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Supplementary Information

Towards Understanding Cell Penetration by Stapled Peptides

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Experimental Methods

Stapled Peptide Synthesis. All peptides were synthesized following an established protocol using standard Fmoc-peptide chemistry on Rink amide MBHA resin. The coupling reactions were generally performed by adding a mixture of 10 equivalents of the amino acids, 9.9 equivalents of HCTU and 20 equivalents of DIPEA in NMP (equivalents relative to initial loading of Rink amide MBHA resin). The reactions were allowed to proceed for one hour, or longer if coupling an amino acid in a difficult stretch of residues. Coupling of non-natural amino acids (*R*/*S*5, *R*/*S*8 or *B*5) was performed with 4 equivalents of the amino acid, 3.9 equivalents of HCTU and 10 equivalents of DIPEA in NMP for two hours. The metathesis reaction of the olefin-containing non-natural amino acids was generally performed using Grubbs I catalyst (benzylidene-bis(tricyclohexylphosphine)-dichlororuthenium) dissolved to approximately 10 mg/mL in 1,2-dichloroethane (DCE) for two hours under nitrogen bubbling. After olefin metathesis, the resin was washed extensively with DCE to remove excess catalyst and coupled with an N-terminal fluorescein isothiocyanate (FITC). Upon completion, peptides were simultaneously cleaved from the resin and deprotected using a cleavage cocktail containing 95% TFA, 2.5% water and 2.5% TIS. Crude peptides were dissolved in 50% acetonitrile/water, passed through a 0.2 µm syringe filter, and purified by reverse phase HPLC using a C-18 column

(Agilent, Palo Alto, CA). Compound identification and purity was assessed using coupled LC/MS (Agilent, Palo Alto, CA). Purified fractions were pooled and evaporated to remove acetonitrile and trace TFA by speedvac and then lyophilized to dryness. Peptides were quantified according to FITC absorbance at 494 nm in pH 8.0 phosphate buffer. After quantification, peptides were stored as DMSO stocks at -20°C.

Cell Penetration Assays. U2OS cells grown in complete growth medium (DMEM, 10% FBS and 1% penicillin/streptomycin) were harvested, counted using a hemocytometer, and seeded in a black, clear bottom 384-well plate (Corning) with 2,000 cells per well. The plate was incubated overnight at 37°C / 5% CO_2 to allow attachment of the cells to the bottom of the well. The next morning, the cells were washed with PBS once and then incubated with the indicated concentrations of fluorescein-labeled peptides in fresh complete growth medium for 12 hours at 37°C. Cells were washed with PBS three times, fixed with 4% (w/v) paraformaldehyde and stained with Hoechst dye (Invitrogen). Then 100 µL PBS was added to each well and the plate was sealed with tape to avoid drying. High-content imaging measurements were performed to determine intracellular access on an IX5000 epifluorescence microscope (Molecular Devices Corporation). Epifluorescence images were taken at 4× magnification in triplicate and exported into MetaXpress software. The relative fluorescence intensity was calculated for positive cells, which were identified based on cell size and signal-to-noise ratio.

- For the dose-dependence assay, peptides were treated at 10 nM, 100 nM, 1 µM, 5 µM, 10 µM and 20 µM for 12 hours before analysis.
- For the time-course assay, peptides were added at a final concentration of 5 µM or 10 µM, and cells were harvested for analysis 2, 4, 8, 16, 20 and 24 hours post-treatment.

- The pulse-chase experiment was performed similar to the normal penetration assay. 12 hours after initial treatment, cell culture medium was aspirated and cells were washed extensively to remove excess FITC-labeled peptides. Fresh medium containing either a new batch of peptide or DMSO vehicle was added to incubate for longer time as indicated. Cells were imaged and analyzed 8, 12, 16, 20 and 24 hours after the initial treatment.
- For the cell penetration assay with small molecule inhibitors, HeLa cells seeded in 384-well clear bottom plates (Corning) were pre-incubated with the indicated small molecules for 1 hour (10 mM NaN_3 and 30 mM 2-DG for ATP depletion, 5 µg/mL filipin or 25 µg/mL nystatin for blocking caveolin-mediated endocytosis, 5 µg/mL chlorpromazine for blocking clathrin-dependent endocytosis, 10 µg/mL cytochalasin D for inhibiting actin polymerization, and 80 mM $NaClO_3$ for disrupting proteoglycan synthesis). Then a final concentration of 1 µM FITC-labeled peptide was added to the cell culture medium containing the small molecule inhibitor and incubated for an additional 4 hours.
- General parameters for image acquisition:

  Wavelength One: DAPI, excitation for 20ms, laser-based and image-based autofocus,

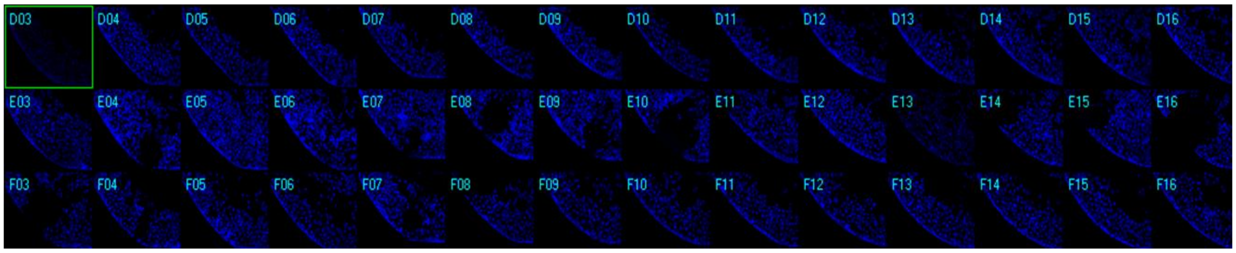
  Wavelength Two: FITC, excitation for 5ms, offset from wavelength one = + 5 µm.
- General parameters for image analysis in MetaXpress:

  Wavelength One: DAPI, minimum cell diameter = 6 µm, maximum cell diameter = 20 µm, minimum intensity above local background = 1,000

  Wavelength Two: FITC, minimum cell diameter = 6 µm, maximum cell diameter = 30 µm, minimum intensity above local background = 500

ATP Depletion Assay. U2OS cells were seeded in a 96-well white opaque plate (Corning) with 10,000 cells per well. The plate was incubated overnight at 37°C and 5% CO_2 to allow attachment of the cells to the bottom of the well. The next morning, the cells were washed with PBS once, followed by incubation with fresh medium containing 10 mM NaN_3 and 30 mM 2-Deoxyglucose (2-DG) for 1 hour. The cellular ATP level was then measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's protocol.

a) Hoechst Channel



b) FITC Channel

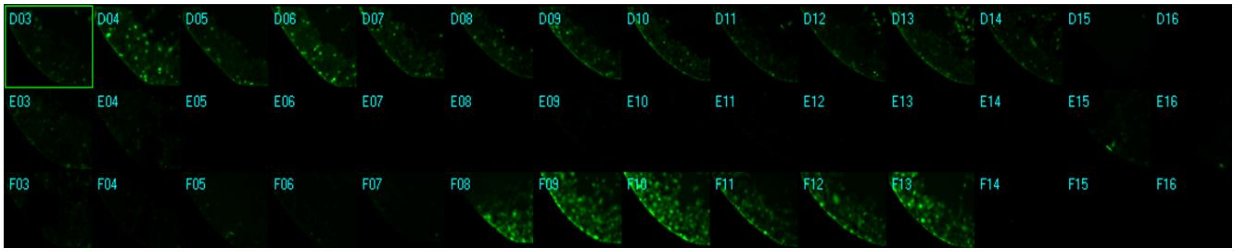


Figure S1. High-throughput epifluorescence microscopy images of stapled peptide intracellular access. a) Hoechst channel of a portion of a 384-well plate indicates the location and number of nuclei. b) The same portion of the 384-well plate shown in a) imaged in the FITC channel reveals different intracellular fluorescence accumulations.

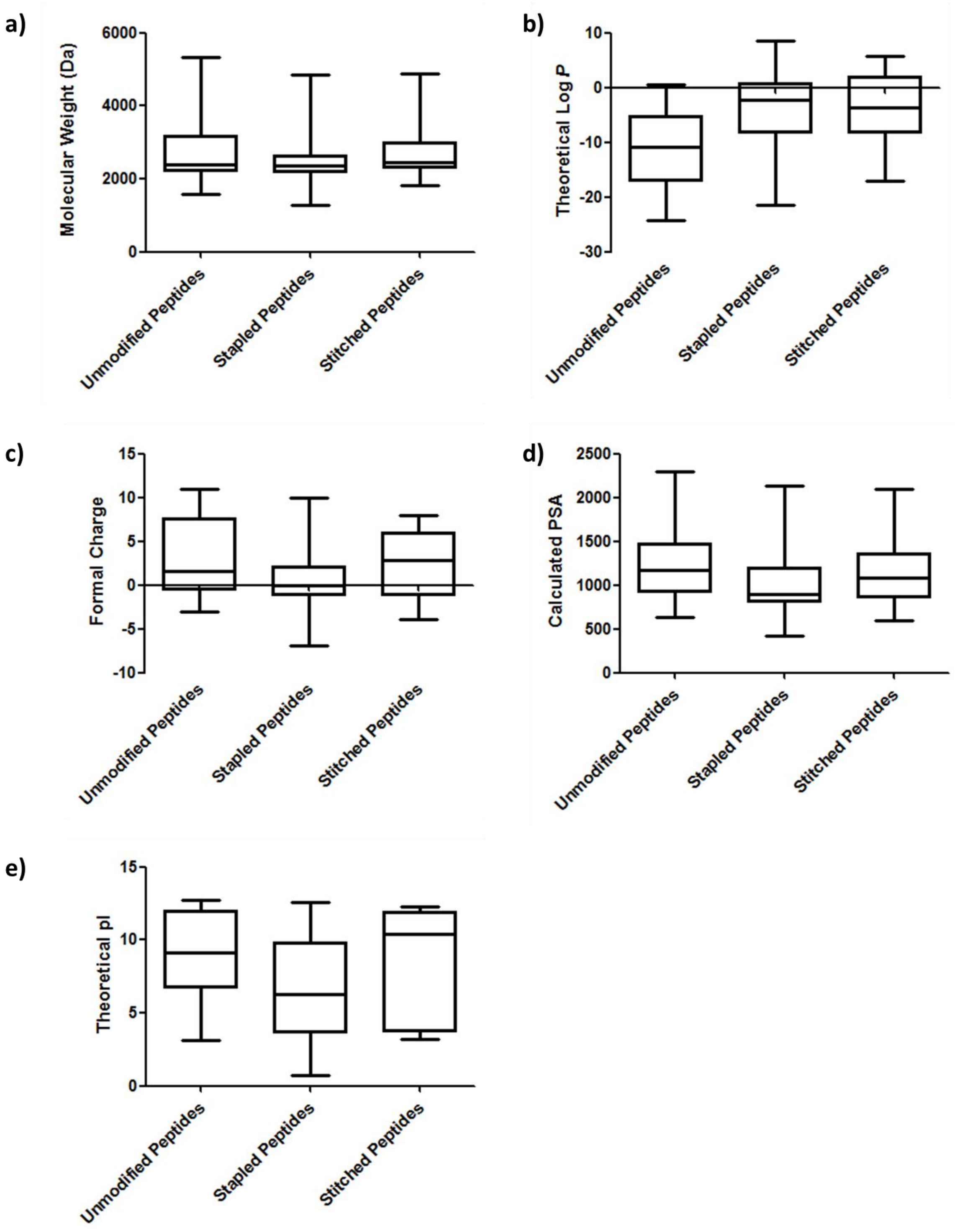


Figure S2. Physicochemical parameters a) molecular weight, b) theoretical log *P*, c) formal charge at pH 7.5, d) calculated PSA and e) theoretical pI of wild-type, stapled and stitched peptides. The data are plotted in box and whiskers format with a line in the middle indicating the median. Box = 25th and 75th percentiles, bars = min and max values.

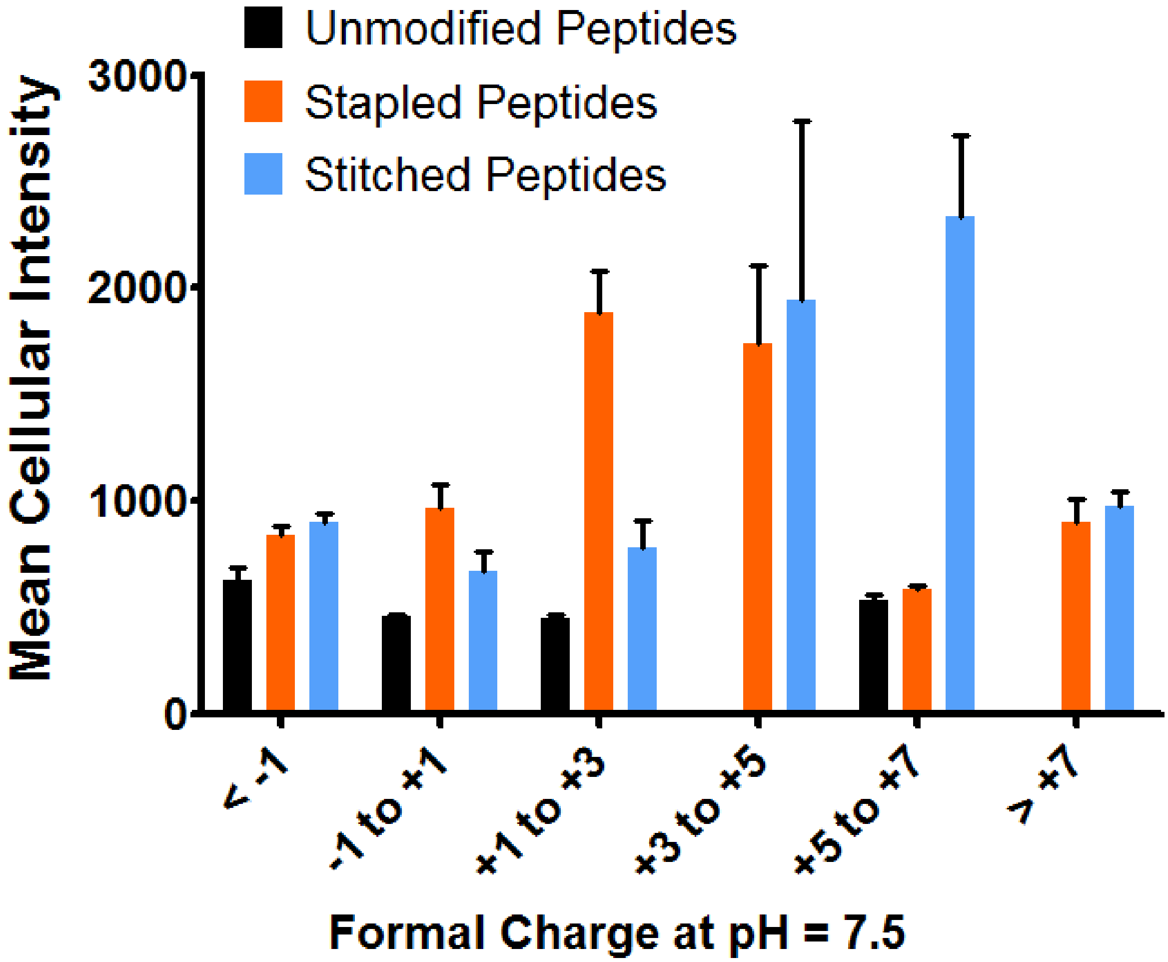


Figure S3. Relationship between mean cellular fluorescence intensity and formal charge at pH 7.5 among different peptide classes (i.e. unmodified, stapled and stitched) showing that the same trend between formal charge and cellular uptakes were observed for both stapled and stitched peptides in general. However, the unmodified peptide class does not have enough samples in several charge groups to draw any meaningful conclusions. Error bars represent the S.E.M. of the mean cellular intensity of the indicated peptide classes.

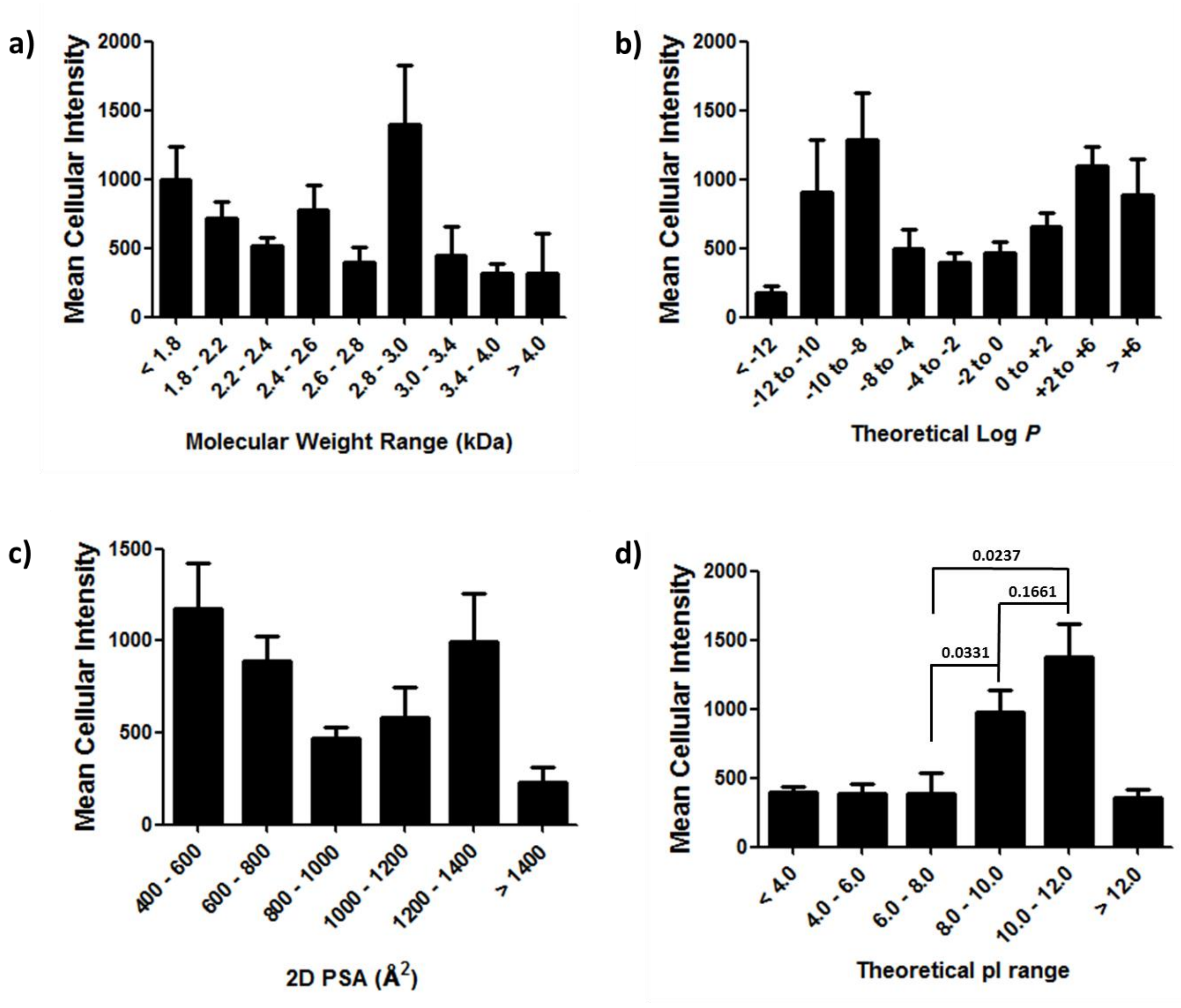


Figure S4. Relationship between mean cellular fluorescence intensity and physicochemical properties of peptides. a) molecular weight, b) theoretical log *P*, c) 2D PSA, and d) theoretical pI. Error bars represent the S.E.M. of the mean cellular intensity of the indicated peptide classes.

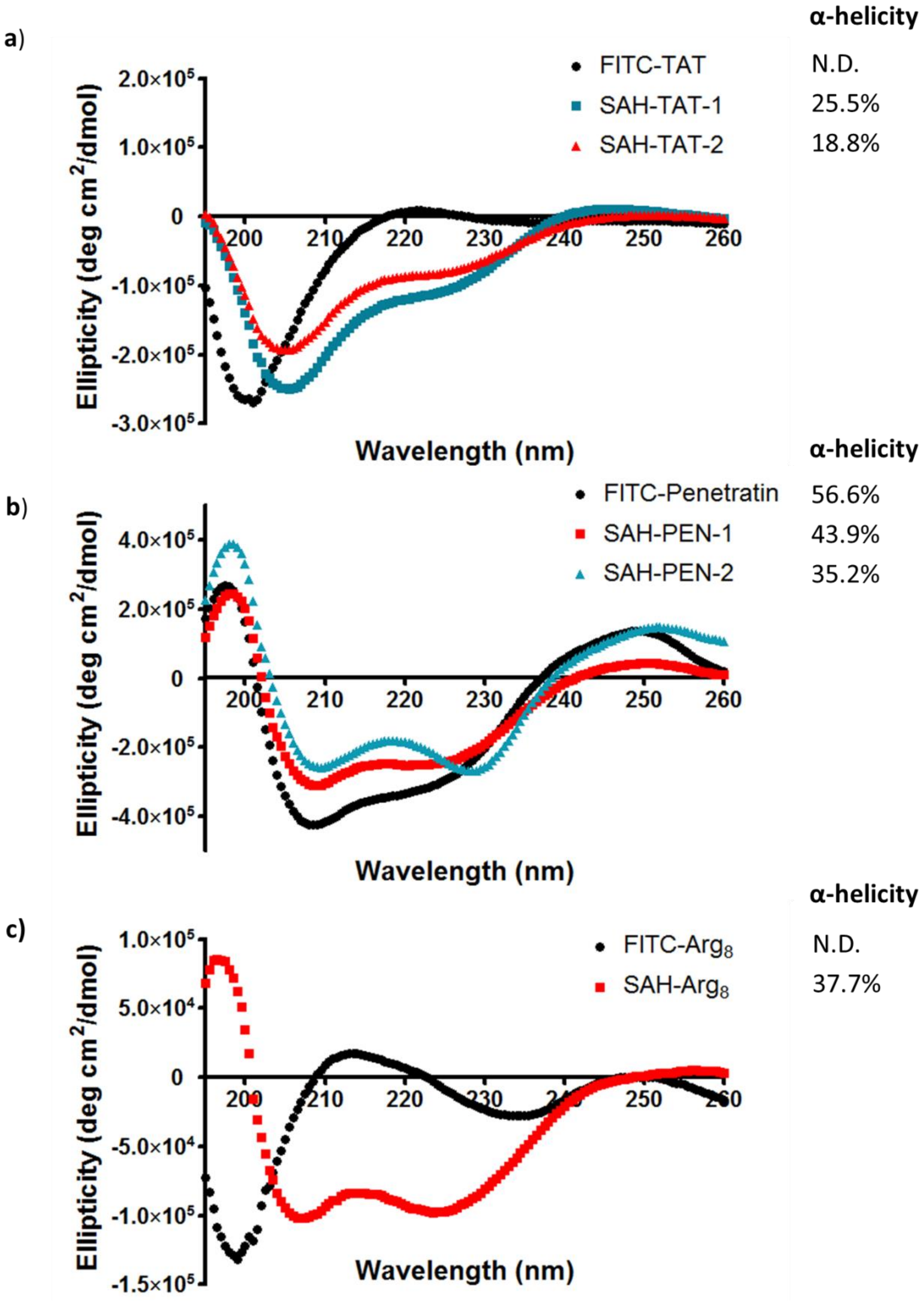


Figure S5. Circular dichroism spectroscopy of a) TAT, b) penetration c) poly-Arg$_8$ and their stapled variants.

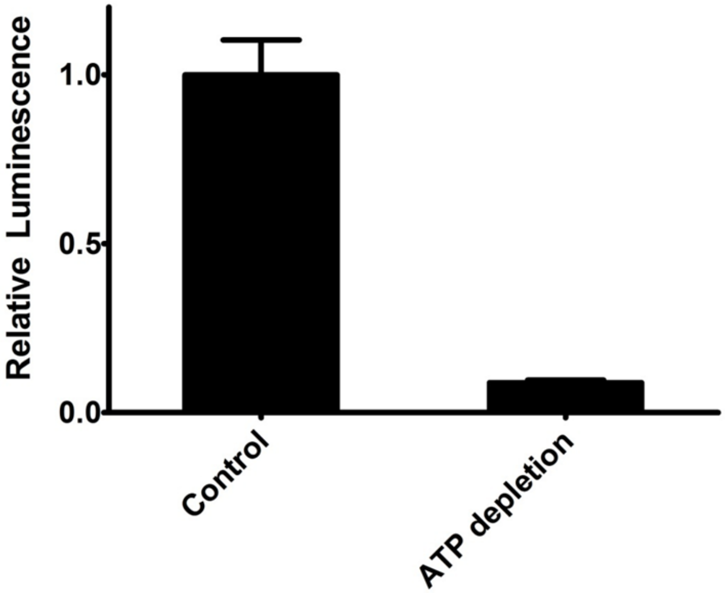


Figure S6. Cellular ATP decreased by approximately 90% in U2OS cells upon NaN_3 and 2-Deoxyglucose (2-DG) treatment. Error bars represent the S.D. of triplicate samples.

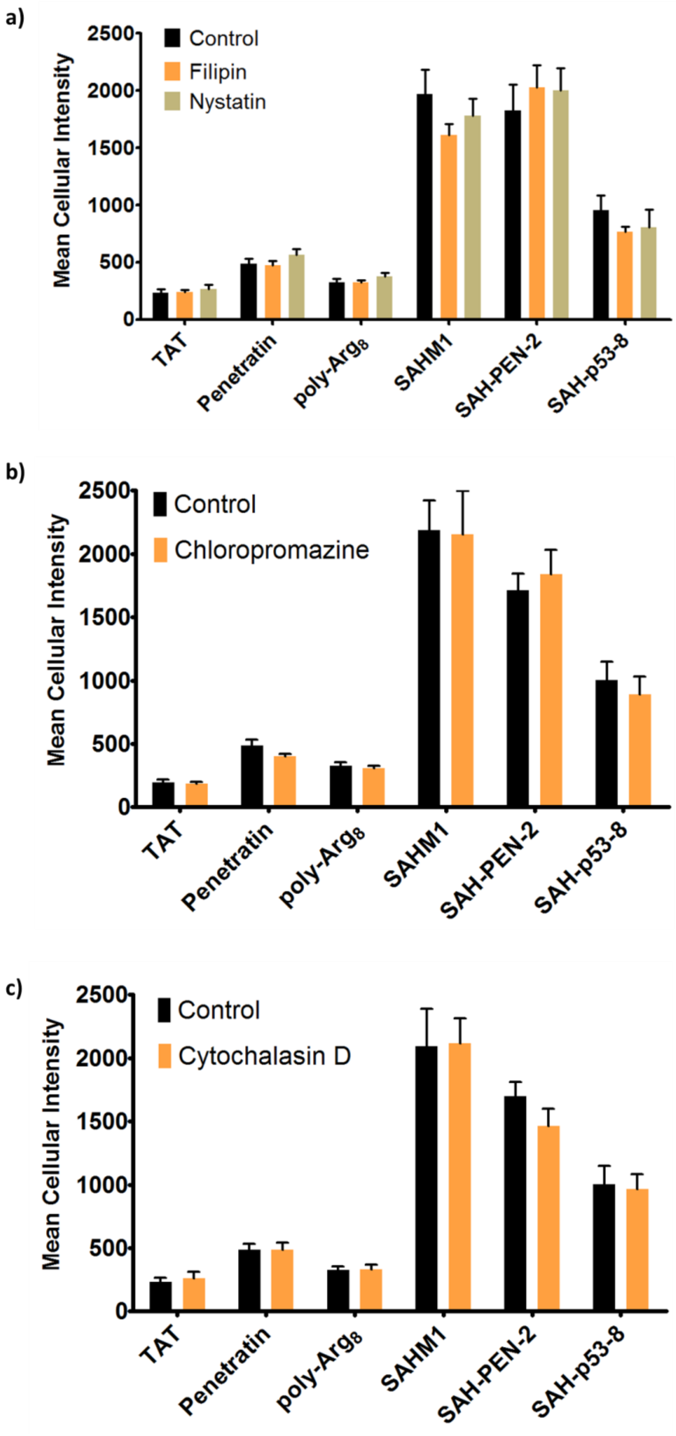


Figure S7. Quantitative illustration showing cellular uptake for peptides under various conditions that each block an endocytosis pathway. a) Filipin and Nystatin prevent assembly of caveolin-coated invaginations, b) Chlorpromazine prevents clathrin-coated pit formation, and c) Cytochalasin D blocks actin polymerization. Experiments were performed in triplicate, and error bars represent S.D. of three measurements.

Table S1. The physiochemical properties and intracellular access of peptides in the cell penetration screen.

Peptide Entry	Peptide sequence*	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type**	Fluorescence Intensity		Mean Fluorescence Intensity
A7	FITC-βAla-TRQARRNRRRRWRRAAAA	2768.095	-17.7	12.22	7.9	1386.17	1	756.557	648.657	702.607
A21	FITC-βAla-TRQARRNRRRRWRERQR	2897.213	-19.59	12.13	7.9	1499.36	1	505.075	495.357	500.216
A38	FITC-βAla-RRRRRRRRWRRR	2381.742	-13.07	12.63	9.9	1228.96	1	518.251	520.961	519.606
A39	FITC-βAla-RQIKIWFQNRRMKWKK	2706.201	-5.81	11.15	5.89	1124.6	1	482.995	500.584	491.789
A45	FITC-βAla-RRRRRRRRRRRR	2351.717	-15.42	12.72	10.9	1275.07	1	525.115	533.447	529.281
A50	FITC-βAla-RRRRRRRRWRRR (same as A38)	2381.742	-13.07	12.63	9.9	1228.96	1	552.135	522.907	537.521
A51	FITC-βAla-YGRKKRRQRRRP	2116.417	-10.33	11.58	6.88	1010.24	1	477.591	473.034	475.312
D8	FITC-βAla-LSQETFSDLWKLLPEN	2379.597	-5.07	3.15	-3.1	940.64	1	460.747	487.38	474.063
G19	FITC-βAla-RKFKRLFQ	1581.84	-1.03	10.17	2.53	634.8	1	433.377	440.598	436.9875
J11	FITC-(PEG)$_5$-NQLKRSFFALRDQI	2459.772	-6.11	8.9	0.9	982.47	1	476.233	481.78	479.006
J19	FITC-βAla-NELKRSFFALRDQI	2196.442	-5.02	6.33	-0.1	921.3	1	482.644	473.349	477.996
J23	FITC-βAla-NQLKRSFFALRDQI	2195.457	-5.83	8.9	0.9	927.09	1	468.336	474.074	471.205
J37	FITC-βAla-NQL-R8-RSFFAL-S5-DQI (ummetathesized)	2231.569	0.52	3.67	-1.1	839.17	1	889.65	863.598	876.624
J44	FITC-βAla-TILKASVDYIRKLQREQQRAKEL	3246.696	-11.37	9.29	1.87	1417.99	1	472.653	472.79	472.722

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J45	FITC-βAla-FNINDRIKELGTLI	2105.371	-3.55	4	-1.1	859.4	1	529.736	508.954	519.345
J56	FITC-βAla-KNHTHQQDI	1579.65	-8.27	6.72	-0.77	715.15	1	435.554	449.736	442.645
J60	FITC-βAla-DHIKDSFHSLRDSVPSLQGEKASRAQILDKATEYIQYN$_{L}$RRK	5260.81	-24.26	8.53	1.18	2291.33	1	431.504	437.145	434.325
J63	FITC-βAla-NELKRSFFALRDQIPSLQGEKASRAQILDKATEYIQYN$_{L}$RRK	5341.024	-20.04	9.45	2.84	2292.26	1	496.388	443.158	469.773
J69	FITC-βAla-EYIQYN$_{L}$RRKNHTHQQDIDDLKRQNALLEQQVRALGG	4807.281	-23.39	7.71	0.19	2167.55	1	439.364	440.488	439.926
J74	FITC-βAla-KATEYIQYN$_{L}$RRKNHTHQQDIDDL	3345.613	-14.46	6.71	-0.82	1488.05	1	427.211	428.277	427.744
R17	FITC-βAla-SSLFERFYNLVTPAGG	2217.98	-3.19	3.63	-1.11	866.25	1	670.035	650.921	660.478
R20	FITC-βAla-ASTLFETFYLGGLLG	2048.92	0.91	0.71	-2.11	740.95	1	590.445	576.457	583.451
R22	FITC-βAla-NSSFADFFHTVPYN$_{L}$L	2217.95	0.49	3.76	-2.02	803.93	1	699.569	654.147	676.858
A8	FITC-βAla-TRQARRN-S5-RRR-S5-RR	2391.725	-13.23	12.19	6.9	1192.08	2	524.128	653.377	588.752
A11	FITC-βAla-RRGSRPSGA-S5-RRR-S5-R	2217.576	-12.58	12.44	5.9	1084.54	2	545.937	506.065	526.001
A12	FITC-βAla-RRGSRPSGA-S5-RRR-S5-RAAAA	2561.88	-14.72	12.44	5.9	1200.94	2	562.223	583.499	572.861

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
A16	FITC-βAla-DRRQRRR-S5-RQR-S5-RRR	2661.028	-15.31	12.21	7.9	1362.05	2	751.496	726.077	738.786
A17	FITC-βAla-S5-RRQ-S5-RRDRQRRRRR	2661.028	-15.31	12.23	7.9	1362.05	2	655.747	1015.37	835.558
A18	FITC-βAla-S5-RRQ-S5-RRRRQRRRRR	2702.127	-15.58	12.49	9.9	1386.65	2	1562.8	947.344	1255.072
A23	FITC-βAla-TRQ-S5-RRQ-S5-RRRWRERQR	2934.312	-15.53	12.08	6.9	1437.46	2	575.099	577.835	576.467
B1	FITC-βAla-S-S5-ELV-S5-EAHNLCTLLENAIQDTVREQ	3435.792	-12.24	3.26	-4.96	1488.6	2	572.779	693.676	633.227
B5	FITC-βAla-SEELV-S5-EAH-S5-LCTLLENAIQDTVREQ	3450.803	-11.14	3.1	-5.96	1482.21	2	760.544	588.222	674.383
B6	FITC-βAla-SEELVAEA-S5-NLC-S5-LLENAIQDTVREQ	3397.741	-11.19	2.68	-6.1	1476.99	2	535.12	512.897	524.008
B7	FITC-βAla-SEELVAEAH-S5-LCT-S5-LENAIQDTVREQ	3408.724	-12.39	3.09	-5.96	1482.81	2	579.51	522.387	550.948
B9	FITC-βAla-SEELVAEAHNLC-S5-LLE-S5-AIQDTVREQ	3420.777	-10.51	3.1	-5.96	1462.58	2	850.458	647.007	748.732
B12	FITC-βAla-SEELVAEAHNLCTLLE-S5-AIQ-S5-TVREQ	3406.794	-10.5	3.28	-4.96	1445.51	2	644.214	779.029	711.621
B14	FITC-βAla-SEELVAEAHNLCTLLENAI-S5-DTV-S5-EQ	3351.669	-10.52	2.67	-6.96	1420.91	2	767.472	695.511	731.491

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
D1	FITC-βAla-FS-S5-LWK-S5-L	1502.774	4.94	5.96	-0.1	477.91	2	1644.55	929.536	1287.043
D2	FITC-βAla-FM-S5-YWK-S5-L	1596.909	6.74	5.92	-0.13	503.21	2	1942.4	1780.12	1861.26
D3	FITC-βAla-AM-S5-YWK-S5-L	1520.813	5.08	5.92	-0.13	503.21	2	1319.53	830.63	1075.08
D6	FITC-βAla-QTFS-S5-LWK-S5-L	1732.008	2.08	5.97	-0.1	599.43	2	1007.14	869.611	938.375
D7	FITC-βAla-QTFSD-R5-WK-S5-L	1733.937	0.18	3.68	-1.1	636.73	2	702.898	519.423	611.160
G1	FITC-βAla-PPKKFR-S5-LFF-S5-S	1976.343	2.91	9.59	1.53	648.86	2	2755.21	3091.25	2923.23
G2	FITC-βAla-KKFR-S5-LFF-S5-S	1782.113	3.44	9.59	1.53	608.24	2	2690.33	2580.05	2635.19
G3	FITC-βAla-KK-pff-R-S5-LFF-S5-S	1872.065	4.16	9.12	1.51	608.24	2	2282.17	2306.42	2294.295
G4	FITC-βAla-RRLFR-S5-N_LFL-S5-T	1931.307	2.77	10.17	1.54	709.1	2	1289.8	1253.4	1271.6
G6	FITC-βAla-RK-pff-S5-RLF-S5-SY	1916.078	3.07	9.02	1.5	664.35	2	2699.11	2082.09	2390.6
G7	FITC-βAla-RR-pff-S5-RLF-S5-SY	1944.091	2.29	9.1	1.5	700.23	2	2434.69	2363.15	2398.92
G8	FITC-βAla-RKF-S5-RLF-S5-SY	1826.125	2.36	9.27	1.51	664.35	2	2385.36	3255.29	2820.325
G9	FITC-βAla-RKA-S5-RLF-S5-SY	1750.029	0.7	9.27	1.51	664.35	2	1420.46	1588.46	1504.46
G10	FITC-βAla-R-pff-K-S5-RLF-S5-SY	1916.078	3.07	8.98	1.49	664.35	2	2307.2	2333.86	2320.53

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
G11	FITC-(PEG)$_2$-RK-pff-S5-RLF-S5-SY	2048.235	2.93	9.06	1.5	692.04	2	1738.15	1407.6	1572.875
G12	FITC-(PEG)$_2$-RKA-S5-RLF-S5-SY	1882.187	0.56	9.27	1.51	692.04	2	1138.56	1235.54	1187.05
G13	FITC-βAla-RKF-S5-RLF-S5	1575.875	3.12	9.71	1.53	565.69	2	3552.8	2725.19	3138.995
G14	FITC-βAla-KQKRKFS-S5-FFK-S5-L	2166.586	0.41	10.47	3.52	790.67	2	715.925	827.459	771.692
G15	FITC-βAla-KQKRKFS-S5-FFK-S5-LV	2265.717	0.76	10.36	3.52	819.77	2	921.429	927.04	924.234
G16	FITC-βAla-KQKRK-pff-S-S5-FFK-S5-L	2256.539	1.12	9.9	3.51	790.67	2	1946.76	1565.86	1756.31
G17	FITC-(PEG)$_2$-KQ-pff-RKKS-S5-FFK-S5-L	2388.696	0.98	9.95	3.51	818.36	2	822.401	799.392	810.896
G18	FITC-(PEG)$_2$-KQKRK-pff-S-S5-FFK-S5-L	2388.696	0.98	9.82	3.5	818.36	2	847.144	806.476	826.81
G20	FITC-βAla-RK-pff-S5-RLF-S5	1665.827	3.84	9.14	1.51	565.69	2	3576.16	2514.56	3045.36
G21	FITC-βAla-KF-S5-RLF-S5	1419.689	4.57	8.43	0.53	474.69	2	1804.46	2035.57	1920.015
G22	FITC-βAla-F-S5-RLF-S5	1291.517	5.24	5.52	-0.46	419.57	2	1921.42	1400.76	1661.09
G23	FITC-βAla-RKA-S5-RLF-S5	1499.779	1.47	9.71	1.53	565.69	2	838.791	565.454	702.122
G24	FITC-βAla-KTYRGAFQ-S5-LFQ-S5-VRE	2452.785	-2.8	8.26	0.52	962.43	2	999.416	994.049	996.732
I4	FITC-βAla-STALR-S5-LIE-S5-LVNITQNQKAPL	2914.52	-7.53	3.74	-1.1	1146.73	2	604.663	591.09	597.876

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
I5	FITC-βAla-STALRELI-S5-ELV-S5-ITQNQKAPL	2929.52	-6.41	3.33	-2.1	1140.94	2	702.946	788.77	745.858
I6	FITC-βAla-STALRELIEEL-S5-NIT-S5-NQKAPL	2945.48	-7.9	3.14	-3.09	1178.24	2	702.252	733.351	717.801
I7	FITC-βAla-STALRELIEELV-S5-ITQ-S5-QKAPL	2944.52	-5.29	3.14	-3.09	1135.15	2	967.8	1765.12	1366.46
J22	FITC-βAla-NELK-S5-SFF-S5-LRDQIPELENNEKAP	3341.7	-8.9	3.57	-3.11	1356.92	2	489.011	484.439	486.725
J24	FITC-βAla-NELK-S5-SFF-S5-LRDQI	2219.515	-1.25	3.97	-1.1	859.4	2	490.935	489.105	490.02
J42	FITC-βAla-LENRQ-S5-KLE-S5-ANRHLL	2443.78	-6.25	7.59	0.05	1031.04	2	432.028	435.647	433.837
J43	FITC-βAla-LENRQKKLE-S5-ANR-S5-LL	2434.813	-5.61	8.82	0.89	1028.38	2	469.099	485.868	477.484
J47	FITC-βAla-IL-S5-ASV-S5-YIRKLQREQ	2426.832	-2.04	8.69	0.88	962.43	2	785.442	658.812	722.127
J49	FITC-βAla-ILKAS-S5-DYI-S5-KLQREQ	2414.775	-2.79	6.36	-0.13	963.85	2	450.395	434.870	442.632
J53	FITC-βAla-FNI-S5-DRI-S5-ELGTLI	2113.433	0.9	3.39	-2.1	790.29	2	1141.23	819.010	980.124
J55	FITC-βAla-DHIK-S5-SFH-S5-LRDSV	2163.412	-3.54	6.72	-0.77	850.81	2	547.172	480.834	514.003
J57	FITC-βAla-KN-S5-THQ-S5-DI	1564.718	-3.48	5.52	-0.95	643.38	2	473.558	473.338	473.448
J58	FITC-βAla-DHIKDSF-S5-SLR-S5-SV	2113.35	-3.82	5.5	-0.95	842.36	2	456.135	449.155	452.645

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J72	FITC-βAla-S5-YIQ-S5-N$_L$RRKNHTHQQDIDDLLKRQNALLEQQVRALGG	4765.331	-21.53	8.67	1.21	2110.02	2	563.150	539.621	551.386
R1	FITC-βAla-TYRGAAQ-S5-AAQ-S5-VREV	2258.09	-3.54	6.36	-0.11	939.57	2	601.426	583.932	592.679
R2	FITC-βAla-SYRGAFQ-S5-LFQ-S5-VREV	2452.2	1.03	6.36	-0.11	939.57	2	975.55	1072.68	1024.115
R3	FITC-βAla-TY-S5-GAF-S5-NLFQSVREV	2369.12	1.35	3.79	-1.11	854.81	2	1028.58	923.419	975.999
R4	FITC-βAla-SSVFY-S5-YFH-S5-VPYFEL	2536.15	8.47	3.63	-2.06	810.17	2	963.101	1119.88	1041.490
R5	FITC-βAla-A-S5-SVF-S5-NYFHSVPYFEL	2558.16	5.14	3.63	-2.03	862.13	2	756.885	783.888	770.386
R7	FITC-βAla-A-S5-TLF-S5-TFYLGGLLG	2111.05	6.28	0.79	-1.11	683.42	2	1124	1148.39	1136.195
R11	FITC-(PEG)$_2$-GAF-S5-NLF-S5-SV	1839.88	3.22	0.7	-1.08	606.57	2	1871.07	1592.69	1731.88
R12	FITC-(PEG)$_2$-S5-GAF-S5-NLFQSV	1967.94	1.53	1.41	-1.08	678.76	2	1209.35	1289.77	1249.56
R13	FITC-(PEG)$_2$-S5-GAF-S5-NLF-R5-SV	1978.98	4.67	0.7	-1.08	635.67	2	930.5	967.173	948.836
R23	FITC-βAla-A-S5-SSF-S5-DFFHTVPYN$_L$L	2382.11	5.37	3.7	-2.01	789.94	2	1380.97	793.39	1087.18
S1	FITC-βAla-ERLRRRI-S5-LCR-S5-HHST	2542.944	-8.02	10.61	3.23	1132.09	2	2215.46	2090.93	2153.195
S1-4	FITC-βAla-ERLRRRI-S5-N$_L$CR-S5-HHST	2540.27	-9.84	10.71	3.12	N.D.	2	3889.55	4204.69	4047.12

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
S1-5	FITC-βAla-ERLRRRI-S5-FCR-S5-HHST	2575.95	-9.45	10.71	3.12	N.D.	2	1664.76	1713.72	1689.24
S1-7	FITC-βAla-ERLRRRL-S5-LCR-S5-HHST	2541.94	-5.44	10.71	3.12	N.D.	2	2168.68	3144.83	2656.755
S1-8	FITC-βAla-ERLRRRN$_L$-S5-LCR-S5-HHST	2541.94	-8.4	10.71	3.12	N.D.	2	686.611	1068.27	877.440
S1-9	FITC-βAla-ERLRRRF-S5-LCR-S5-HHST	2575.95	-3.05	10.71	3.12	N.D.	2	1115.44	1635.05	1375.245
S1-11	FITC-βAla-ERN$_L$RRRI-S5-LCR-S5-HHST	2541.94	-6.11	10.71	3.12	N.D.	2	1676.22	858.871	1267.545
S1-12	FITC-βAla-ERFRRRI-S5-LCR-S5-HHST	2575.95	-7.23	10.71	3.12	N.D.	2	658.642	603.695	631.168
S1-13	FITC-βAla-ERWRRRI-S5-LCR-S5-HHST	2615.99	-6.84	10.71	3.12	N.D.	2	1864.66	1241.87	1553.265
S1-14	FITC-βAla-ERLARRI-S5-LCR-S5-HHST	2456.53	-3.23	9.72	2.12	N.D.	2	2999.35	1594.68	2297.015
S1-D1	FITC-βAla-RELRREI-S5-LCR-S5-HHST	2515.872	-7.46	8.84	1.12	1107.49	2	4598.86	2355.2	3477.03
T049	FITC-βAla-ENPESILD-S5-HVQ-S5-VM	2220.478	-3.74	3.17	-3.95	883.07	2	456.218	439.275	447.747
T050	FITC-βAla-ENPE-S5-ILD-S5-HVQRVM	2289.587	-3.6	3.8	-2.95	924.74	2	420.593	426.310	423.451
T058	FITC-βAla-PE-S5-ILD-S5-HVQRVM	2046.37	-0.72	4.19	-1.95	786.15	2	584.177	597.006	590.591
T059	FITC-βAla-NPE-S5-ILD-S5-HVQRVM	2160.473	-2.71	4.19	-1.95	858.34	2	428.459	443.523	435.991
T060	FITC-βAla-WPE-S5-ILD-S5-HVQRVM	2232.58	0.49	4.19	-1.95	831.04	2	513.292	490.684	501.988

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
T061	FITC-βAla-KPE-S5-ILD-S5-HVQRVM	2174.542	-1.39	5.58	-0.95	841.27	2	454.726	461.938	458.332
T071	FITC-(PEG)$_1$-PE-S5-ILD-S5-HVRRVMR	2304.691	-2.25	7.6	0.05	914.42	2	558.356	458.181	508.268
T072	FITC-(PEG)$_1$-WPE-S5-ILD-S5-HVRRVMR	2490.901	-1.03	7.6	0.05	959.31	2	560.343	479.275	519.809
T073	FITC-(PEG)$_1$-RPE-S5-ILD-S5-HVRRVMR	2460.876	-3.7	8.99	1.05	1005.42	2	474.352	421.063	447.707
A9	FITC-βAla-TRQA-R8-RNRRRR-S5-RR	2433.805	-11.9	12.21	6.9	1192.08	3	613.807	911.278	762.542
A10	FITC-βAla-RRRR-R8-RQRRRR-S5-RR	2616.077	-12.55	12.59	9.9	1314.46	3	1200.32	911.4	1055.86
A13	FITC-βAla-RRGSRPSGA-R8-RRRRRA-S5	2546.919	-13.23	12.35	6.9	1204.64	3	566.461	631.856	599.158
A14	FITC-βAla-RRGSRPSGA-R8-RRRRRR-S5	2632.027	-14.14	12.33	7.9	1266.54	3	626.639	578.61	602.624
A15	FITC-βAla-RRGSRPSGA-R8-RRRRRA-S5-AA	2689.075	-14.3	12.35	6.9	1262.84	3	661.275	573.647	617.461
A19	FITC-βAla-R8-RRQRRR-S5-RQRRRRR	2744.207	-14.24	12.5	9.9	1386.65	3	1508.86	1590.82	1549.84
A24	FITC-βAla-TRQARRN-R8-RRRWRE-S5-QR	2877.258	-13.57	11.91	5.9	1375.56	3	569	571.396	570.198
A25	FITC-βAla-TRQARRN-R5-RRRWRE-S8-QR	2877.258	-13.57	11.91	5.9	1375.56	3	534.381	547.054	540.717
A37	FITC-βAla-RRRR-R5-RRRWRR-S8	2361.787	-7.05	12.36	7.9	1105.16	3	812.375	706.958	759.666

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
A40	FITC-βAla-RQIKIW-R5-QNRRMK-S8-KK	2665.234	-4.71	11.32	5.89	1108.81	3	570.84	531.727	551.283
A46	FITC-βAla-RRRR-R5-RRRRRR-S8	2331.762	-9.4	12.22	8.9	1151.27	3	660.104	658.93	659.517
A49	FITC-βAla-RRRR-R5-RRRWRR-S8 (same as A37)	2361.787	-7.05	12.36	7.9	1105.16	3	670.339	684.697	677.518
A52	FITC-βAla-YGRK-R5-RRQRRR-S8	2183.546	-6.27	11.6	5.88	993.01	3	562.385	537.512	549.948
B2	FITC-βAla-S-R8-ELVAEA-S5-NLCTLLENAIQDTVREQ (isomer 1)	3411.81	-10.13	2.8	-5.1	1459.92	3	673.145	786.046	729.595
B3	FITC-βAla-S-R8-ELVAEA-S5-NLCTLLENAIQDTVREQ (isomer 2)	3411.81	-10.13	2.8	-5.1	1459.92	3	638.178	1025.9	832.039
B4	FITC-βAla-SE-R8-LVAEAH-S5-LCTLLENAIQDTVREQ	3434.847	-9.45	3.25	-4.96	1445.51	3	751.087	696.879	723.983
B8	FITC-βAla-SEELVAEAH-R8-LCTLLE-S5-AIQDTVREQ	3449.858	-8.36	3.1	-5.96	1439.72	3	619.602	599.81	609.706
B10	FITC-βAla-SEELVAEAHNLC-R8-LLENAI-S5-DTVREQ	3448.83	-9.47	3.11	-5.96	1462.58	3	633.623	649.646	641.634
B11	FITC-βAla-SEELVAEAHNLCT-R8-LENAIQ-S5-TVREQ	3449.819	-11.87	3.26	-4.96	1488.6	3	815.385	657.586	736.485

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
B13	FITC-βAla-SEELVAEAHNLCTLLE-R8-AIQDTV-S5-EQ	3407.775	-8.9	2.66	-6.96	1420.91	3	781.2	523.513	652.356
D9	FITC-βAla-LSQETF-R8-DLWKLL-S5-EN	2487.821	-0.1	3.19	-3.1	929.02	3	575.609	595.273	585.441
F1	FITC-(EEA)-ILR-R8-AVSHMK-S5-LRGT (isomer 1)	2392.882	-2.46	9.85	2.05	911.39	3	1437.47	1065.54	1251.505
F3	FITC-(EEA)-ILR-R8-AVSHMK-S5-LRGT (isomer 2)	2392.882	-2.24	9.85	2.05	885.37	3	986.649	1525.75	1256.199
F4	FITC-(EEA)-IL-R8-MAVSHM-S5-SLRGT	2326.797	-1.82	7.6	0.05	869	3	1710.44	1711.45	1710.945
J1	FITC-βAla-NEL-R8-RSFRSL-S5-DSI	2188.46	-3.28	4.01	-1.1	892.65	3	624.782	630.873	627.827
J2	FITC-βAla-NEL-R8-RSFRAL-S5-DSI	2172.46	-2.24	4.01	-1.1	872.42	3	910.781	765.676	838.228
J3	FITC-βAla-NEL-R8-RSFRAL-S5-DQI	2213.512	-2.35	4	-1.1	895.28	3	651.648	814.064	732.856
J4	FITC-βAla-NEL-R8-RSFFSL-S5-DQI	2220.5	-0.83	3.3	-2.1	853.61	3	1032.71	950.423	991.566
J5	FITC-βAla-NEL-R8-RSFFAL-S5-DSI	2163.448	0.33	3.31	-2.1	810.52	3	895.491	1804.1	1349.795
J6	FITC-βAla-WNEL-R8-RSFRSL-S5-DQI	2374.67	-2.37	4.01	-1.1	937.54	3	624.778	772.729	698.753
J7	FITC-βAla-NEL-R8-RSFFAL-S5-DQI	2204.5	0.22	3.3	-2.1	833.38	3	798.9	731.273	765.086
J8	FITC-βAla-NQR-R8-LSFFAL-S5-DQI	2203.516	-0.59	3.67	-1.1	839.17	3	1314.17	1264.92	1289.545

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J9	FITC-(PEG)$_5$-NQL-R8-RSFFAL-S5-DQI	2467.831	-0.87	3.67	-1.1	894.55	3	916.016	1292.76	1104.388
J10	FITC-(PEG)$_5$-NQR-R8-LSFFAL-S5-DQI	2467.831	-0.87	3.67	-1.1	894.55	3	598.879	635.706	617.2925
J12	FITC-(PEG)$_5$-NQL-R8-RSFFAL-S5-DQI (D-amino acids)	2467.831	-0.87	3.67	-1.1	894.55	3	2040.37	2004.04	2022.205
J13	FITC-(PEG)$_5$-NQL-R8-LSFFAR-S5-DQI	2467.831	-0.87	3.67	-1.1	894.55	3	585.671	554.415	570.043
J14	FITC-(PEG)$_5$-IQD-S5-LAFFSR-R8-LQN (D-amino acids)	2467.831	-0.87	3.67	-1.1	894.55	3	1079.38	1195.99	1137.685
J15	FITC-βAla-NKL-R8-RS FFAL-S5-DQI	2203.559	0.44	6.27	-0.1	822.1	3	1160.5	1998.67	1579.585
J16	FITC-βAla-NKL-R8-RS FKAL-S5-KQI	2196.654	0.32	10.12	2.89	824.81	3	3446.2	3242.54	3344.37
J17	FITC-βAla-NEL-R8-RS FFAL-S5-DQI	2204.5	0.22	3.3	-2.1	833.38	3	1114.13	981.989	1048.059
J18	FITC-βAla-NELK-R8-S FFALR-S5-QI	2217.585	0.73	6.26	-0.1	822.1	3	3017.35	3513.49	3265.42
J20	FITC-βAla-NELK-R8-S FFALR-S5-QIPELENNE KAP	3339.771	-6.93	3.84	-2.11	1319.62	3	471.842	488.486	480.164
J21	FITC-βAla-NQL-R8-RS FFAL-S5-DQIPELENNE KAP	3325.701	-8.25	3.46	-3.1	1336.69	3	647.768	631.281	639.524
J25	FITC-(PEG)$_1$-AHL-R8-LCLEKL-S5-GLV	2134.601	3.68	5.39	-0.96	739.73	3	827.712	928.865	878.288

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J26	FITC-(PEG)$_1$-NQL-R8-RSFFAL-S5-DQI	2277.594	-0.92	3.67	-1.1	857.63	3	963.093	981.505	972.299
J27	Ac-AHL-R8-LCLEKL-S5-GLV-(K-(PEG)$_1$-FITC)	2304.81	2.83	5.39	-0.96	797.93	3	725.679	665.469	695.574
J29	FITC-(PEG)$_2$-NQL-R8-RSFFAL-S5-DQI	2335.673	-0.73	3.67	-1.1	866.86	3	1195.69	1030.08	1112.885
J30	FITC-βAla-KV-R8-ILKKAT-S5-YILS	2128.616	3.96	9.2	1.86	729.22	3	1059.86	2442.36	1751.11
J31	FITC-βAla-EENAKRR-R8-HNALER-S5-RR	2687.006	-11.14	9.83	2.05	1240.05	3	475.395	485.988	480.691
J32	FITC-βAla-EEA-R8-KRRAHA-S5-AERARR	2558.877	-9.5	9.83	2.05	1153.87	3	469.684	463.521	466.602
J33	FITC-βAla-NQL-R8-FSRFAL-S5-DQI (D-amino acids)	2203.516	-0.59	3.67	-1.1	839.17	3	1347.67	1235.88	1291.775
J34	FITC-βAla-IQD-S5-LAFFSR-R8-LQN (D-amino acids)	2203.516	-0.59	3.67	-1.1	839.17	3	1317.22	1322.91	1320.065
J35	FITC-βAla-NQL-R8-LSFFAR-S5-DQI	2189.489	-1.04	3.67	-1.1	839.17	3	622.526	633.052	627.789
J36	FITC-βAla-NQL-R8-RSFFAL-S5-DQI (D-amino acids)	2203.516	-0.59	3.67	-1.1	839.17	3	1287.33	1343.84	1315.585
J38	FITC-βAla-NQL-R8-FSRFAL-S5-DQI	2203.516	-0.59	3.67	-1.1	839.17	3	540.274	717.801	629.037
J39	FITC-βAla-NQL-R8-RSFFAL-S5-DQI (isomer 1)	2203.516	-0.59	3.67	-1.1	839.17	3	789.287	676.368	732.827

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J40	FITC-βAla-NQL-R8-RS FFAL-S5-DQI (isomer 2)	2203.516	-0.59	3.67	-1.1	839.17	3	1063.44	1064.19	1063.815
J46	FITC-βAla-TILKASVDYI RKL-R8-REQQRA-S5-EL	3282.81	-5.88	8.64	0.87	1348.88	3	488.058	465.789	476.924
J48	FITC-βAla-RNI-R8-DRI KEL-S5-TLI	2235.645	0.1	6.4	-0.1	878.21	3	1983.27	1443.82	1713.55
J50	FITC-βAla-FNI-R8-DRI KEL-S5-TLI	2226.634	2.67	4.09	-1.1	816.31	3	1015.99	1064.62	1040.30
J51	FITC-βAla-FNIN-R8-RI KELG-S5-LI	2181.596	1.92	6.25	-0.1	801.87	3	579.161	567.719	573.440
J52	FITC-βAla-RNI-R8-DRI KEL-S5-TRI	2278.673	-2.06	8.9	0.9	940.11	3	1294.82	1124.28	1209.553
J54	FITC-βAla-FNI-R8-DRI KEL-S5-TRI	2269.662	0.5	6.4	-0.1	878.21	3	1087.41	839.551	963.484
J59	FITC-βAla-KATEYIQY N$_{L}$RRKN-R8-THQQDI-S5-DL	3385.803	-8.85	7.48	-0.01	1422.07	3	495.963	464.831	480.397
J65	FITC-βAla-NQL-R8-RS FRAL-S5-DQI	2212.527	-3.16	6.28	-0.1	901.07	3	632.060	689.194	660.627
J67	FITC-βAla-NEL-R8-RS FFAL-S5-DQIDQIPAA KRVKLD	3540.052	-6.09	4.47	-1.11	1405.42	3	845.168	1229.82	1037.494
J68	FITC-βAla-NEL-R8-RS FFAL-S5-DQIDQIPKK KRKV	3425.996	-5.9	9.33	1.88	1361.96	3	1263.61	1865.90	1564.760

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
J71	FITC-βAla-EYIQ-R5-N$_L$RRKNH-S8-HQQDIDDLKRQNALLEQQVRALGG	4835.42	-19.92	7.74	0.21	2127.09	3	462.646	445.234	453.940
T048	FITC-βAla-ENPE-R8-ILDEHV-S5-RVM	2332.651	-1.46	3.5	-3.95	918.95	3	420.578	428.829	424.703
A1	FITC-βAla-S8-RQARRN-B5-RRRWRE-S8-QR (reduced)	2987.498	-8.45	11.88	5.9	1355.33	4	3043.84	5604.59	4324.215
A2	FITC-βAla-S8-RQARRQ-B5-RRRWRE-S8-QR (reduced)	3001.524	-8.17	11.71	5.9	1355.33	4	3817.07	4041.81	3929.44
A3	FITC-βAla-TRQ-S5-RRN-B5-RRRWRE-S8-QR	2971.412	-11.14	11.93	5.9	1375.56	4	3009.63	4414.14	3711.885
A4	FITC-βAla-TRQ-S5-RRQ-B5-RRRWRE-S8-QR	2985.439	-10.85	11.89	5.9	1375.56	4	3069.98	4593.74	3831.86
A5	FITC-βAla-TRQ-S5-RRA-B5-RRRWRE-S8-QR	2928.387	-9.69	11.75	5.9	1332.47	4	2989.85	3872.72	3431.285
A6	FITC-βAla-TRQ-S5-RRN-B5-RRRWRE-S8-QR (reduced)	2975.444	-10.42	11.93	5.9	1375.56	4	4484.2	5423.02	4953.61
A22	FITC-βAla-S5-RRN-B5-RRRWRE-S8	2301.678	-3.69	11.65	3.9	999.85	4	1085.04	1118.66	1101.85
A30	FITC-βAla-R8-RQARRN-B5-RRRWRE-S8-QR (reduced)	2987.498	-8.45	11.88	5.9	1355.33	4	1137.32	1374.89	1256.105

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
A31	FITC-βAla-R8-RQARRQ-B5-RRRWRE-S8-QR (reduced)	3001.524	-8.17	11.71	5.9	1355.33	4	1257.53	1361.26	1309.395
A32	FITC-βAla-S5-RRN-B5-RRRWRR-S8	2328.75	-4.25	12.09	5.9	1024.45	4	1247.29	1492.27	1369.78
A33	FITC-βAla-S5-RRQ-B5-RRRWRR-S8	2342.777	-3.96	11.85	5.9	1024.45	4	1168.67	1147.77	1158.22
A34	FITC-βAla-S5-RRA-B5-RRRWRR-S8	2285.725	-2.8	11.94	5.9	981.36	4	1238.57	1523.29	1380.93
A35	FITC-βAla-S5-RRR-B5-RRRWRR-S8	2370.833	-3.71	12.05	6.9	1043.26	4	1359.59	1300.68	1330.135
A36	FITC-βAla-S5-RRR-B5-RRRRRR-S8	2340.809	-6.06	12.25	7.9	1089.37	4	793.942	1039.95	916.946
A41	FITC-βAla-RQ-S5-KIW-B5-QNRRMK-S8-KK	2717.309	-3.62	11.32	5.89	1108.81	4	658.971	675.52	667.245
A43	FITC-βAla-S5-KIW-B5-QNRRN$_{L}$K-S8	2158.611	1.62	10.37	2.89	810.08	4	961.253	959.043	960.148
A44	FITC-βAla-S5-KIW-B5-QNRRAK-S8	2116.531	0.21	10.3	2.89	810.08	4	809.83	890.876	850.353
A47	FITC-βAla-S5-RRR-B5-RRRRRR-S8 (same as A36)	2340.809	-6.06	12.25	7.9	1089.37	4	1052.81	1035.36	1044.085
A48	FITC-βAla-S5-RRR-B5-RRRWRR-S8 (same as A35)	2370.833	-3.71	12.05	6.9	1043.26	4	1408.85	1270.41	1339.63
A53	FITC-βAla-S5-GRK-B5-RRQRRR-S8	2185.605	-5.19	11.93	5.9	972.78	4	970.934	1027.99	999.462

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
C1	FITC-βAla-L-S5-ILQ-B5-AVQ-R5-ILGLEQQVRER	2981.511	-2.31	4.09	-1.1	1164.93	4	686.475	697.567	692.021
C2	FITC-βAla-L-S5-ILQ-B5-AVQVIL-S8-LEQQVRER	3065.671	0.48	4.09	-1.1	1164.93	4	932.295	942.385	937.34
C3	FITC-βAla-LLILQQAV-S5-VIL-B5-LEQ-R5-VRER	3008.62	1.56	4.09	-1.1	1121.84	4	785.527	896.131	840.829
C4	FITC-βAla-LLILQQAV-S5-VIL-B5-LEQQVR-S8-R	3049.715	2.08	6.27	-0.1	1127.63	4	790.587	781.239	785.913
C5	FITC-βAla-LLIL-S5-QAV-B5-VIL-R5-LEQQVRER	3008.62	1.56	4.09	-1.1	1121.84	4	972.677	1090.05	1031.363
D4	FITC-βAla-S5-DFS-B5-YWK-R5-L	1833.11	5.76	3.75	-1.12	593.64	4	1129.77	882.068	1005.919
D5	FITC-βAla-R5-DFS-B5-YWK-S5-L	1833.11	5.76	3.75	-1.12	593.64	4	1384.76	1269.1	1326.93
D10	FITC-βAla-LS-S5-ETF-B8-DLWKLL-S8-EN	2524.924	3.49	3.2	-3.1	885.93	4	981.586	700.025	840.805
D11	FITC-βAla-LS-S5-ETA-B8-DLWKLL-S8-EN	2448.828	1.84	3.2	-3.1	885.93	4	1271.7	778.68	1025.19
D12	FITC-βAla-LSQ-S5-TFS-B8-LWKLLA-S8-N	2437.893	2.64	5.97	-0.1	837.35	4	800.699	676.661	738.68
E1	FITC-βAla-EDIIRNIA-S5-HLA-B5-VGDWN$_{L}$D-S8-SI	3065.53	0.07	3.56	-3.95	1142.16	4	1030.43	599.24	814.835

Table S1 (Continued)

Peptide Entry	Peptide sequence	Molecular Weight	Log *P*	Theoretical pI	Formal Charge	PSA	Peptide Type*	Fluorescence Intensity		Mean Fluorescence Intensity
E2	FITC-βAla-NIA-S5-HLA-B5-VGDWN$_L$D-S8-SI (isomer 1)	2440.81	1.19	3.39	-2.95	860.16	4	1087	747.6	917.3
E3	FITC-βAla-NIA-S5-HLA-B5-VGDWN$_L$D-S8-SI (isomer 2)	2440.81	1.99	3.39	-2.95	860.16	4	764	922.23	843.115
E4	FITC-βAla-NIA-S5-HLA-B5-VGDWN$_L$D-S8	2240.576	2.78	3.41	-2.95	781.73	4	948.928	853.246	901.087
E5	FITC-βAla-S5-HLA-B5-VGDWN$_L$D-S8 (isomer 1)	1942.237	4.5	3.41	-2.95	651.34	4	524.418	665.808	595.113
E6	FITC-βAla-S5-HLA-B5-VGDWN$_L$D-S8 (isomer 2)	1942.237	4.5	3.41	-2.95	651.34	4	849.054	743.958	796.506
J41	FITC-βAla-NVKRR-R8-HNVLER-S5-RRNEL-R8-RSFFAL-S5-DQI	4211.896	-9.31	10.62	3.05	1757.56	4	2494.60	3076.09	2785.350
J70	FITC-βAla-EYIQYN$_L$R-S5-KNH-B5-HQQDID-S8-LKRQNALLEQQVRALGG	4892.553	-14.58	7.7	0.19	2048.12	4	493.486	498.443	495.965
J73	FITC-βAla-S5-YIQ-B5-N$_L$RRKNH-S8-HQQDIDDLLKRQNALLEQQVRALGG	4871.539	-17.14	8.67	1.21	2089.79	4	516.662	549.448	533.055

* Structure of non-natural amino acids (besides *S*/*R*5, *S*/*R*8 and *B*5) and linkers are shown in Figure S8

** Peptide Type: 1 = Wild-Type, 2 = *i*, *i*+4 stapled, 3 = *i*, *i*+7 stapled, 4 = stitched

Table S2. List of peptides (sequence and type) in dose-dependent and time-course cell penetration assays.

Peptide	Peptide sequence*	Peptide Type**
FITC-TAT	FITC-βAla-GRKKRRQRRRPPQ	1
FITC-Penetratin	FITC-βAla-RQIKIWFQNRRMKWKK	1
FITC-Arg$_8$	FITC-βAla-RRRRRRRR	1
SAH-TAT-1	FITC-βAla-GRKK-S5-RQR-S5-RPPQ	2
SAH-TAT-2	FITC-βAla-GRK-S5-RRQ-S5-RRPPQ	2
SAH-PEN-1	FITC-βAla-RQI-S5-IWF-S5-NRRMKWKK	2
SAH-PEN-2	FITC-βAla-RQIKIWFQ-S5-RRM-S5-WKK	2
SAH-Arg$_8$	FITC-βAla-R-S5-RRR-S5-RR	2
SAHM1	FITC-βAla-ERLRRRI-S5-LCR-S5-HHST	2
TNG90	FITC-(PEG)$_1$-RWPQ-S5-ILD-S5-HVRRVWR	2
TNG147	FITC-βAla-RRWPQ-S5-ILD-S5-HVRRVWR	2
SAH-p53-8	FITC-βAla-QSQQTF-R8-NLWRLL-S5-QN	3

* Structure of βAla and PEG$_1$ linkers are shown in Figure S8

** Peptide Type: 1 = Wild-Type, 2 = *i*, *i*+4 stapled, 3 = *i*, *i*+7 stapled

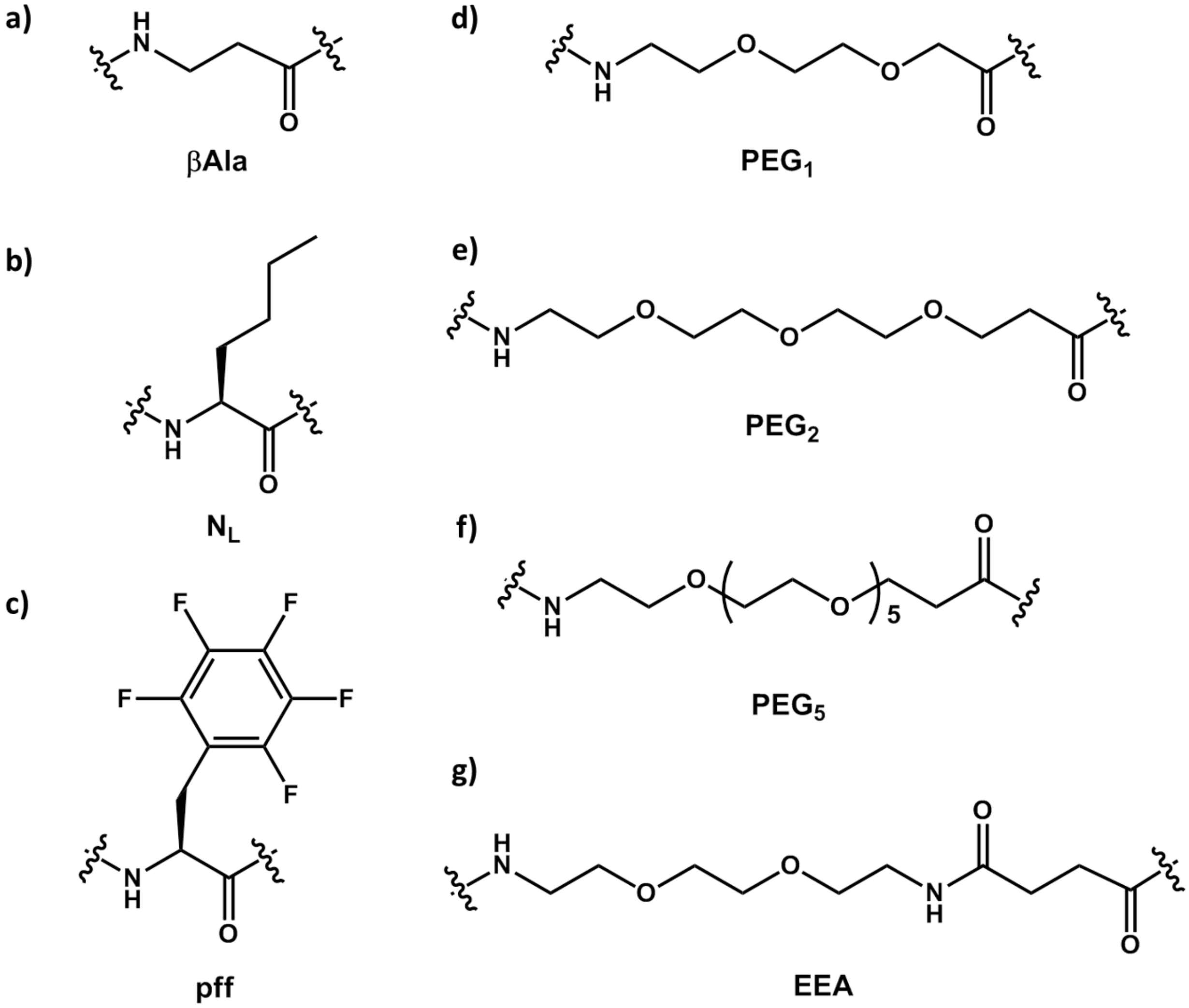


Figure S8. Structure of non-natural amino acids and linkers used in this study. a) βAla (β-alanine), b) N_L (L-Norleucine), c) pff (pentafluoro-L-phenylalanine), d) PEG_1, e) PEG_2, f) PEG_5, and g) EEA.

Table S3. Small-molecule modulators of energy-dependent endocytosis.

Modulator	**Target**	**Mechanism**
NaN_3	ATP production	Inhibitor of mitochondrial oxidative phosphorylation
2-Deoxyglucose	ATP production	Inhibitor of glycolysis
Filipin	Caveolin-mediated endocytosis	Inhibitor of caveolin-coated invagination
Nystatin	Caveolin-mediated endocytosis	Inhibitor of caveolin-coated invagination
Chlorpromazine	Clathrin-mediated endocytosis	Inhibitor of clathrin-coated pit formation
Cytochalasin D	Actin polymerization	Inhibitor of actin polymerization
$NaClO_3$	Proteoglycan synthesis	Inhibitor of ATP sulfurylase