SUPPORTING INFORMATION

PKA-type I Selective Constrained Peptide Disruptors of AKAP Complexes

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METHODS

Chemicals and Materials. The N-α-Fmoc protected amino acids and Rink Amide MBHA resin used for peptide synthesis were purchased from Novabiochem. (S)-N-Fmoc-2-(4'-pentenyl)alanine was purchased from Okeanos. All other reagents and organic solvents used in this study were purchased from Fisher Scientific except where noted. All reagents and solvents used in preparation or analysis of peptides such as methanol, acetonitrile, and trifluoroacetic acid were HPLC grade.

Peptide Synthesis. 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis was used to synthesize all of the peptides used in this study. First, Rink Amide MBHA resin was equilibrated in 1-methyl-2-pyrrolidinone (NMP) for 15 min. Deprotection was then performed using a 25% (v/v) solution of piperidine in NMP for 30 min, followed by three brief washes in NMP. For coupling of amino acid, 10 equiv. of N-α-Fmoc-protected amino acids (0.25 M final concentration in NMP) were added, followed by addition of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, 0.23 M final concentration) in NMP and 8% (v/v) N,N-diisopropyl ethylamine (DIEA). Another three washes in NMP were performed after 45 min of coupling. Deprotection and coupling steps described above were sequentially performed.

For peptides that were designed to incorporate a peptide staple, olefin metathesis was performed using 0.4 equiv. bis-(tricyclohexylphosphine) benzylidene ruthenium(IV) dichloride (Grubbs' first generation catalyst, Sigma Aldrich) relative to resin substitution while the peptide was still protected and bound to resin. The reaction was performed in 1,2-dichloroethane at room temperature with agitation for 1 hour. To ensure complete

reaction, the metathesis was repeated once more using the same conditions. Next, 11-Amino-3,6,9-trioxaundecanoic acid (NH-PEG3-CH2COOH, ChemPep Inc.) was added to the N-terminus using standard coupling conditions with 4 equivalents. Finally, the addition of an N-terminal biotin or 5(6)-carboxyfluorescein (5/6 FAM) was performed. For fluorescein labeling, 2 equivalents of 5/6 FAM (Acros Organics) along with 0.046 M HCTU and 2% (v/v) DIEA in N,N-dimethylformamide (DMF) were added and agitated overnight. For biotin labeling, 10 equivalents of D-biotin (Anaspec), 0.14 M HCTU, and 4% (v/v) DIEA in a 1:1 mixture of DMF and dimethyl sulfoxide (DMSO) were added and agitated overnight. Peptides were cleaved from resin by using 95% trifluoroacetic acid, 2.5% water, and 2.5% of triisopropylsilane (Sigma Aldrich) for 4-5 h. Upon completion, the products were precipitated in methyl-tert-butyl ether at 4 °C and lyophilized. Peptides were suspended in methanol and purified by high-performance liquid chromatography (HPLC). Products were verified by mass spectrometry (MS). Fluorescein-labeled peptides were quantified by measuring the absorbance of 5/6 FAM at 495 nm using a Synergy 2 microplate reader (Bio-Tek). Biotin-labeled peptides were quantified by measuring decreased absorbance of the 2-(4'-hydroxybenzeneazo)benzoic acid (HABA)-avidin complex (VWR) at 500 nm.

The molecular weights of the purified peptides are as follows: (5/6 FAM)-$(PEG)_3$-RIAD = 2637.4 (expected mass = 2637.8); (5/6 FAM)-$(PEG)_3$-st.1 = 2631.0 (expected mass = 2631.9); (5/6 FAM)-$(PEG)_3$-st.2 = 2631.2 (expected mass = 2631.9); (5/6 FAM)-$(PEG)_3$-st.3 (RI-STAD-1) = 2616.4 (expected mass = 2616.9); biotin-$(PEG)_3$-st.3 (RI-STAD-1) = 2484.2 (expected mass = 2484.9); (5/6 FAM)-$(PEG)_3$-RI-STAD-1-scr = 2616.2 (expected mass = 2616.9); (5/6 FAM)-$(PEG)_3$-st.4 = 2615.6 (expected mass = 2616.9);

(5/6 FAM)-$(PEG)_3$-st.5 (RI-STAD-2) = 2758.2 (expected mass = 2759.1); unstapled (5/6 FAM)-$(PEG)_3$-st.5 (unstapled RI-STAD-2) = 2787.0 (expected mass = 2787.1); biotin-$(PEG)_3$-st.5 (RI-STAD-2) = 2626.4 (expected mass = 2627.1); (5/6 FAM)-$(PEG)_3$-RI-STAD-2-scr = 2758.4 (expected mass = 2759.1); (5/6 FAM)-$(PEG)_3$-st.6 (RI-STAD-3) = 2745.4 (expected mass = 2745.4); biotin-$(PEG)_3$-st.6 (RI-STAD-3) = 2613.2 (expected mass = 2614.1); (5/6 FAM)-$(PEG)_3$- RI-STAD-3-scr = 2745.6 (expected mass = 2746.1); (5/6 FAM)-$(PEG)_3$-st.7 = 2725.8 (expected mass = 2726.0).

Protein Expression and Purification. The RIα docking/dimerization (D/D) domain (residues 1-61) of *Bos taurus*, RIIα D/D (1-44) of *Rattus norvegicus* and human PKA regulatory subunits (hRIα, hRIβ, hRIIα, hRIIβ) were expressed as previously described (*1, 2*). For RIα or RIIα D/D expression, cells were induced using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 3 hours. Cells were subsequently lysed in buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, and 0.1 mM phenylmethanesulfonylfluoride (PMSF) before purification. The protein constructs were subsequently purified using a Talon cobalt-affinity resin (Clontech) and a Superdex 75 size exclusion column (AKTA) on an AKTA Purifier UPC 10 (AKTA). Proteins were concentrated using Vivaspin 6 columns with a 3 kDa molecular weight cutoff (GE Healthcare). 20% glycerol was added to concentrated proteins before being snap frozen in liquid nitrogen and stored at -80 °C.

Recombinant full length human PKA regulatory subunits (hRIα, hRIβ, hRIIα, hRIIβ) were expressed and purified via Sp-8-AEA-cAMPS agarose according the procedure of

Bertinetti et al (*3*). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor protein expression and ensure purity to ≥95% homogeneity.

Fluorescence Polarization Using PKA-R D/D Domain. On a 96-well plate (Corning), 10 nM fluorescein-labeled peptides were plated with either RIα D/D or RIIα D/D. The protein constructs were 10-fold serially diluted from 100 μM to 0.1 nM in buffer containing 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20. The plates were incubated in the dark at room temperature for 30 minutes before measured using a Synergy 2 microplate reader (Biotek). Experiments were performed in triplicates. Binding curves were generated, and dissociation constants (K_D) were calculated from the nonlinear regression curve using GraphPad Prism.

Fluorescence Polarization Using Human Full-length PKA-R. Interaction analysis between the PKA regulatory subunit isoforms and the RI-STAD peptides was measured using a fluorescence polarization (FP) assay as previously described (*1*). A range of concentrations (from 60 pM to 10 μM) of the four full-length different PKA regulatory subunits were incubated with 4 nM of fluorescently labeled RI-STAD-1, -2 or -3 peptides in buffer containing 20 mM MOPS pH 7, 150 mM NaCl, 0.005% (v/v) CHAPS. Data was obtained using a Fusion™ alpha-FP plate reader at room temperature and a data acquisition of 2 sec at Ex 485 nm/Em 535 nm in a 384 well microtiterplate (Perkin-Elmer Optiplate, black). Data represents the mean ± standard error of the mean of triplicate measurements (n = 3 per data point) for a single experiment. K_D determination was performed as described above.

Surface Plasmon Resonance Measurements. Interaction studies were performed using a Biacore 3000 instrument (Biacore^{TM} 3000 Control Software, version 4.1.2, GE Healthcare) (*5*). Measurements were performed in running buffer containing 20 mM MOPS, 150 mM NaCl, 0.05 mM EDTA, 0.005% (v/v) Tween 20, pH 7.0, at 25 °C instrument temperature. In brief, 8-AHA-cAMP (BioLog, Bremen, Germany) was covalently coupled to CM5 sensor chips (research grade) using NHS/EDC chemistry as described previously (*2*). Full-length RI or RII subunits were injected in running buffer containing 1 mg/ml bovine serum albumin and reversibly captured on an 8-AHA-cAMP surface (surface concentration of 800–3500 resonance units). To determine unspecific binding, blank runs were performed with identical samples and solutions using a 8-AHA-cAMP surface without immobilized R-subunit and these values were subtracted.

Binding Kinetics of RI-STAD-2. RI-STAD-2 (PEG-L*QYA*QLADQIIKEATEK) was injected over a concentration range (4.8 to 15000 nM) at a flow rate of 60 µL/min. Both association and dissociation phases were recorded for 180 s. Rate constants (k_a and k_d) and equilibrium binding constants (K_D) were calculated based on nonlinear regression analysis assuming a 1:1 Langmuir binding model (1:1 Langmuir binding model with mass transfer in case of RI-STAD-2) using the BIAevaluation software, version 4.1.1 (GE Healthcare). The same software package was used to analyze steady-state equilibrium binding data.

Surface competition experiments with RIAD and RI-STAD-2. The AKAP149 (285-387) construct was prepared using GST-chromatography with subsequent thrombin cleavage of the GST-tag (*5*). AKAP149 (500 nM) was injected at a flow rate of 30 μL/min in the presence or absence of 5 μM RIAD or RI-STAD-2. Both association and dissociation phases were recorded for 300 s. Since AKAP disruptor peptides bind to the immobilized R-subunits, a small increase in mass was expected. Therefore control experiments were performed using 5 μM of RIAD or RI-STAD-2 without AKAP149 (285-387) present and these values were subtracted from the readings. For regeneration after each injection, the entire protein-peptide (R subunits and RIAD or RI-STAD-2) or protein-protein (R subunits and AKAP149 (285-387)) complex was removed by three short injections (15 s each) of 0.1% (w/v) SDS, followed by a single injection of 4 M guanidinium hydrochloride (30 s) and an injection of running buffer (including 1 M NaCl, 30 s) until baseline was reached.

Ht31 Competition Assay. Ht31 (DLIEEAASRIVDAVIEQVKAAGAY) and Ht31P (DLIEEAASRPVDAVPEQVKAAGAY) were obtained from Biaffin GmbH and Co KG. Increasing concentrations of Ht31 or Ht-31P (ranging from 30 μM to 15 nM) were mixed with 4 nM of the indicated fluorescent labeled peptides. The mixture was incubated with human full length RIα whose concentration was adapted to 70% of the maximum value derived from the previous FP assays. Fluorescence polarization was measured after 5 minutes. Data was obtained as described above. Data was analyzed using GraphPad Prism by plotting the resulting polarization signals or the normalized polarization signals against the logarithmic scale of Ht31 concentrations.

Peptide Internalization in Live Cells. MDA-MB-231 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) Medium with L-glutamine (Lonza), 10% fetal bovine serum (Thermo Scientific), and penicillin/streptomycin (Amresco) and seeded (~10×10^3) into 96-wells and allowed to attach overnight prior to treatment with peptides where indicated. Cells were incubated for 6 hours in complete growth medium with 5 μM 5(6)-carboxyfluorescein-labeled RI-STAD peptides at 37 °C, followed by DNA staining using 5 μg/mL Hoescht 33342 (Pierce) for 20 min. As a control, the unstapled version of RI-STAD-2 was used. This peptide contains the olefinic amino acids, but remains in the uncyclized state without undergoing the ring-closing metathesis step. After DNA staining, cells were subsequently rinsed three times with PBS. Live cells were fluorescently imaged using a Thermo Scientific ArrayScanVTI HCS Reader (Thermo Fisher Scientific/Cellomics). Data was automatically generated by the ArrayScan software and was graphed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Experiments were performed using triplicate samples and at least 10 field images per well were incorporated into the analysis (n = 28-32 fields per data point and representing 1410 to 1670 cells per condition). Representative fluorescent images are shown and internalization is quantified by bar graphs. The experiment was repeated three times. Statistical differences measuring the significance of experimental data were determined using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison post test. * $p < 0.05$ and *** $p < 0.001$ indicate levels that are considered statistically significant.

Biotin Pull-down Assays. MDA-MB-231 cells grown to sub-confluency were treated with 5 μM biotin-labeled peptides for 12 h prior to lysis. Cells were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5. Lysates were incubated with 100 μL avidin resin slurry (G-biosciences) while rocking at 4 °C for 6 hours. Resin was collected by centrifugation at 1000*g for 2 min, washed three times with phosphate buffered saline (PBS) before boiling in laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) at 95 °C for 5 min. PKA-RI (1:450, BD Biosciences) and RII (1:2000, Abcam) antibodies were used for the immunoblot analysis. Anti-rabbit (1:25,000) and anti-mouse were used correspondingly as secondary antibodies (1:30,000, LI-COR Biosciences). Blots were imaged using Odyssey Fc imaging system (LI-COR Biosciences).

Generation of U2OS RIIΔ cells. U2OS osteosarcoma cells were cultured in DMEM-H containing 10% FBS and antibiotics (Life Technologies). Cells were co-transfected (Transit LT-1, Mirus) with plasmids that express sgRNAs against human *PRKAR2A* and *PRKAR2B* genes as well as a humanized Cas9-NLS protein, and a plasmid expressing the puromycin resistance gene. After two days, media containing 2 μg/ml puromycin was added. Three days later, cells were trypsinized, counted, passed through a 40 μm filter, and plated at low density. When single colonies appeared, clones were expanded and tested for expression of RIIα and RIIβ by western blotting. PCR was used to confirm disruption of the appropriate genomic regions. Full characterization of CRISPR knockout cell lines will be published elsewhere.

PKA reporter assay. The PKA reporter assay was performed as previously described (*6*). Briefly, the RI-selective inhibitor was tested using U2OS RIIΔ cells that were transiently transfected with cDNA encoding for AKAR-18RBS for 48 hours. Cells were then preincubated with 1 μM of R1-STAD-2 (n=26 cells) or its scrambled control (n= 24 cells) in serum-free media for 1 hour. The cells were monitored using a DMI6000B inverted microscope (Leica) with a 37 C chamber for 150 seconds. Images were taken every 5 seconds using an exposure time of 300 ms. After 40 seconds, cells were stimulated with 1 μM isoproterenol. FRET changes within a region of interest were calculated as the ratio of measured fluorescent intensities from two channels (Mdonor, MIndirectAcceptor) after subtracting the background signal. FRET ratio (YFP/CFP) changes were normalized to the average basal FRET values measured before stimulation. As a control, the RII-selective inhibitor was tested in HEK293 cells that were transiently transfected with cDNA encoding for the same AKAR reporter and wild-type RII as described (*6*). Cells were pre-incubated with either 1 μM of STAD-2 or its scrambled control. Cells were then stimulated with isoproterenol (100 nM) and imaged over the course of 400 seconds.

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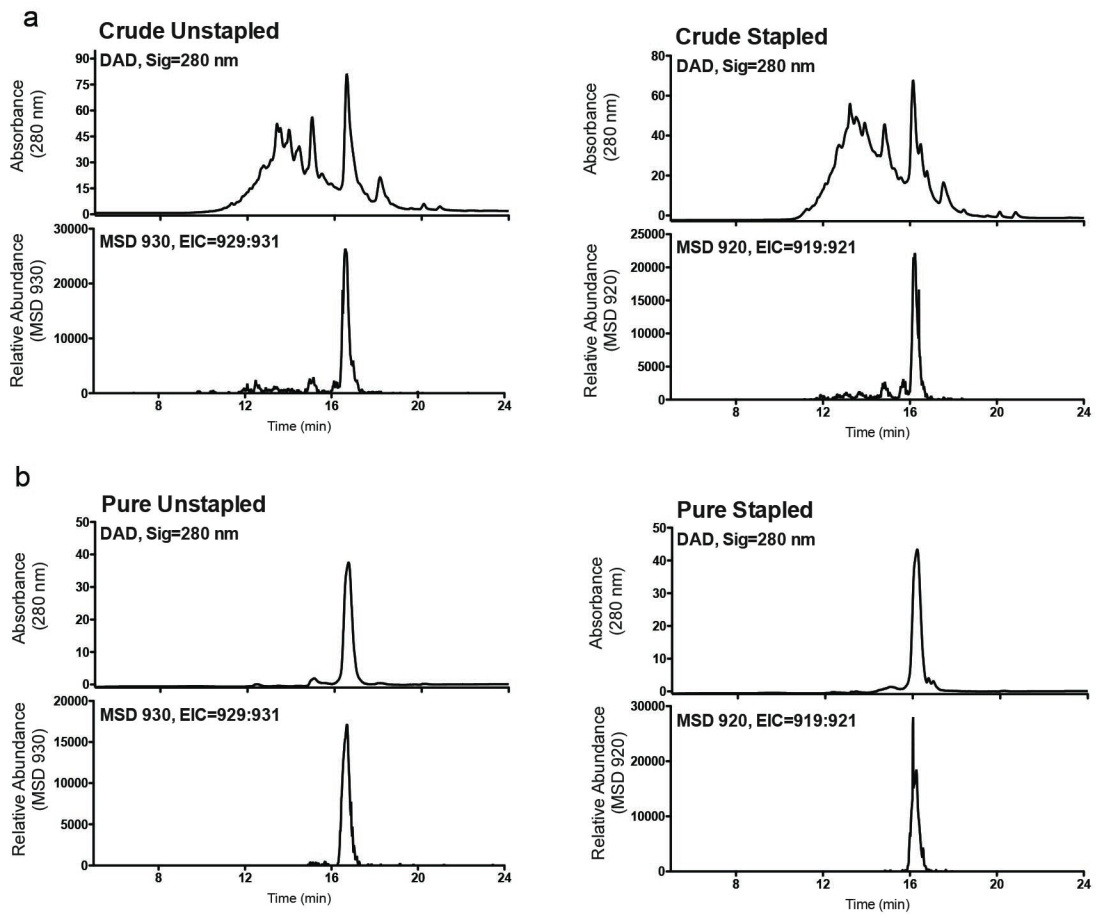

Figure S1. LC/MS spectra of unstapled and stapled RI-STAD-2

(a) Spectra of the crude unpurified product for the unstapled and stapled versions of Fluorescein-labeled RI-STAD-2 are shown. The unstapled version contains the olefinic amino acids but has not undergone the RCM reaction. Absorbance profiles (280 nm) and extracted ion current (EIC) mass spectrometric detection (MSD) are shown for each apparent product (unstapled expected mass: (M+3)/3=930.0, stapled expected mass: (M+3)/3=920.7). Only one major product peak was detected for the stapled product after the RCM reaction. (b) Analytical MS spectra after purification shows that a single peak was isolated during purification for RI-STAD-2.

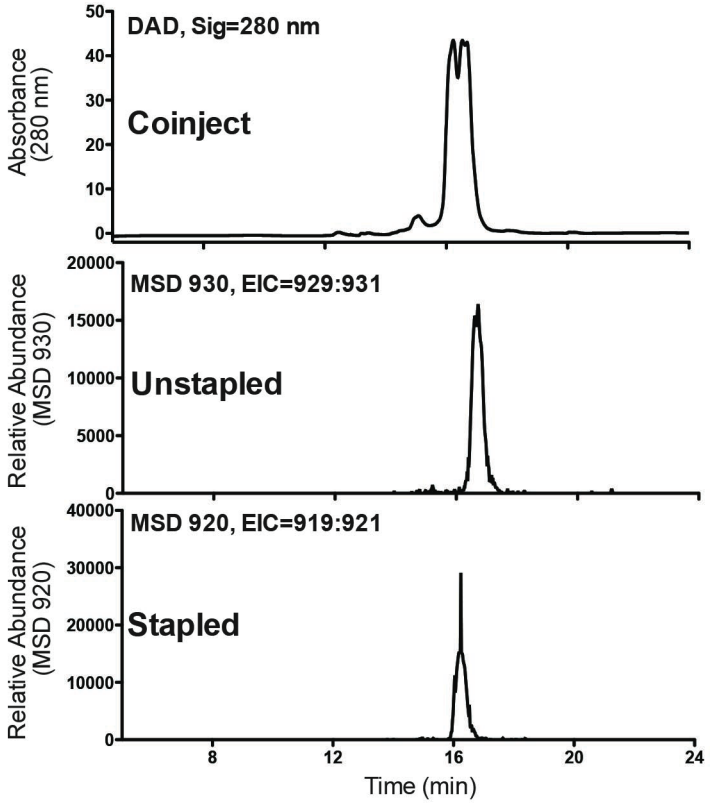

Figure S2. Coinjection of unstapled and stapled RI-STAD-2

The purified products from unstapled and stapled RI-STAD-2 were coinjected for LC/MS analysis. The stapled peptide demonstrates a shift in retention time (retention time of the unstapled product=16.9 min; retention time of the stapled product=16.2 min).

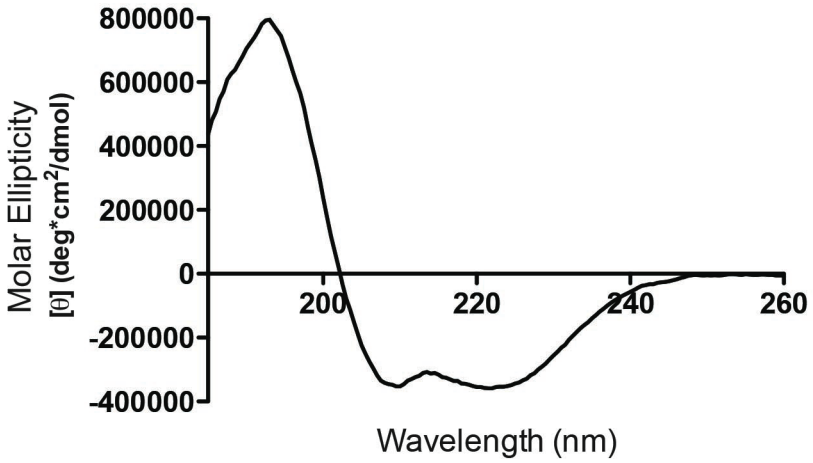

Figure S3. Circular dichroism of purified stapled RI-STAD-2

The peak isolated for RI-STAD-2 (as shown in Figure S1b) was analyzed by circular dichroism (CD). The molar ellipticity is shown for purified RI-STAD-2 which has an apparent alpha-helical secondary structure with minima at 208 and 222 nm.

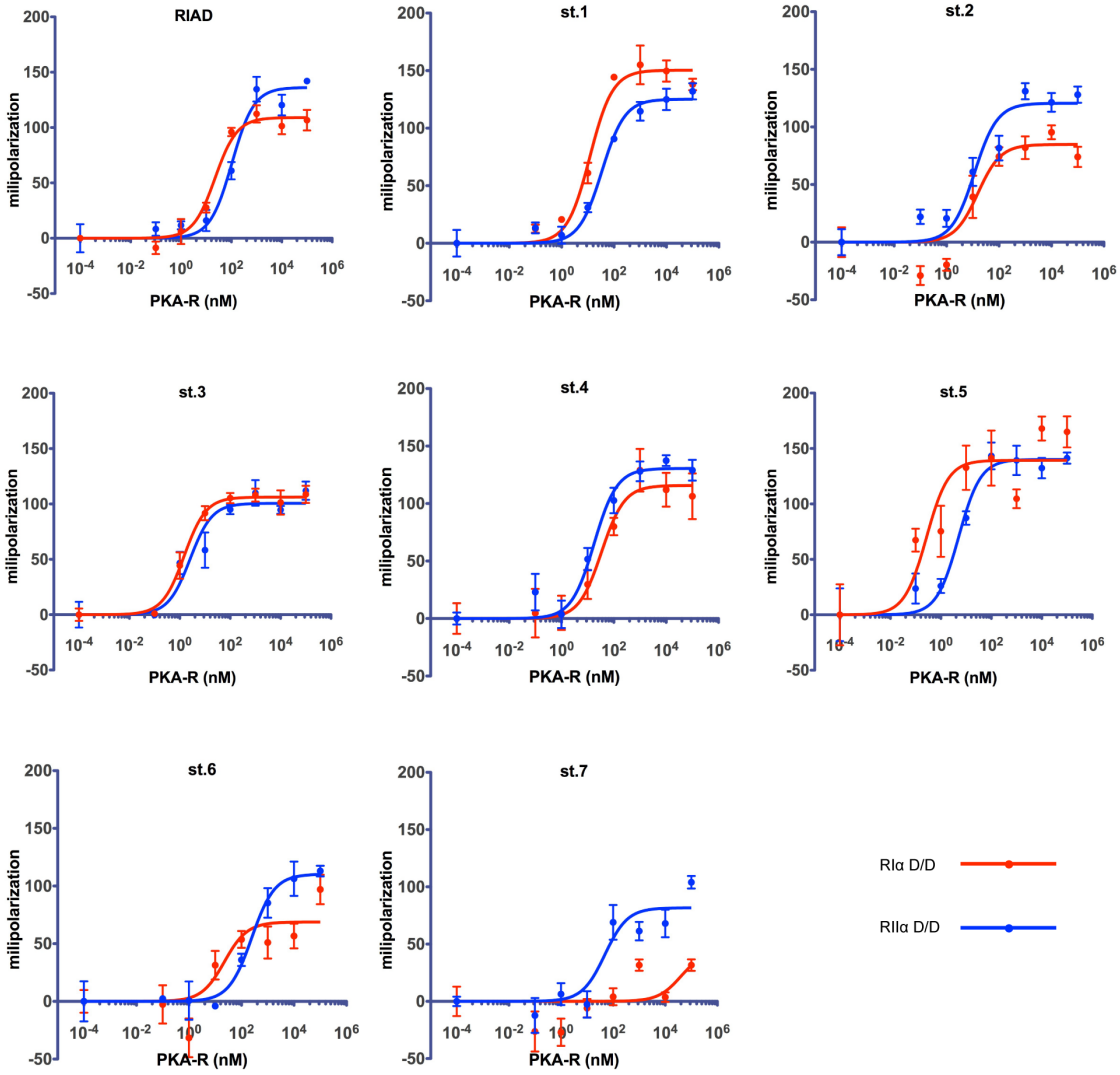

Figure S4. FP spectra of RI disruptor peptides

Fluorescence polarization (FP) was performed on each of the peptides using purified protein constructs of the D/D domains from either PKA-RI (red) or PKA-RII (blue). Peptides were plated at a final concentration of 10 nM and the D/D domains were tested over a concentration range of 0.1 nM to 100 μM. The experiment was performed in triplicate for each concentration tested.

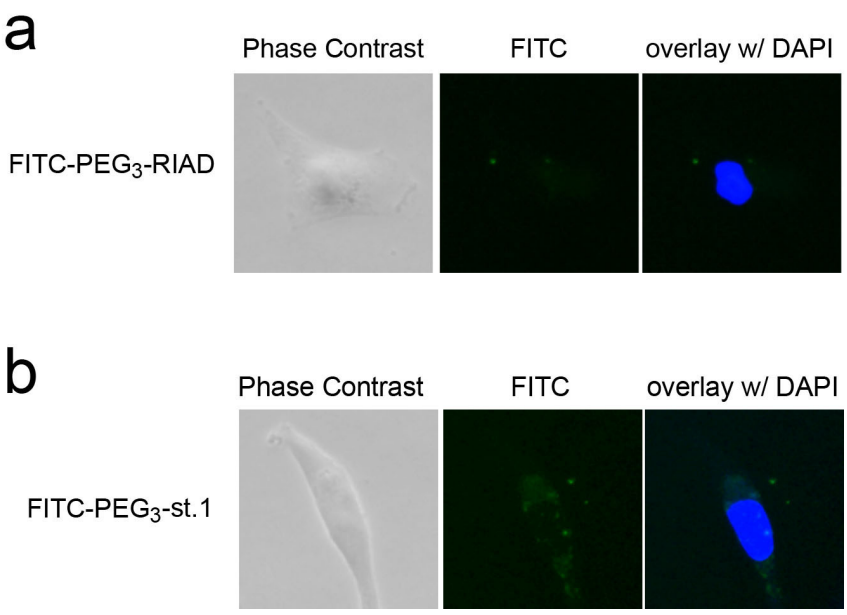

Figure S5. Limited cell permeability is evident for some peptides

MDA-MB-231 cells were treated with 5 μM FITC-labeled PEG_3-RIAD (a) or st.1 (b) for 21 hours. No apparent uptake was observed for PEG_3-RIAD. A faint amount of st.1 was observed in cells at this time point.

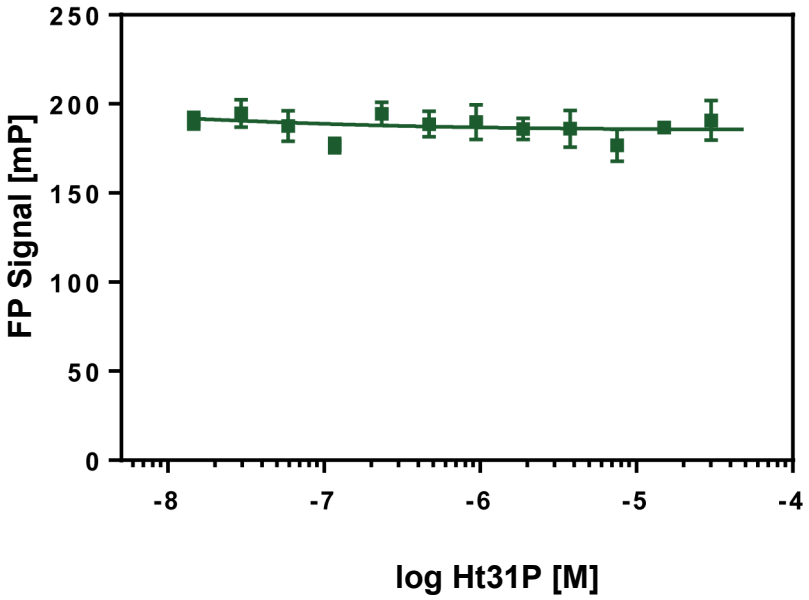

Figure S6. The Ht31 control peptide, Ht31P, cannot out-compete RI-STAD-2

FP competition spectrum using the negative control peptide for Ht31 (Ht31P). The RIα isoform remains in a bound state with RI-STAD-2 peptide since Ht31P could not displace this interaction.

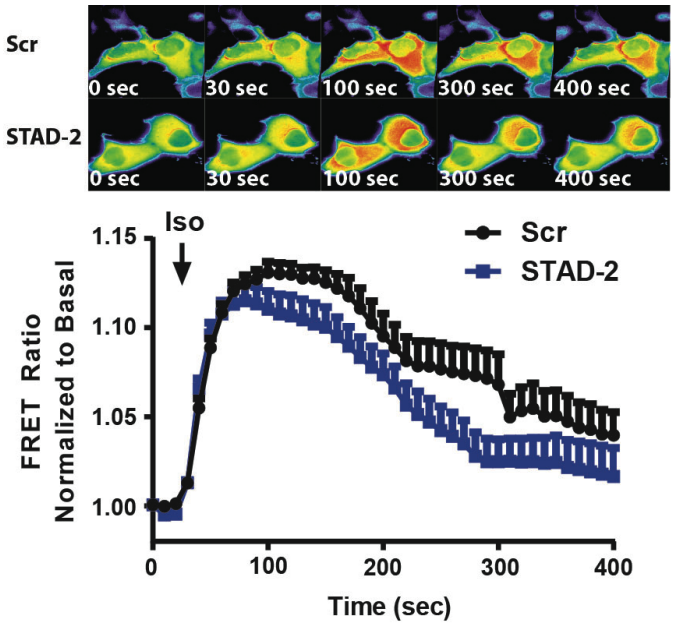

Figure S7. The RII disruptor also inhibits anchored PKA activity

AKAP-18$_{RBS}$ AKAR activity was monitored and FRET signal was measured in HEK293 cells pre-incubated with 1 μM of STAD-2 or its scrambled control for 1 hour (n=30, 3 cells each). AKAP-18$_{RBS}$ AKAR activity was monitored over 400 seconds after stimulation with 100 nM Isoproterenol. Data was normalized relative to unstimulated basal FRET levels. STAD-2 reduces RII-anchored AKAP signaling in cells. Warmer colors indicate increasing phosphorylation.