence obtained from screening the activated β2-adrenergic receptor with a Gαs library, and there is either no binding or very low-affinity binding. There is also little or no binding with RHO8, a peptide identified from a Gαt library for its ability to bind activated Rhodopsin. This supports the selective nature of the binding of peptide sequences to PAR_1. The curves were generated and IC_{50} values were derived by fitting the dose-response curves with a non-linear regression program. The position of substituted amino acids in proprietory peptide sequences are marked x.

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