Supporting Information

A peptide for transcellular cargo delivery: structure-function relationship and mechanism of action

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Contents:

Supplemental methods 1

Synthesis scheme 1:

The peptides Fmoc-GRLLRLLR_8-Rink, Fmoc-grllrllr_8-Rink, Fmoc-GRLLrLLR_8-Rink, Fmoc-GRLLLRLLLR_8-Rink, Fmoc-GRLLLLLR_8-Rink, Fmoc-GRLLRLLLR_8-Rink, Fmoc- GLRLRLR_8-Rink, Fmoc-GRLLRLLR_2-Rink, Fmoc-GRLLRLLR_4-Rink were synthesized on a 0.25 mmol scale on the Focus XC automated peptide synthesizer (AAPPTEC, Louisville, KY). For all peptides, Fmoc was removed by incubating with 20% 4-methylpiperidine in DMF for 15 minutes (repeated twice). K(Mtt) was manually conjugated to the N-terminus of peptides at a ratio peptide/K(Mtt)/HBTU/DIPEA (1:4:4:10) overnight. The Mtt group was removed by incubating with DCM/TIS/TFA (94:5:1) (v/v) for 5 minutes (repeated 5 times). 5FAM was manually conjugated to the peptide following the same procedure as for K(Mtt). Following the Fmoc removal, the α-amino group of the N-terminus was acetylated by incubating with 20% acetic anhydride in DMF with 80 µL of DIPEA for 20 minutes (repeated twice). The reactions were monitored by the ninhydrin test (Anaspec Inc., Fremont, CA) for free amines. The peptides were cleaved from the resin using a mixture of TFA/TIS/H_2O at a ratio of 95:2.5:2.5 for 2.5 hours. The crude products were precipitated in cold diethyl ether, centrifuged down, and placed under vacuum overnight. The peptide conjugates were purified using a Varian ProStar Model 325 HPLC (Agilent Technologies, Santa Clara, CA) equipped with Varian PLRP-S column (100 Å, 10 µm, 150 × 25 mm) and a fraction collector. Collected fractions were analyzed by ESI-MS (LDQ Deca iontrap mass spectrometer, Thermo Finnigan, USA) or MALDI-TOF (Bruker Autoflex III MALDI-TOF/TOF, Billerica, MA). The fractions with the target molecules were combined, rotovaped to remove acetonitrile, lyophilized (FreeZone −105 °C, Labconco, Kansas City, MO), and stored at −4 °C. For IR800CW NHS ester dye conjugation, the acetylated H-KGRLLRLLR_8-NH_2 was HPLC purified after cleavage from the resin and conjugated to the dye as described in [1]. Briefly, 0.43 µmol of the peptide was reacted with 0.26 µmol of IR800CW NHS ester in 100 µL DMSO in the presence of 11.4 µmol DIPEA at room temperature overnight, while stirring.

Synthesis scheme 2:

Fmoc-R_8-Rink was synthesized manually on a 1.0 mmol scale following procedure described for K(Mtt) and 5FAM conjugation (see Synthesis scheme 1). The peptides H-GGRLLRLLR_8-Rink, H-GGRLLRLLR_{12}-Rink, H-GGRLLRLLR_{16}-Rink, H-GGRLLRLYR_8-Rink, H-GGRLLRLWR_8-Rink, H-GGRLLRVLR_8-Rink, H-GGRLLRLLRLLR_8-Rink, H-GGRLLRLLRLLRLLR_8-Rink were synthesized on R_8-preloaded Rink resin on a 0.1 mmol scale using Liberty Blue automated microwave peptide synthesizer (CEM Corporation, Matthews, NC). The peptides were conjugated to carboxylated dyes 5FAM or 5(6)TAMRA following the protocol modified from [2]. The peptides were conjugated on a 0.7-2 µmol scale to dyes at a ratio peptide/dye/PyAOP/DIPEA (1:6:6:12) for 1.5 hours, while stirring. The dyes were preactivated by stirring with PyAOP in DMF for 30 min prior to adding them to the resin. After 5FAM conjugation, the resin was washed with 20% 4-methylpiperidine in DMF to remove excess dye precipitates, followed by DMF and DCM washes. After 5(6)TAMRA conjugation, the resin was washed with DMF and DCM only. The peptides were cleaved from the resin using a mixture of TFA/phenol/water/TIS (88:5:5:2) (v/v) (Reagent B) for 2 hours starting with freezer-cold temperature and allowing to warm up to room temperature. The crude products were precipitated in cold diethyl ether for 15 minutes on ice, centrifuged down, and placed under vacuum overnight. The peptides were purified on Agilent 1260 Infinity Quaternary HPLC system equipped with Agilent Zorbax 300SB C18 analytical column (300 Å, 5µm, 4.6 mm x 250 mm).

Synthesis scheme 3:

The peptides H-ggrllrllr_8-Rink and H-GGRLLrLLR_8-Rink were synthesized by conjugating a glycine using Liberty Blue synthesizer to Fmoc-grllrllr_8-Rink and Fmoc-GRLLRrLLR_8-Rink (synthesized using *Synthesis scheme 1*).

For all peptides, the peptide purity was assessed by HPLC using Agilent 1260 Infinity Quaternary HPLC system by running a binary gradient of water with 0.1%TFA (Solution A) and acetonitrile with 0.1%TFA (Solution B): 0-18 min gradient 5% to 95% B, 18-19 min gradient 95% to 5% B, 19-20 min isocratic 95% A, 5% B; flow rate: 1 ml/min; Agilent Zorbax 300SB C18 analytical column (300 Å, 5µm, 4.6 mm x 250 mm); monitored wavelength was 220 nm. The peptide molecular weight was measured using MALDI-TOF assisted with CHCA matrix.

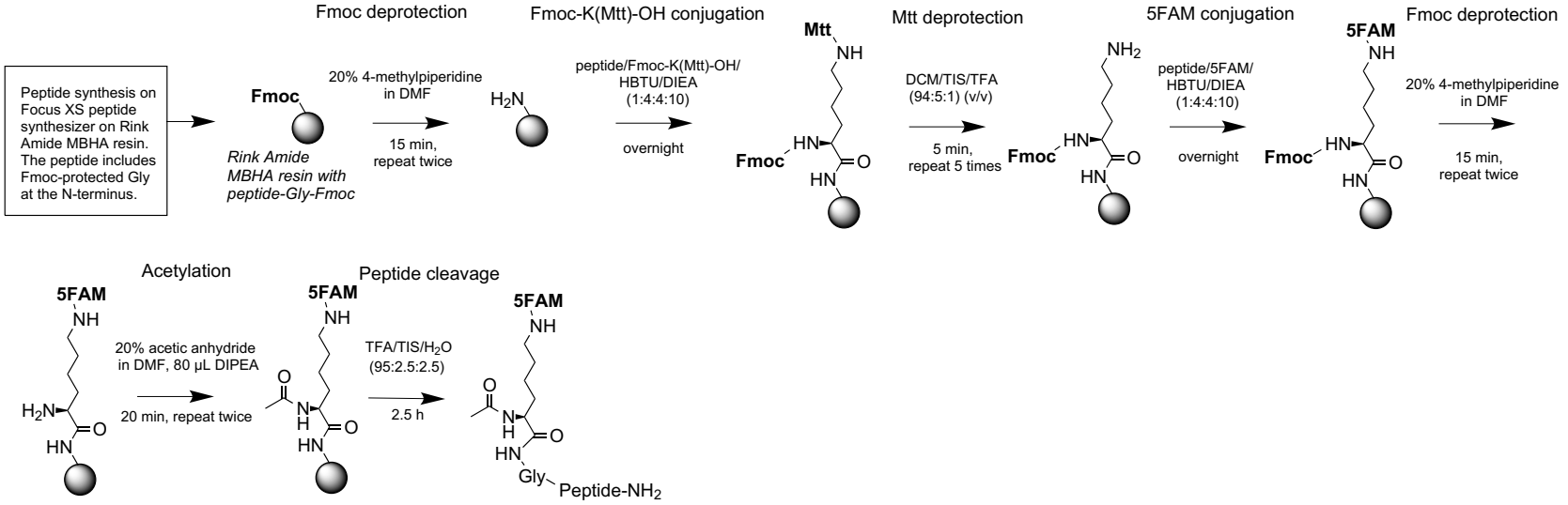
Chemical abbreviations:
CHCA: α-Cyano-4-hydroxycinnamic acid; DCM: dichloromethane; DIPEA: N,N-diisopropylethylamine; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; Fmoc: fluorenylmethyloxycarbonyl; HBTU: hexafluorophosphate benzotriazole tetramethyl uranium; Mtt: 4-methyltrityl; PyAOP: 7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TIS: triisopropylsilane; TFA: trifluoroacetic acid; 5FAM: 5-carboxyfluorescein; 5(6)TAMRA: 5(6)carboxytetramethylrhodamine isomer mixture.

References:
[1] Chen, Y. *et al*. A low molecular weight PSMA-based fluorescent imaging agent for cancer, *Biochem Biophys Res Commun* **390**, 624-629, doi: 10.1016/j.bbrc.2009.10.017 (2009).

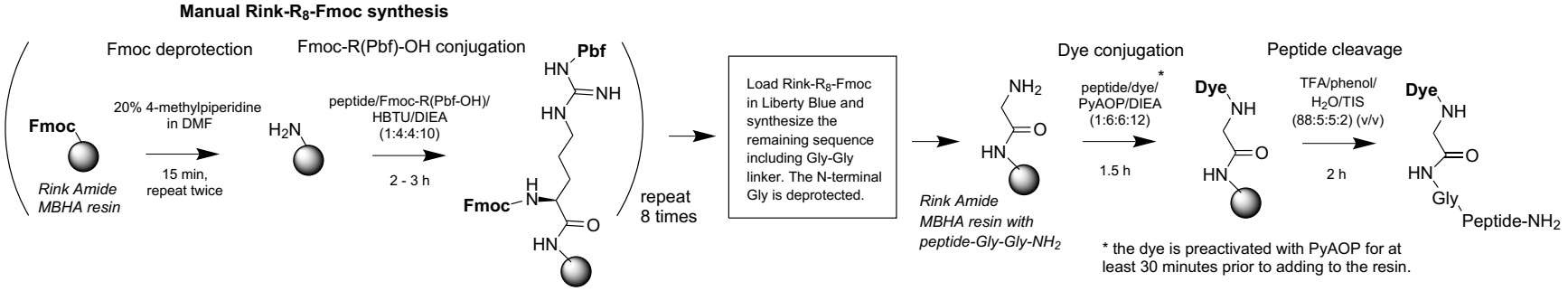
[2] Stahl, P.J. *et al.* On-the-resin N-terminal modification of long synthetic peptides, *Anal Biochem* **424**, 137-139, doi: 10.1016/j.ab.2012.02.032 (2012).

Supplemental methods 1 (continued)

Scheme 1: Synthesis of peptide variants with a Lys(Acetyl)Gly linker and 5FAM cargo.



Scheme 2: Synthesis of peptide variants with a Gly-Gly linker and 5FAM or 6TAMRA cargo.



Scheme 3: Modification of the peptides originally synthesized on Focus XS (Scheme 1) to add a Gly and a cargo.

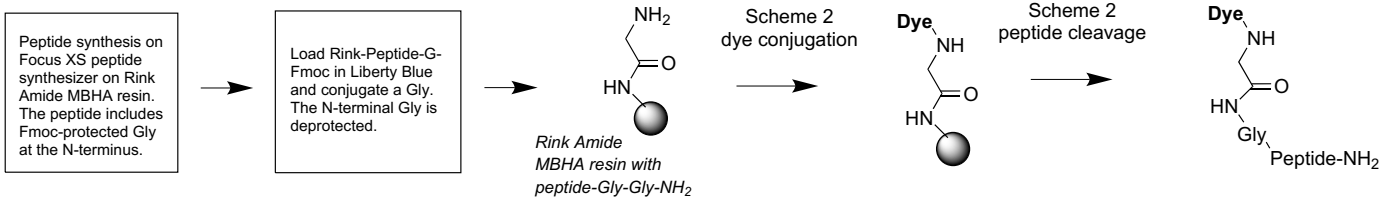
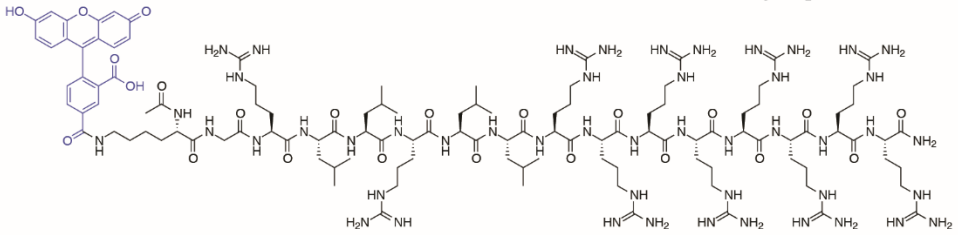


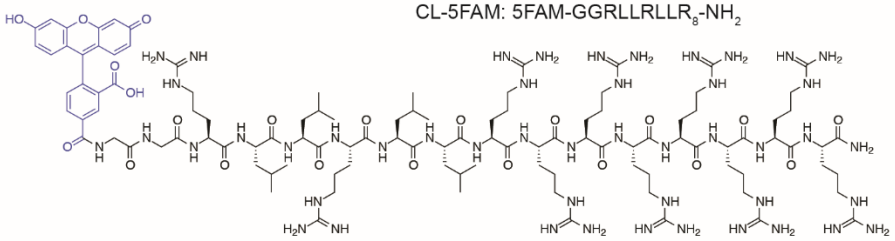
Figure S1: Chemical structures of peptide-cargo conjugates.

CL-5FAM: 5FAM-K(Acetyl)GRLLRLLR$_8$-NH$_2$
DCL-5FAM: 5FAM-k(Acetyl)grllrllr$_8$-NH$_2$
CLr-5FAM: 5FAM-K(Acetyl)GRLLrLLR$_8$-NH$_2$



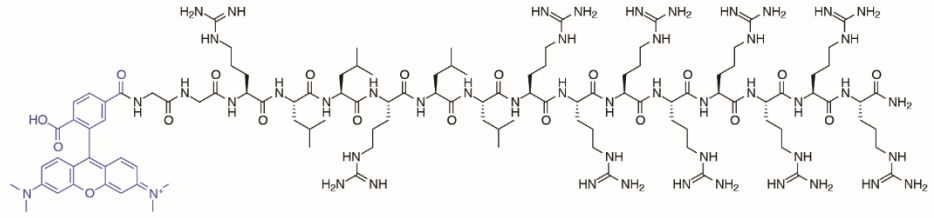
Chemical Formula: $C_{115}H_{194}N_{48}O_{23}$
Exact Mass: 2615.55
Molecular Weight: 2617.13

CL-5FAM: 5FAM-GGRLLRLLR$_8$-NH$_2$



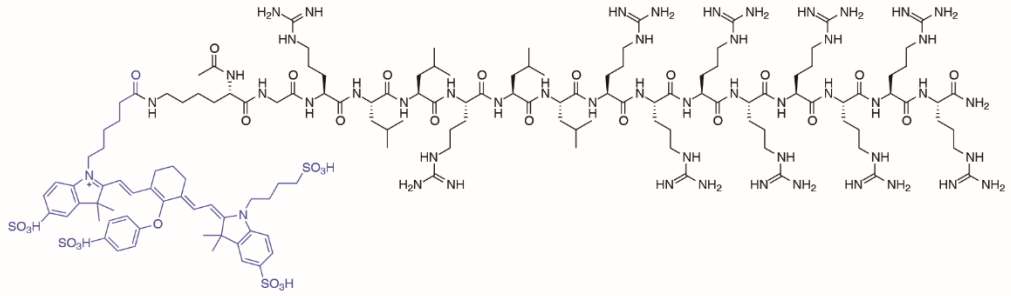
Chemical Formula: $C_{109}H_{183}N_{47}O_{22}$
Exact Mass: 2502.46
Molecular Weight: 2503.97

CL-6TAMRA: 6TAMRA-GGRLLRLLR$_8$-NH$_2$
DCL-6TAMRA: 6TAMRA-ggrllrllr$_8$-NH$_2$
CLr-6TAMRA: 6TAMRA-GGRLLrLLR$_8$-NH$_2$



Chemical Formula: $C_{113}H_{194}N_{49}O_{20}^+$
Exact Mass: 2557.57
Molecular Weight: 2559.12

CL-IR800CW: IR800CW-K(Acetyl)GRLLRLLR$_8$-NH$_2$



Chemical Formula: $C_{140}H_{237}N_{50}O_{31}S_4^+$
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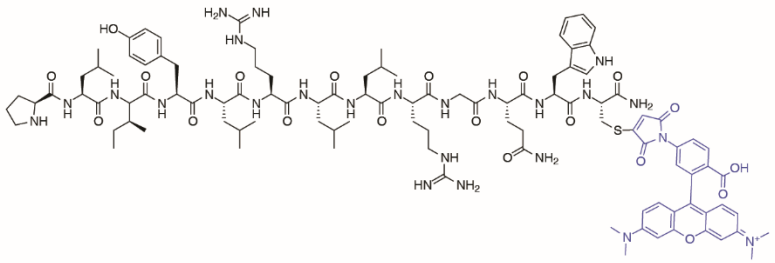
Molecular Weight: 3244.99

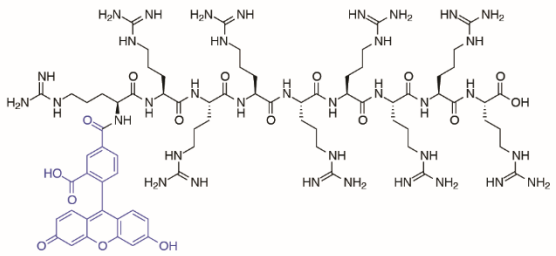
TP2-6TAMRA maleimide: PLIYLRLLRGQWC-6TAMRA maleimide



Chemical Formula: $C_{105}H_{146}N_{25}O_{20}S^+$
Exact Mass: 2109.09
Molecular Weight: 2110.54

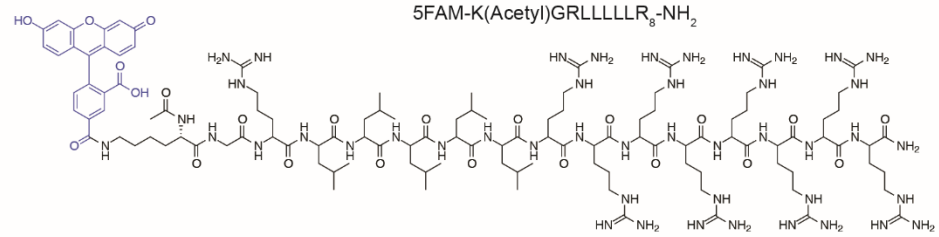
Figure S1: Chemical structures of peptide-cargo conjugates (continued).

Arg_9-5FAM: 5FAM-R_9-OH



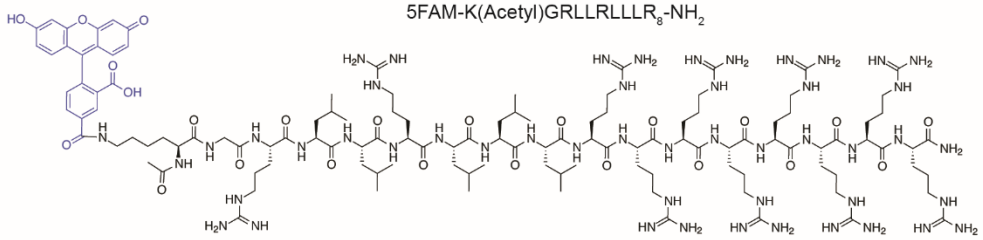
Chemical Formula: $C_{75}H_{120}N_{36}O_{16}$
Exact Mass: 1780.97
Molecular Weight: 1782.02

5FAM-K(Acetyl)GRLLLLLR$_8$-NH_2



Chemical Formula: $C_{115}H_{193}N_{45}O_{23}$
Exact Mass: 2572.53
Molecular Weight: 2574.10

5FAM-K(Acetyl)GRLLRLLLR$_8$-NH_2



Chemical Formula: $C_{121}H_{205}N_{49}O_{24}$
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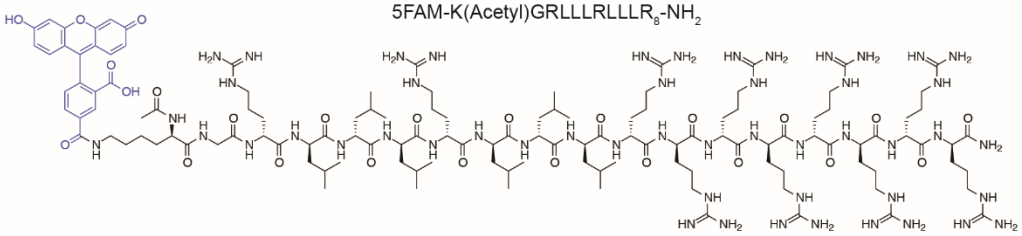
Molecular Weight: 2730.29

5FAM-K(Acetyl)GRLLLRLLLR$_8$-NH_2



Chemical Formula: $C_{127}H_{216}N_{50}O_{25}$
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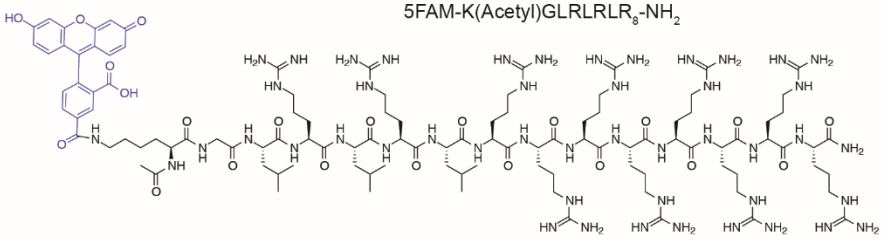
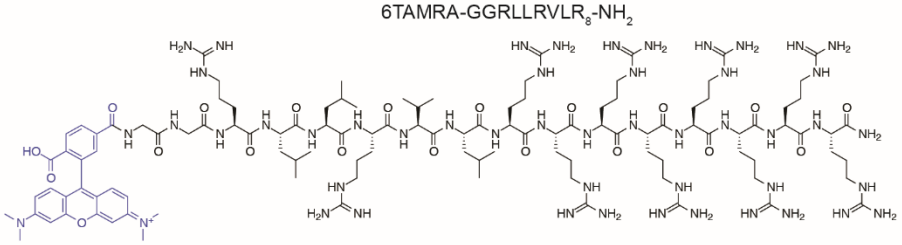
Molecular Weight: 2843.45

5FAM-K(Acetyl)GLRLRLR$_8$-NH_2



Chemical Formula: $C_{109}H_{183}N_{47}O_{22}$
Exact Mass: 2502.46
Molecular Weight: 2503.97

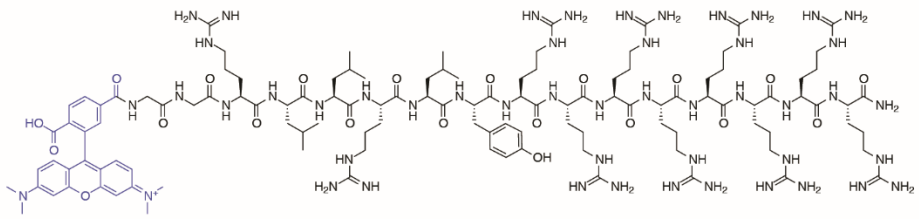
6TAMRA-GGRLLRVLR$_8$-NH_2



Chemical Formula: $C_{112}H_{192}N_{49}O_{20}^{+}$
Exact Mass: 2543.55
Molecular Weight: 2545.09

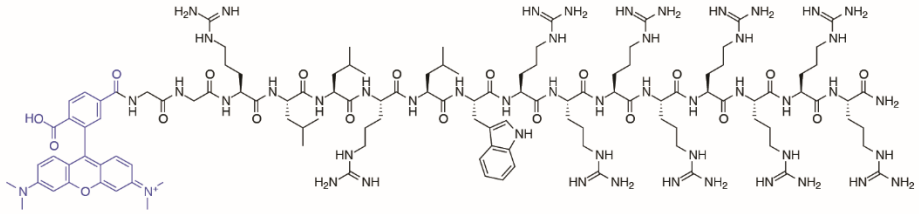
Figure S1: Chemical structures of peptide-cargo conjugates (continued).

6TAMRA-GGRLLRLYR$_8$-NH$_2$



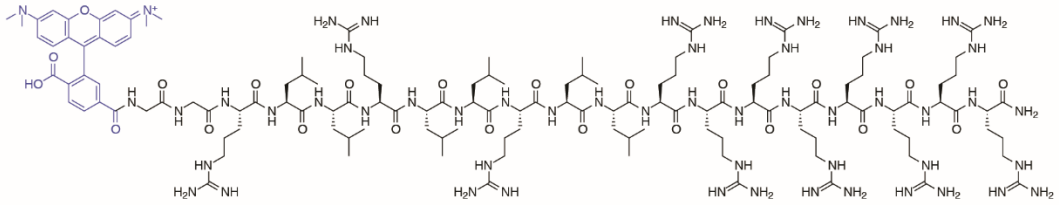
Chemical Formula: $C_{116}H_{192}N_{49}O_{21}^+$
Exact Mass: 2607.55
Molecular Weight: 2609.13

6TAMRA-GGRLLRLWR$_8$-NH$_2$



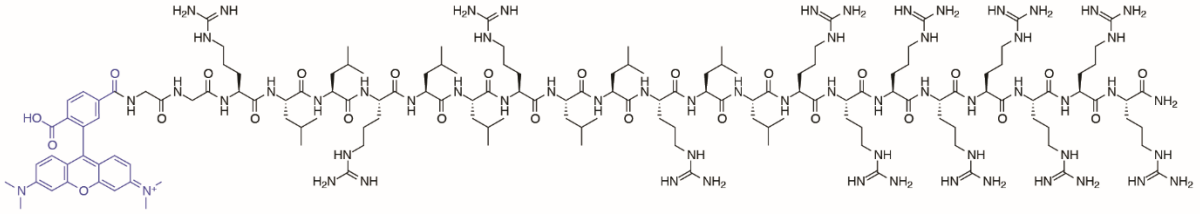
Chemical Formula: $C_{118}H_{193}N_{50}O_{20}^+$
Exact Mass: 2630.56
Molecular Weight: 2632.17

6TAMRA-GGRLLRLLRLLR$_8$-NH$_2$



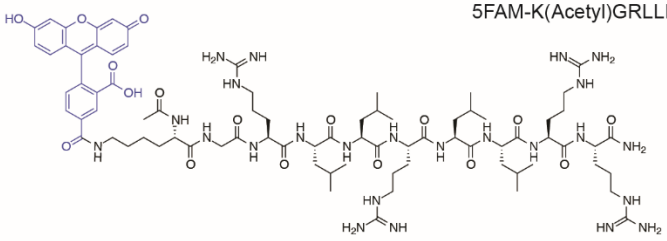
Chemical Formula: $C_{131}H_{228}N_{55}O_{23}^+$
Exact Mass: 2939.84
Molecular Weight: 2941.63

6TAMRA-GGRLLRLLRLLRLLR$_8$-NH$_2$



Chemical Formula: $C_{149}H_{262}N_{61}O_{26}^+$
Exact Mass: 3322.10
Molecular Weight: 3324.14

5FAM-K(Acetyl)GRLLRLLR$_2$-NH$_2$



Chemical Formula: $C_{79}H_{122}N_{24}O_{17}$
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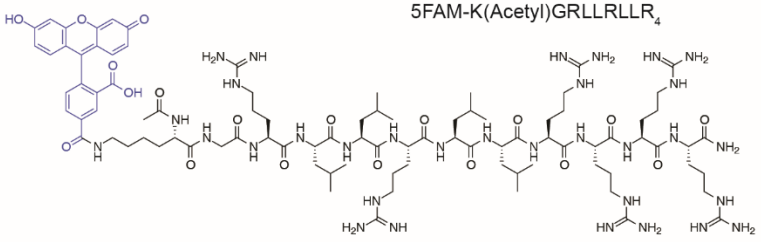
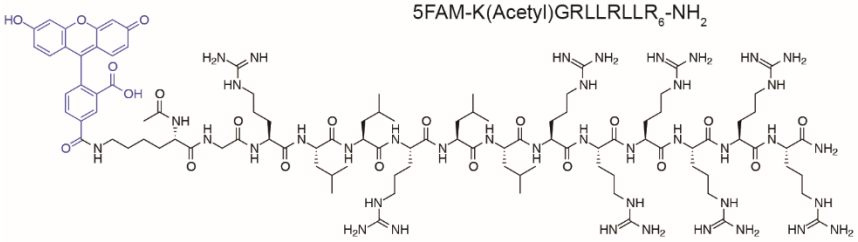
5FAM-K(Acetyl)GRLLRLLR$_4$



Chemical Formula: $C_{91}H_{146}N_{32}O_{19}$
Exact Mass: 1991.14
Molecular Weight: 1992.37

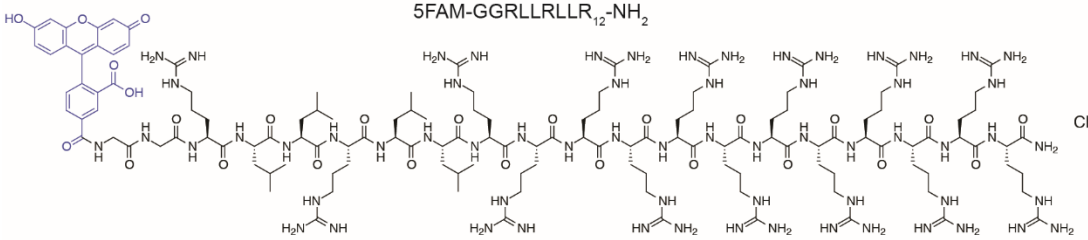
Figure S1: Chemical structures of peptide-cargo conjugates (continued).

5FAM-K(Acetyl)GRLLRLLR$_6$-NH$_2$



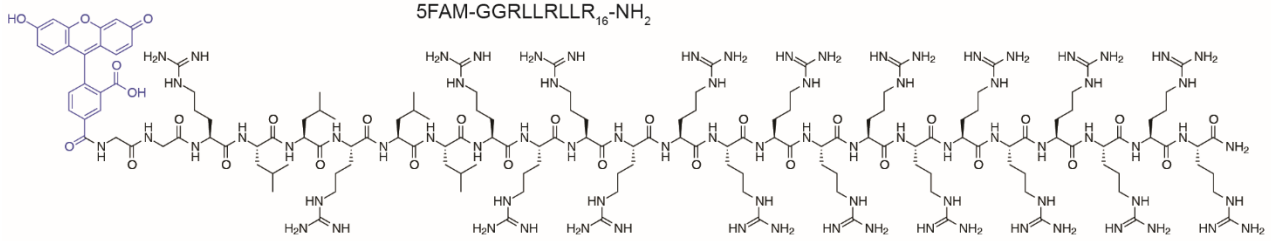
Chemical Formula: $C_{103}H_{170}N_{40}O_{21}$
Exact Mass: 2303.35
Molecular Weight: 2304.75

5FAM-GGRLLRLLR$_{12}$-NH$_2$



Chemical Formula: $C_{133}H_{231}N_{63}O_{26}$
Exact Mass: 3126.87
Molecular Weight: 3128.73

5FAM-GGRLLRLLR$_{16}$-NH$_2$



Chemical Formula: $C_{157}H_{279}N_{79}O_{30}$
Exact Mass: 3751.27
Molecular Weight: 3753.48

Figure S2: Purity and molecular weight characterization of peptide-cargo conjugates.

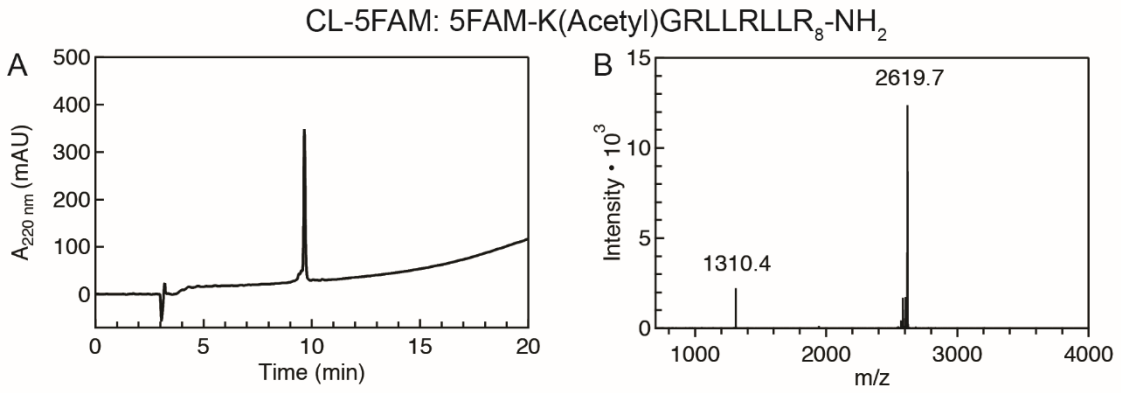


Figure S2A. (A) RP-HPLC and (B) MALDI-TOF characterization of CL-5FAM (5FAM-K(Acetyl)GRLLRLLR$_8$-NH$_2$). m/z is 2619.7 for $[M+H]^+$, 1310.4 for $[M+2H]^{2+}$. $C_{115}H_{194}N_{48}O_{23}$ calculated molecular weight 2617.13.

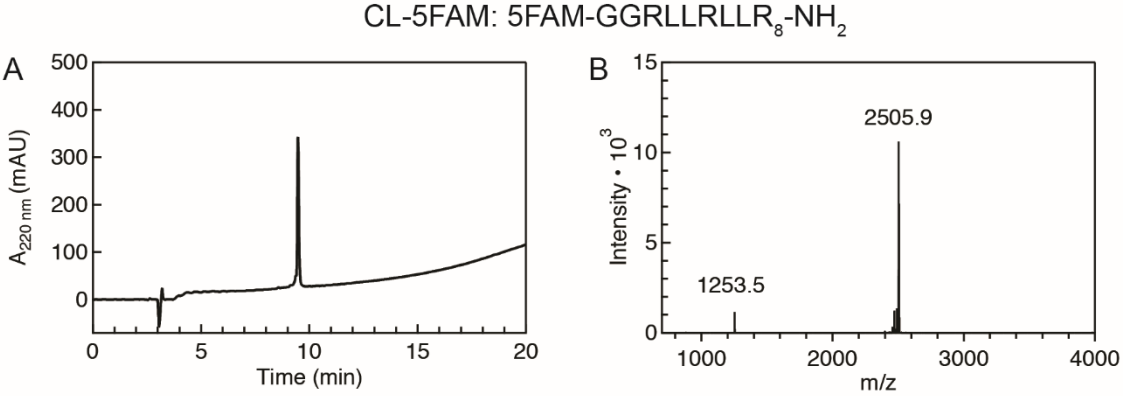


Figure S2B. (A) RP-HPLC and (B) MALDI-TOF characterization of CL-5FAM (5FAM-GGRLL-RLLR$_8$-NH$_2$). m/z is 2505.9 for $[M+H]^+$, 1253.5 for $[M+2H]^{2+}$. $C_{109}H_{183}N_{47}O_{22}$ calculated molecular weight 2503.97.

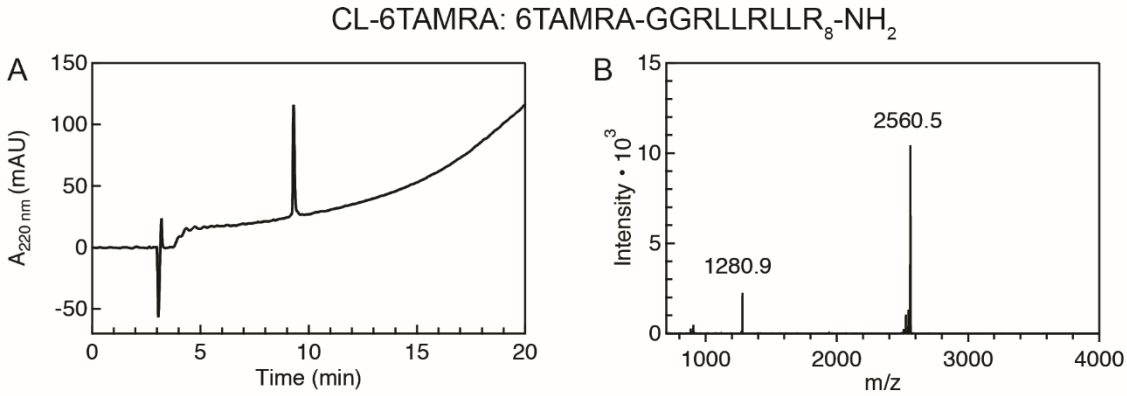


Figure S2C. (A) RP-HPLC and (B) MALDI-TOF characterization of CL-6TAMRA (6TAM-RA-GGRLLRLLR$_8$-NH$_2$). m/z is 2560.5 for $[M+H]^+$, 1280.9 for $[M+2H]^{2+}$. $C_{113}H_{194}N_{49}O_{20}$ calculated molecular weight 2559.12.

CL-IR800CW: IR800CW-K(Acetyl)GRLLRLLR$_8$-NH$_2$

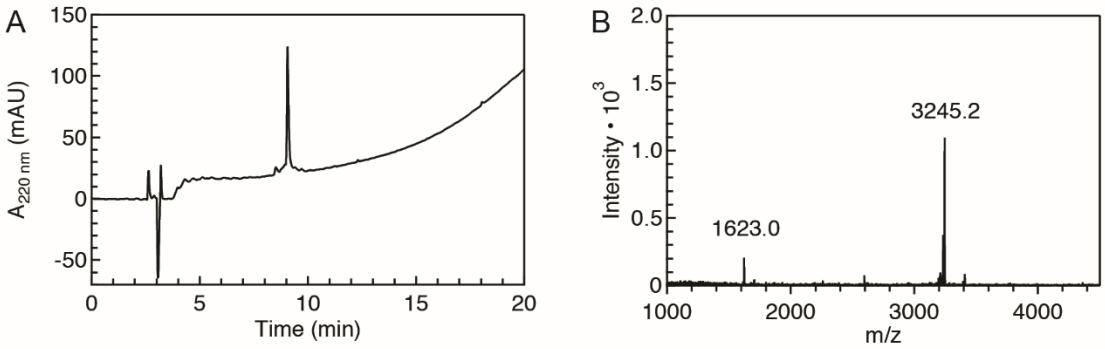


Figure S2D. (A) RP-HPLC and (B) MALDI-TOF characterization of CL-IR800CW (IR800CW-K(Acetyl)GRLLRLLR$_8$-NH$_2$). m/z is 3245.2 for $[M+H]^+$, 1623.0 for $[M+2H]^{2+}$. $C_{140}H_{233}N_{50}O_{31}S_4$ calculated molecular weight 3244.99.

Arg$_9$-5FAM: 5FAM-R$_9$-OH

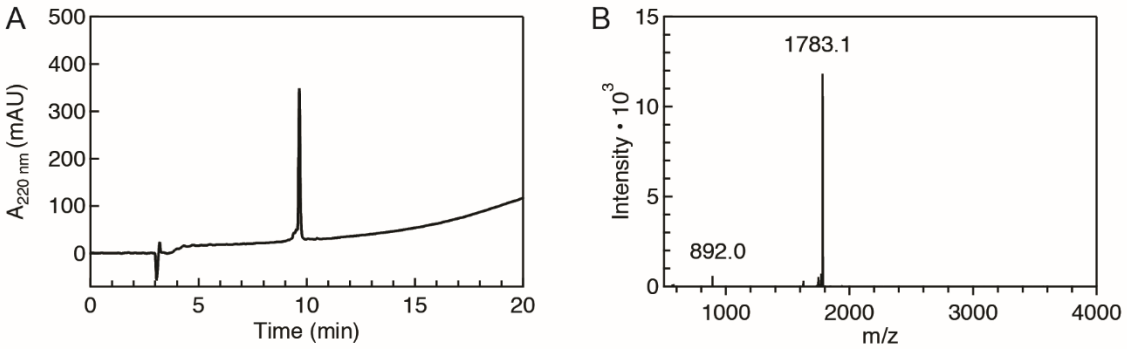


Figure S2E. (A) RP-HPLC and (B) MALDI-TOF characterization of Arg$_9$-5FAM (5FAM-R$_9$-OH). m/z is 2852.7 for $[M+H]^+$, 1426.9 for $[M+2H]^{2+}$. $C_{75}H_{120}N_{36}O_{16}$ calculated molecular weight 1782.02.

5FAM-K(Acetyl)GRLLLLLR$_8$-NH$_2$

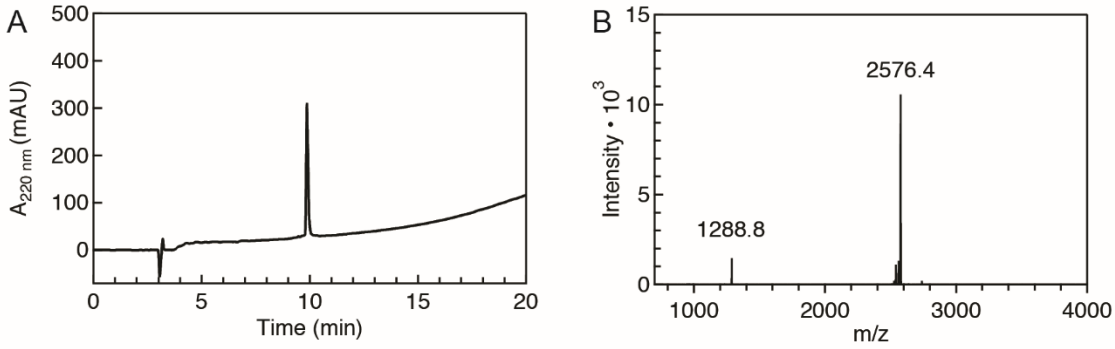


Figure S2F. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-K(Acetyl)GRLLLLLR$_8$-NH$_2$). m/z is 2576.4 for $[M+H]^+$, 1288.8 for $[M+2H]^{2+}$. $C_{115}H_{193}N_{45}O_{23}$ calculated molecular weight 2574.10.

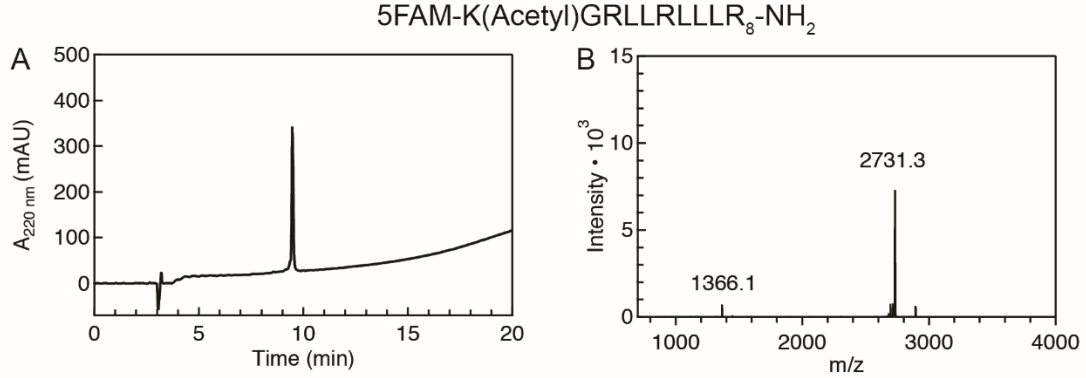


Figure S2G. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-K(Acetyl)GRLL-RLLLR$_8$-NH$_2$. m/z is 2731.3 for $[M+H]^+$, 1366.1 for $[M+2H]^{2+}$. $C_{121}H_{205}N_{49}O_{24}$ calculated molecular weight 2730.29.

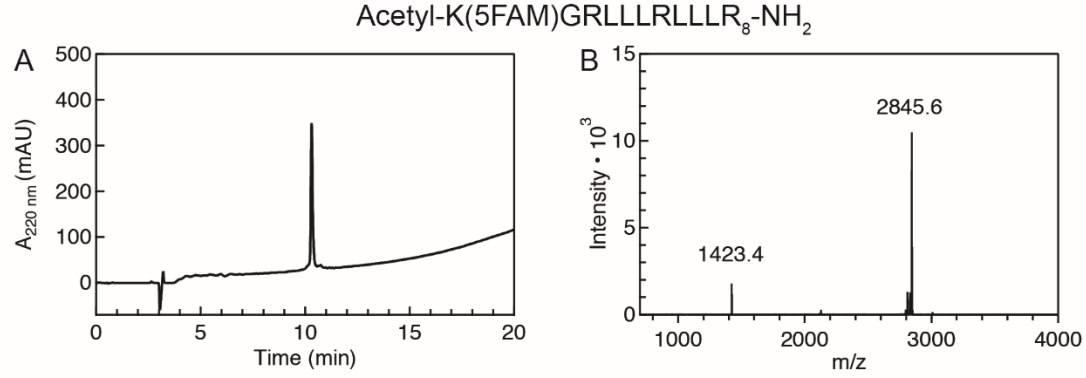


Figure S2H. (A) RP-HPLC and (B) MALDI-TOF characterization of Acetyl-K(5FAM)GRLLL-RLLLR$_8$-NH$_2$. m/z is 2845.6 for $[M+H]^+$, 1423.4 for $[M+2H]^{2+}$. $C_{127}H_{216}N_{50}O_{25}$ calculated molecular weight 2843.45.

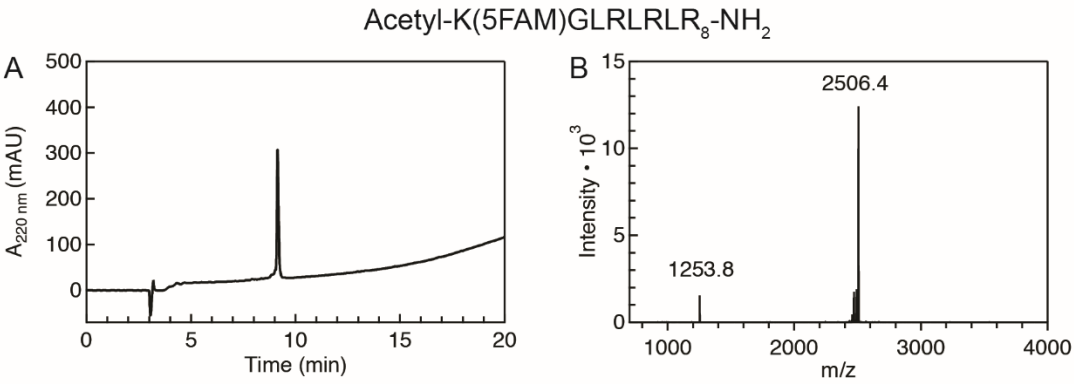


Figure S2I. (A) RP-HPLC and (B) MALDI-TOF characterization of Acetyl-K(5FAM)GLRL-RLR$_8$-NH$_2$. m/z is 2506.4 for $[M+H]^+$, 1253.8 for $[M+2H]^{2+}$. $C_{109}H_{183}N_{47}O_{22}$ calculated molecular weight 2503.97.

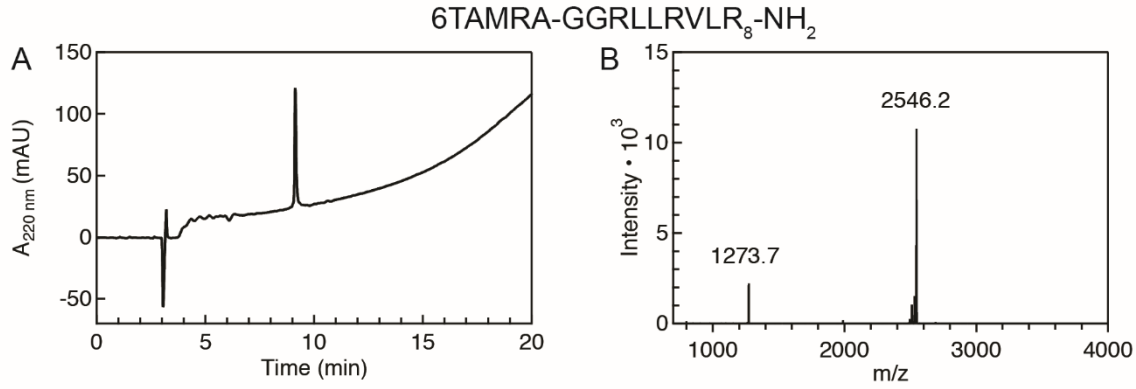


Figure S2J. (A) RP-HPLC and (B) MALDI-TOF characterization of 6TAMRA-GGRLLRVLR$_8$-NH$_2$. m/z is 2546.2 for $[M+H]^+$, 1273.7 for $[M+2H]^{2+}$. $C_{112}H_{192}N_{49}O_{20}$ calculated molecular weight

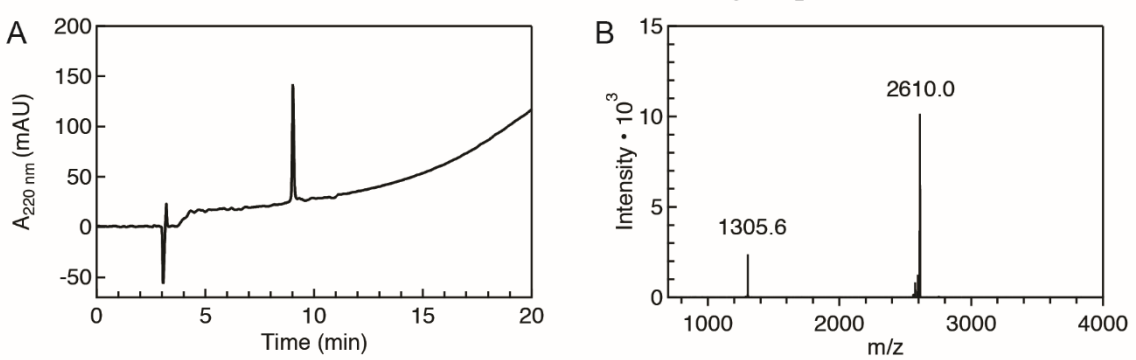


Figure S2K. (A) RP-HPLC and (B) MALDI-TOF characterization of 6TAMRA-GGRLLR-LYR$_8$-NH$_2$. m/z is 2610.0 for $[M+H]^+$, 1305.6 for $[M+2H]^{2+}$. $C_{116}H_{192}N_{49}O_{21}$ calculated molecular weight 2609.13.

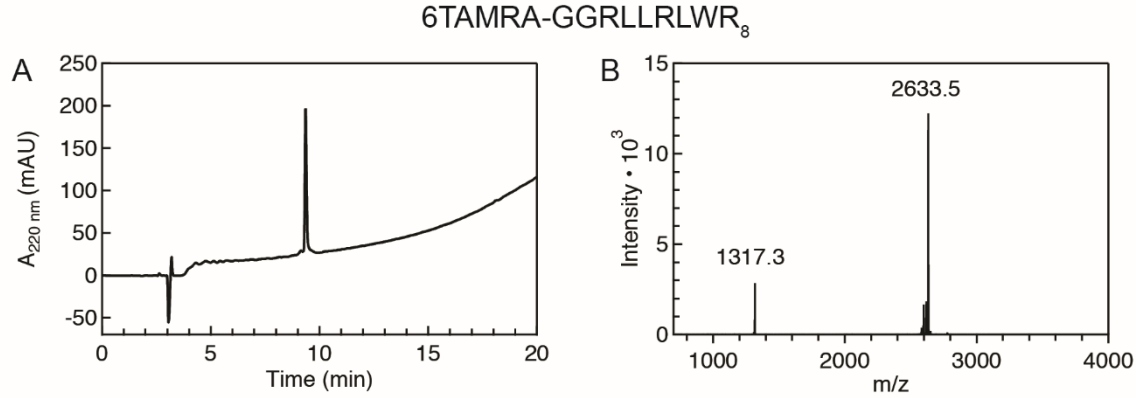


Figure S2L. (A) RP-HPLC and (B) MALDI-TOF characterization of 6TAMRA-GGRLLRL-WR$_8$-NH$_2$. m/z is 2633.5 for $[M+H]^+$, 1317.3 for $[M+2H]^{2+}$. $C_{118}H_{193}N_{50}O_{20}$ calculated molecular weight 2632.17.

6TAMRA-GGRLLRLLRLLR$_8$-NH$_2$

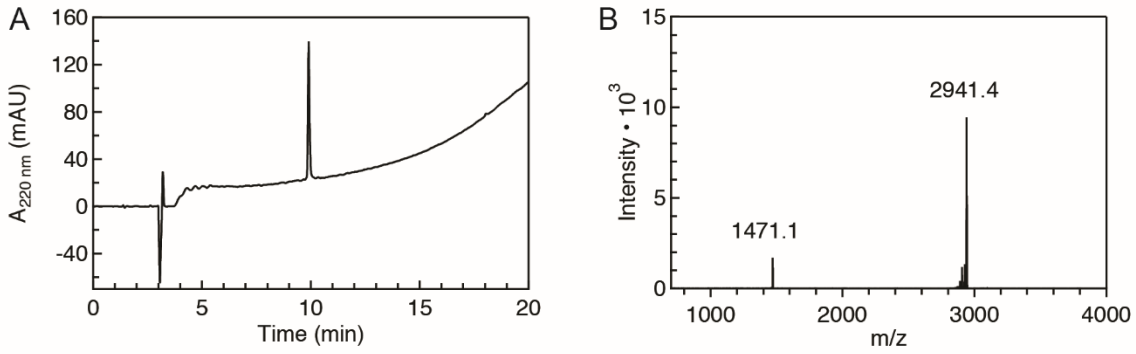


Figure S2M. (A) RP-HPLC and (B) MALDI-TOF characterization of 6TAMRA-GGRLLRLL-RLLR$_8$-NH$_2$. m/z is 2941.4 for $[M+H]^+$, 1471.1 for $[M+2H]^{2+}$. $C_{131}H_{228}N_{55}O_{23}$ calculated molecular weight 2941.63

6TAMRA-GGRLLRLLRLLRLLR$_8$-NH$_2$

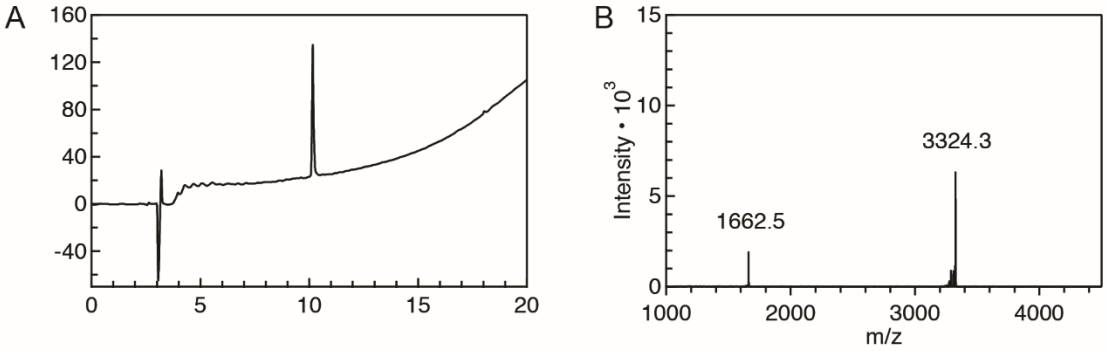


Figure S2N. (A) RP-HPLC and (B) MALDI-TOF characterization of 6TAMRA-GGRLLRLLRLL-RLLR$_8$-NH$_2$. m/z is 3324.3 for $[M+H]^+$, 1662.5 for $[M+2H]^{2+}$. $C_{149}H_{262}N_{61}O_{26}$ calculated molecular weight 3324.14.

5FAM-K(Acetyl)GRLLRLLR$_2$-NH$_2$

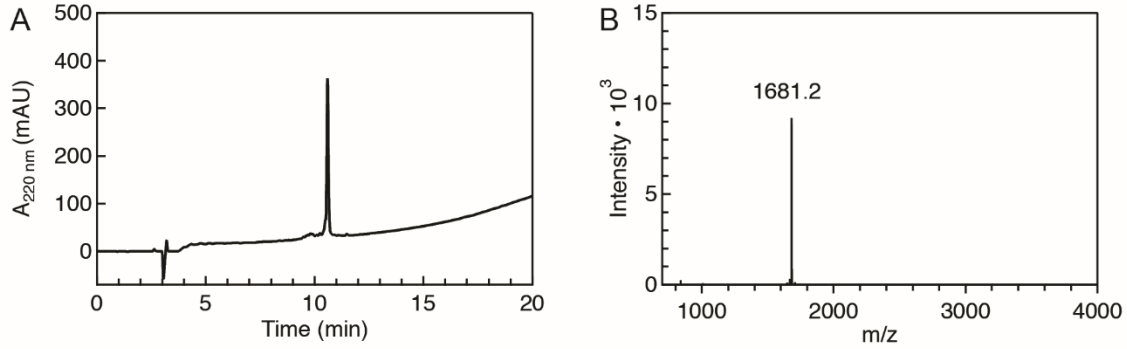


Figure S2O. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-K(Acetyl)GRLL-RLLR$_2$-NH$_2$. m/z is 1681.2 for $[M+H]^+$. $C_{79}H_{122}N_{24}O_{17}$ calculated molecular weight 1680.00.

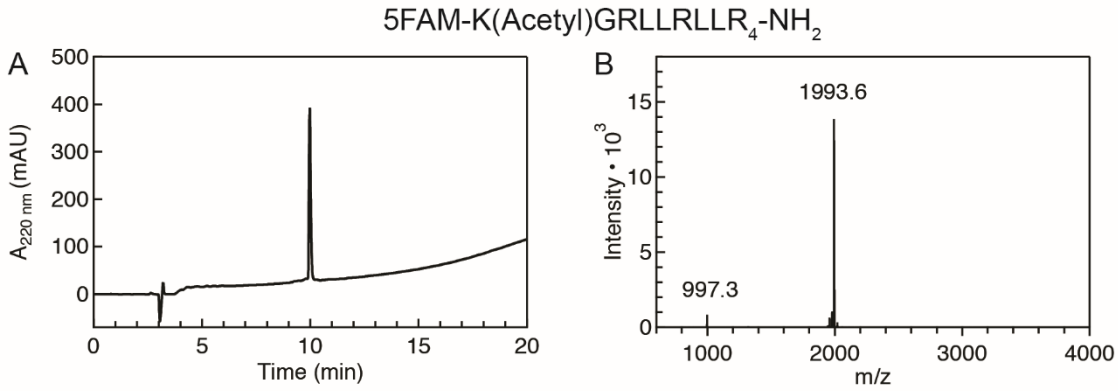


Figure S2P. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-K(Acetyl)GRLL-RLLR$_4$-NH$_2$. m/z is 1993.6 for $[M+H]^+$, 997.3 for $[M+2H]^{2+}$. $C_{91}H_{146}N_{32}O_{19}$ calculated molecular weight 1992.37.

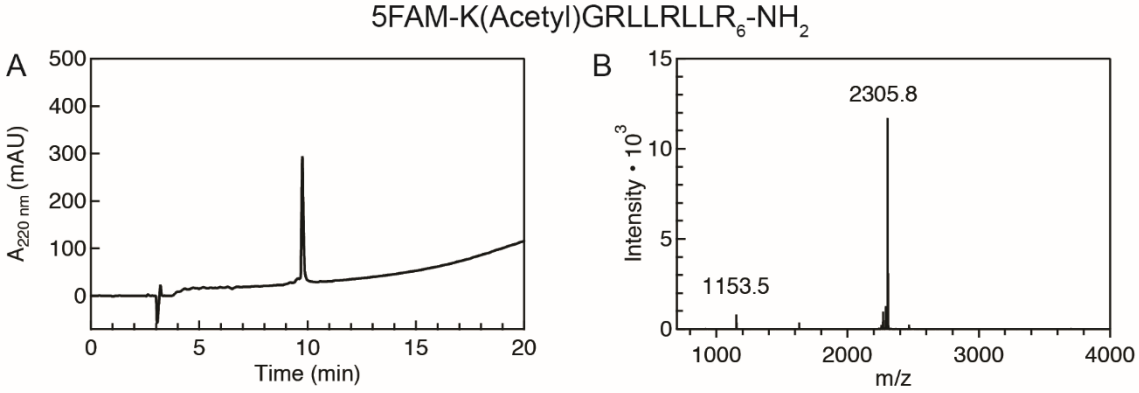


Figure S2Q. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-K(Acetyl)GRLL-RLLR$_6$-NH$_2$. m/z is 2305.8 for $[M+H]^+$, 1153.5 for $[M+2H]^{2+}$. $C_{103}H_{170}N_{40}O_{21}$ calculated molecular weight 2304.75.

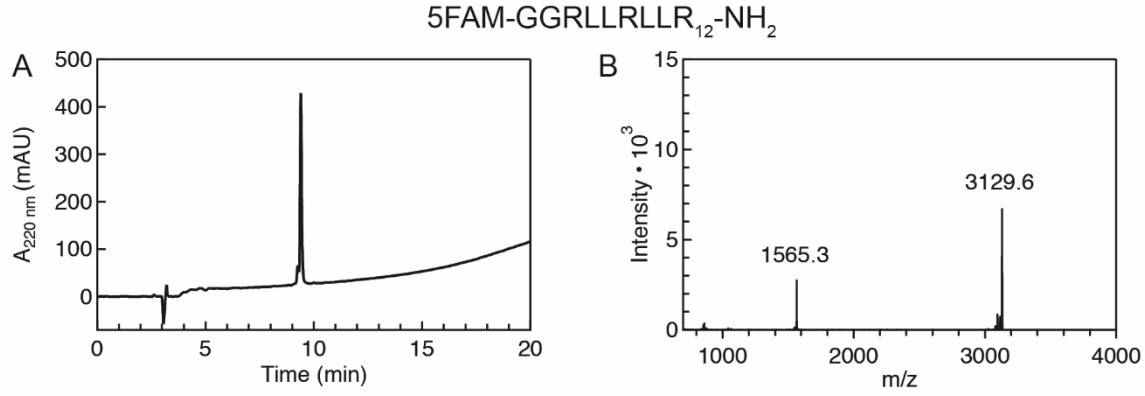


Figure S2R. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-GGRLLRLLR$_{12}$-NH$_2$. m/z is 3129.6 for $[M+H]^+$, 1565.3 for $[M+2H]^{2+}$. $C_{133}H_{231}N_{63}O_{26}$ calculated molecular weight 3128.73.

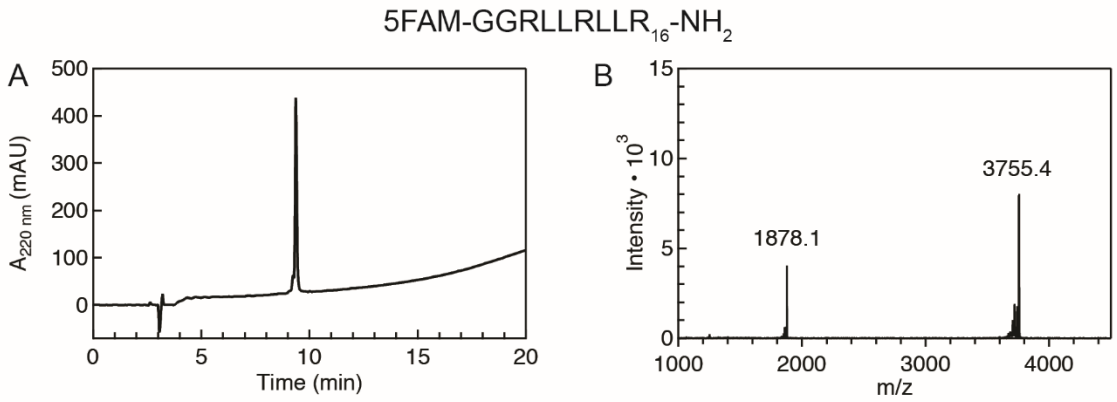


Figure S2S. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-GGRLLRLLR$_{16}$-NH$_2$. m/z is 3755.4 for $[M+H]^+$, 1878.1 for $[M+2H]^{2+}$. $C_{157}H_{279}N_{79}O_{30}$ calculated molecular weight 3753.48.

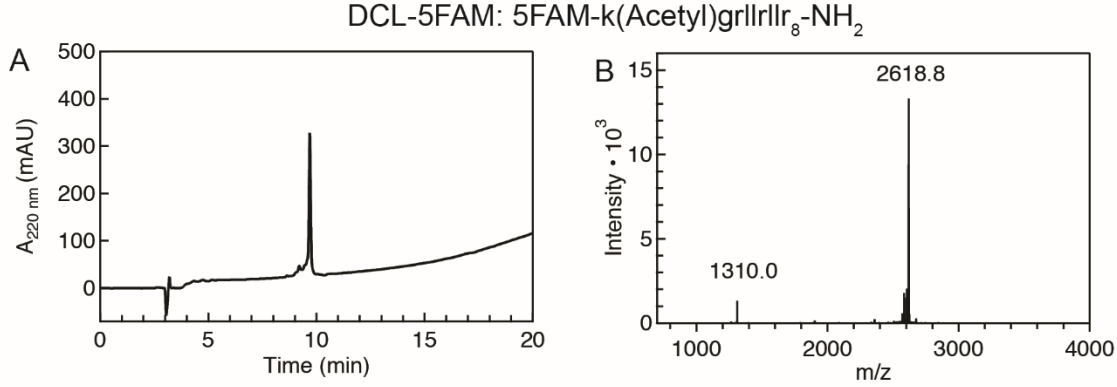


Figure S2T. (A) RP-HPLC and (B) MALDI-TOF characterization of 5FAM-DCL (5FAM-k(Acetyl)grllrllr$_8$-NH$_2$). m/z is 2618.8 for $[M+H]^+$, 1310.0 for $[M+2H]^{2+}$. $C_{115}H_{194}N_{48}O_{23}$ calculated molecular weight 2617.13.

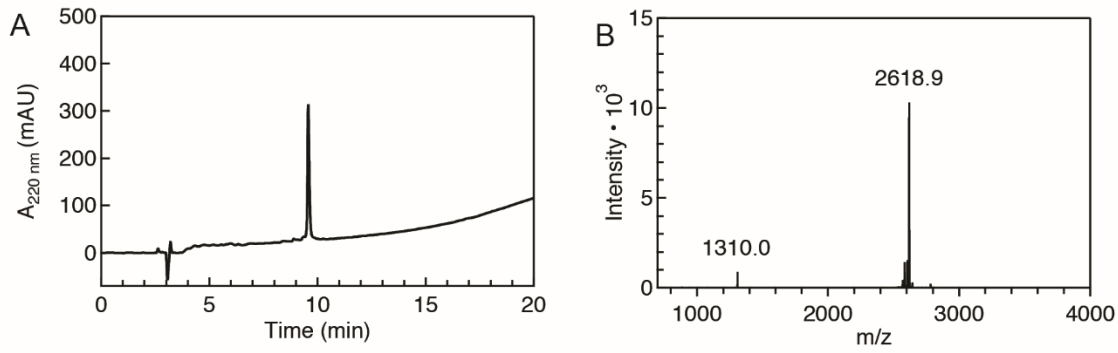


Figure S2U. (A) RP-HPLC and (B) MALDI-TOF characterization of CLr-5FAM (5FAM-K(Acetyl) GRLLrLLR$_8$-NH$_2$). m/z is 2618.9 for $[M+H]^+$, 1310.0 for $[M+2H]^{2+}$. $C_{115}H_{194}N_{48}O_{23}$ calculated molecular weight 2617.13.

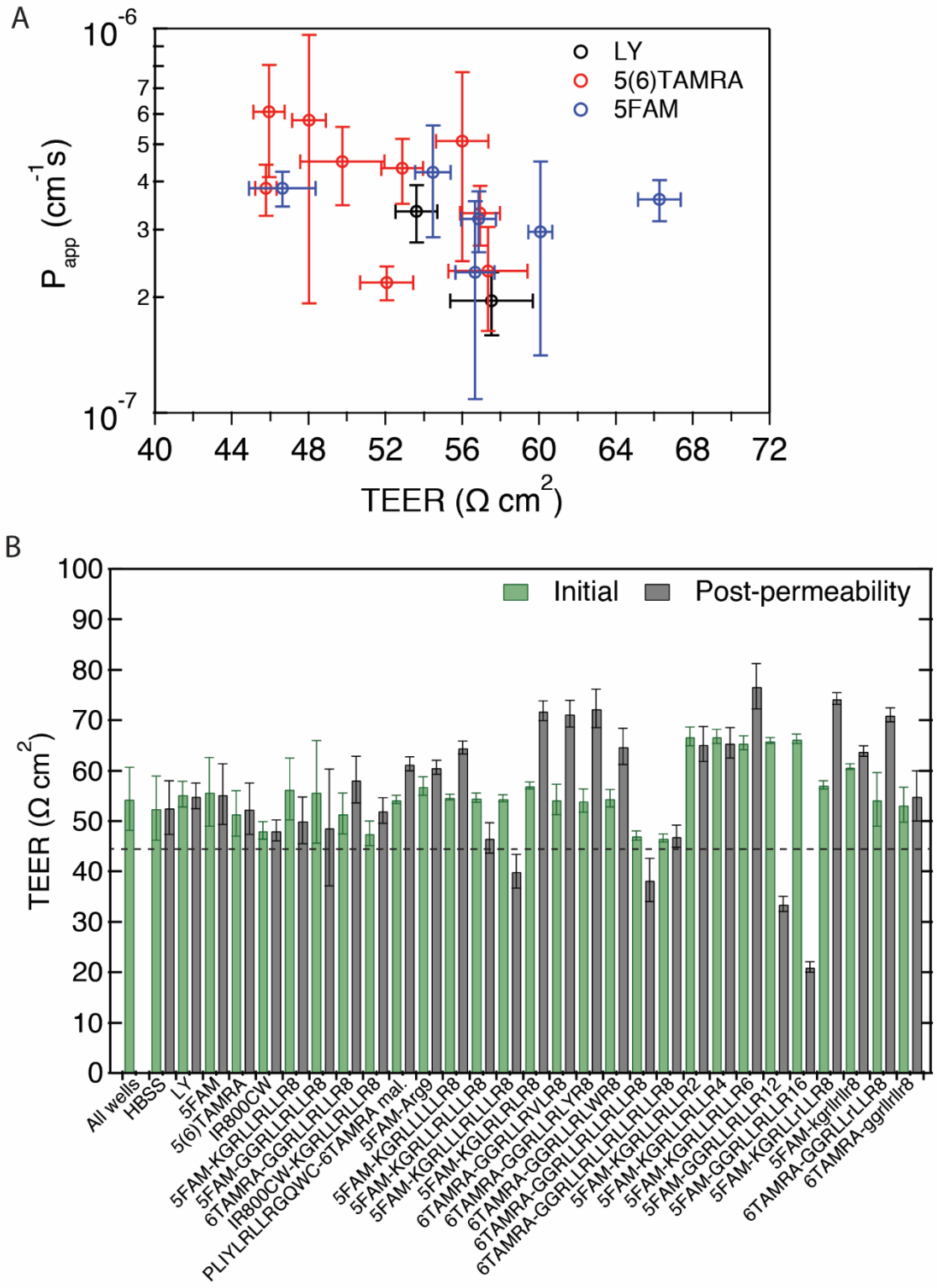


Figure S3. Barrier integrity assessment of MDCKII monolayers in a transwell assay. **A.** The permeability (P_{app}) of fluorescent dyes (cargoes) versus transepithelial electrical resistance (TEER) across MDCKII cells in a transwell assay. Lucifer yellow (LY), a dye commonly used to validate the barrier integrity, and free cargoes have low P_{app} and were used to assess the cell monolayer integrity. We measure low P_{app} for dyes in the 45-68 Ω cm^2 TEER range. **B.** TEER across MDCKII cells in a transwell assay before and after the 1-hour permeability experiment. The apical concentrations were 2.5 μM for peptide-cargo conjugates, 10 μM for 5FAM, 20 μM for 5(6)TAMRA, and 100 μM for LY. Data represent mean ± SD.

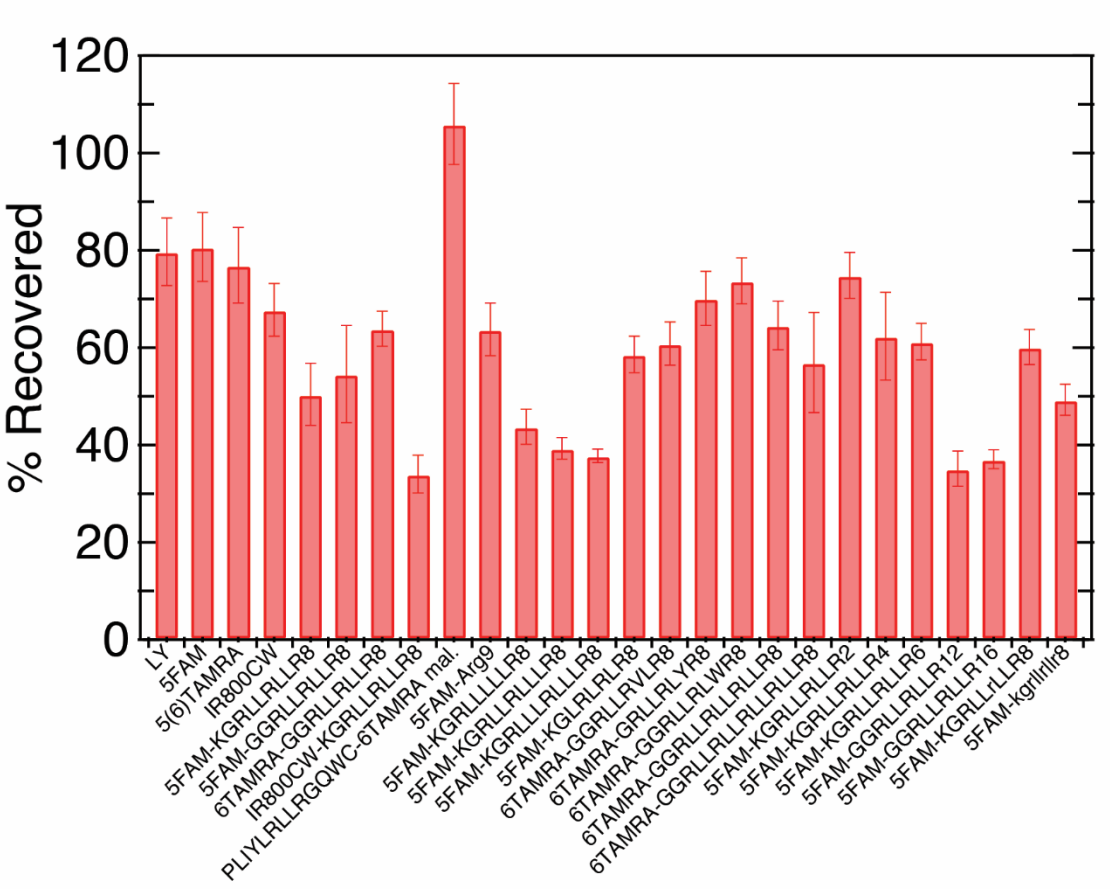


Figure S4. Mass balance (%recovered) for the free cargo and the peptide-cargo conjugates after the permeability experiment in a transwell assay. The majority of peptide-cargo conjugates exhibit 40-80% recovery and cargo exhibit 70-80% recovery. The %recovered was calculated using the equation %recovered = $(n_{bas} + n_{ap})/n_o$ x 100%, where n_{bas} and n_{ap} are the number of moles recovered from the basolateral and apical chambers, respectively, after the permeability experiment, and n_o is the initial number of moles added to the apical chamber. The number of moles sequestered by cells was not included in the mass balance calculations.

Table S1. Peptide-cargo conjugate panel and its calculated physico-chemical properties.

Name	Sequence [a]	Cargo	MW (g/mol) [b]	Peptide charge [c]	ΔG INF (kcal/mol) [d]	ΔG INF alpha helix (kcal/mol) [d, e]	μ alpha helix (kcal/mol) [e, f]
CL-5FAM	KG**RLLRLLR**R_7	5FAM	2617.13	10	2.7	-2.61	2.87
CL-5FAM	GG**RLLRLLR**R_7	5FAM	2503.97	10	2.7	-2.61	2.87
CL-6TAMRA	GG**RLLRLLR**R_7	6TAMRA	2559.12	10	2.7	-2.61	2.87
CL-IR800CW	KG**RLLRLLR**R_7	IR800CW	3244.99	10	2.7	-2.61	2.87
TP2-6TAMRA maleimide	PLIYLRLLRGQWC	6TAMRA maleimide	2110.54	1.9	2.39	N/A	N/A
Arg_9-5FAM	R_9	5FAM	1782.02	9	1.06	N/A	N/A
	KG**RLLLLLR**R_7	5FAM	2574.10	9	1.33	-3.98	1.5
	KG**RLLRLLLR**R_7	5FAM	2730.29	10	3.17	-3.57	3.28
	KG**RLLLRLLLR**R_7	5FAM	2843.45	10	2.7	-4.53	2.74
	KGLRLRLRR_7	5FAM	2503.97	10	0.77	N/A	N/A
	GG**RLLRVLR**R_7	6TAMRA	2545.09	10	2.68	-1.98	2.83
	GG**RLLRLYR**R_7	6TAMRA	2609.13	10	3.06	-2.99	3.23
	GG**RLLRLWR**R_7	6TAMRA	2632.17	10	3.95	-3.9	4.1
	GG**RLLRLLRLLR**R_7	6TAMRA	2941.63	11	2.74	-4.12	2.84
	GG**RLLRLLRLLRLLR**R_7	6TAMRA	3324.14	12	2.04	-5.63	2.06
	KG**RLLRLLR**R	5FAM	1680.00	4	3.53	-2.61	2.87
	KG**RLLRLLR**R_3	5FAM	1992.37	6	2.49	-2.61	2.87
	KG**RLLRLLR**R_5	5FAM	2304.75	8	3.48	-2.61	2.87
	GG**RLLRLLR**R_{11}	5FAM	3128.73	14	3.06	-2.61	2.87
	GG**RLLRLLR**R_{15}	5FAM	3753.48	18	3.38	-2.61	2.87
CLr-5FAM	KGRLLrLLRR_7	5FAM	2617.13	10	2.7	N/A	N/A
CLr-6TAMRA	GGRLLrLLRR_7	6TAMRA	2559.12	10	2.7	N/A	N/A
DCL-5FAM	kg**rllrllr**r_7	5FAM	2617.13	10	2.7	-2.61	2.87
DCL-6TAMRA	gg**rllrllr**r_7	6TAMRA	2559.12	10	2.7	-2.61	2.87

[a] The peptides have amidated C-terminus ($CONH_2$). The N-terminal lysine is acetylated and the cargo is conjugated to the side chain. The bolded sequence is assumed to form an alpha helix.
[b] Calculated for peptide-cargo conjugates from their chemical structures using ChemDraw 13.0 (PerkinElmer, Inc., Waltham, MA).
[c] Calculated using the Peptide Property Calculator PepCalc.com (http://pepcalc.com; Innovagen AB, Lund, Sweden). The charge calculation excluded N-terminal lysine and cargo.
[d] Gibbs free energy of peptide partitioning (ΔG) into the lipid bilayer interface from water was calculated using MPEX 3.2.15 (Stephen White Laboratory, University of California, Irvine, USA). The calculation excluded the N-terminal lysine or glycine.
[e] The calculation was made for the sequence in bold assuming a 100% helix and no end groups.
[f] Hydrophobic moment (μ) was calculated for the sequence in bold based on ΔG of partitioning into the lipid bilayer interface using MPEX 3.2.15.

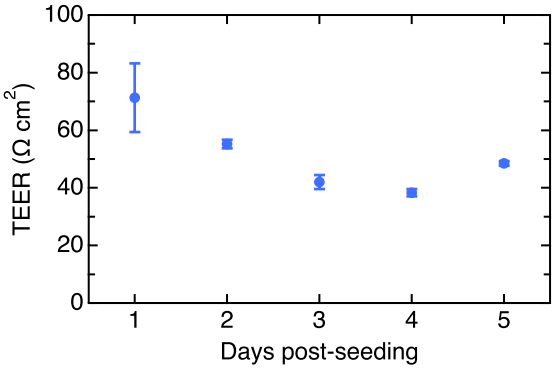
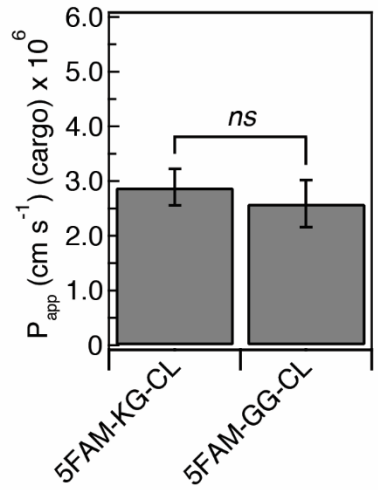
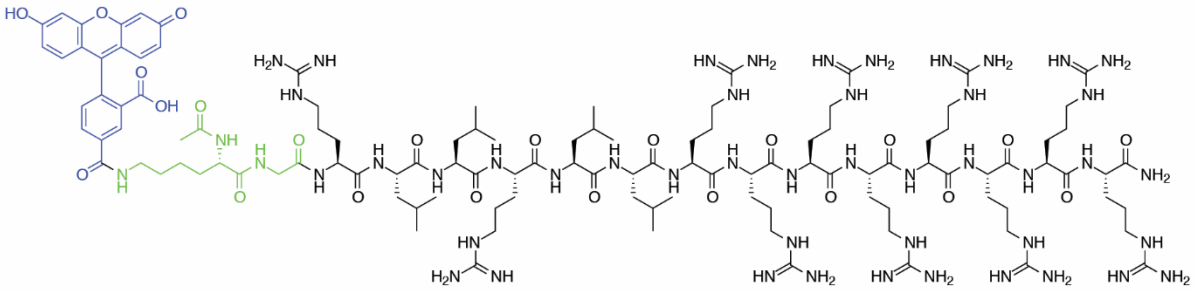


Figure S5. Transepithelial electrical resistance measurements across MDCKII cells on different days after seeding in a transwell. We observe that TEER reaches stable values of 40 – 50 Ω cm^2 on the third day after seeding. The higher TEER on Day 1 is likely due to the presence of nonadherent cells on the epithelial surface, which are subsequently removed during medium changes. Data represent mean ± SD. Sample size n represents the number of transwell replicates: n=5.



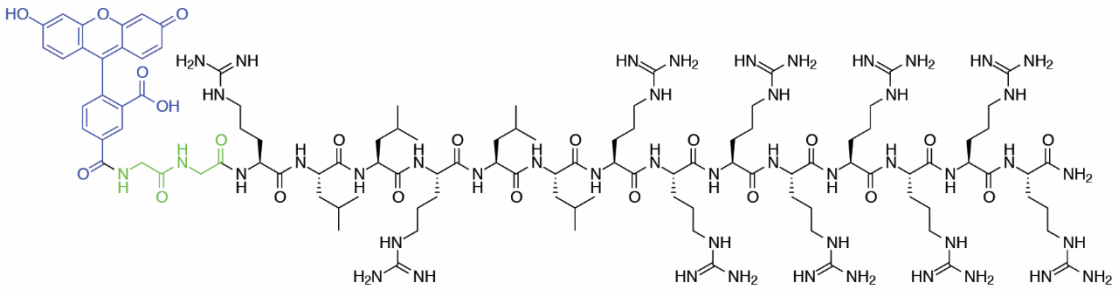
5FAM-KG-CL: 5FAM-K(Acetyl)GRLLRLLR$_8$-NH$_2$
5FAM-GG-CL: 5FAM-GGRLLRLLR$_8$-NH$_2$

5FAM-KG-CL



5FAM linker CL peptide

5FAM-GG-CL



5FAM linker CL peptide

Figure S6. The effect of the linker on permeability across MDCKII monolayers in a transwell assay. **A.** The permeability of CL-5FAM conjugate with either Lys(Acetyl)-Gly or Gly-Gly linker between the cargo and the peptide. There is no statistically significant difference between the permeabilities. p = 0.18 (Student's t-test, two-tailed, unequal variance). Sample size n represents the number of transwell replicates: n=7. Data represent mean ± SD. **B.** Chemical structures of CL-5FAM conjugates with Lys(Acetyl)-Gly or Gly-Gly linkers.

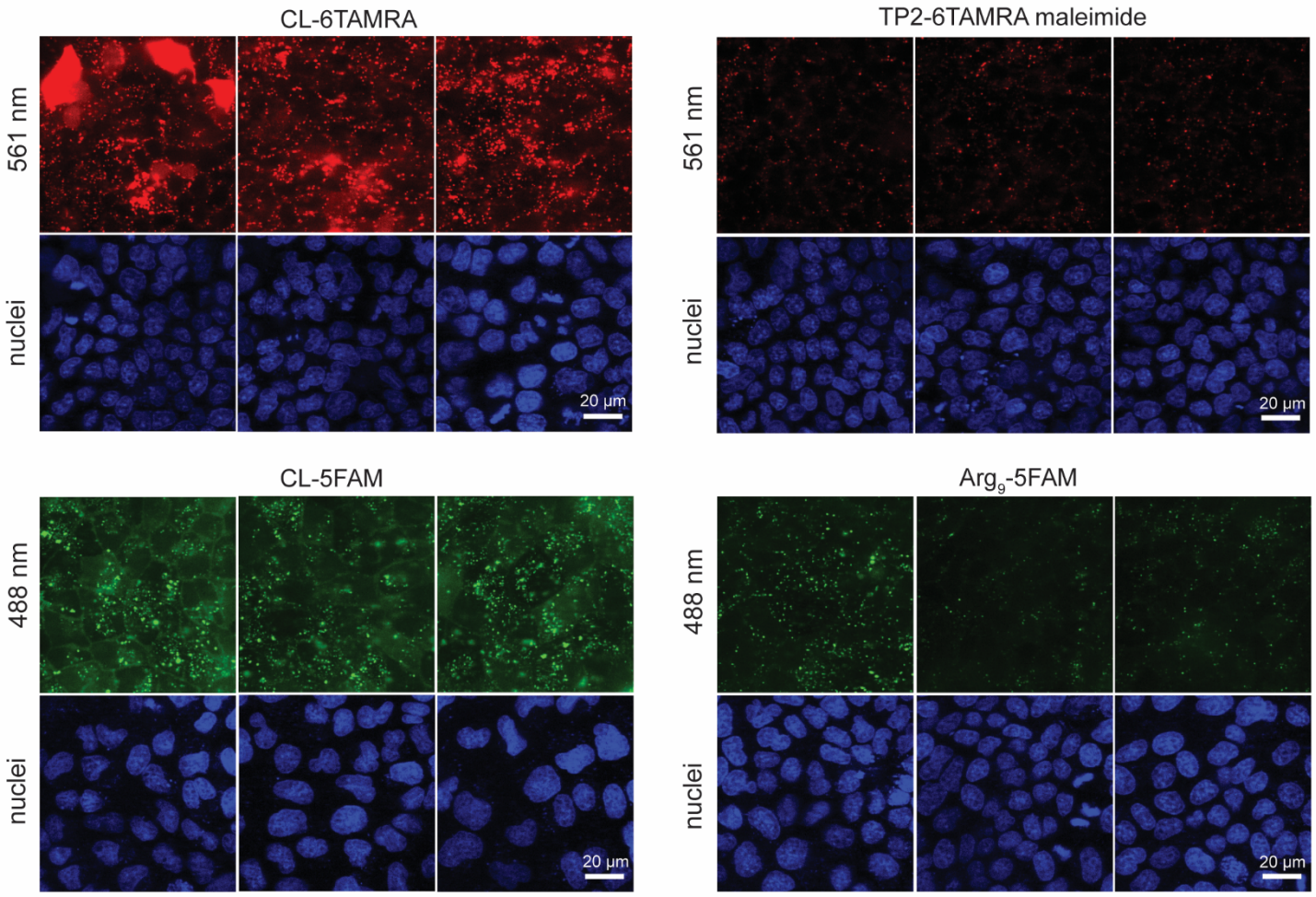


Figure S7. Confocal microscopy images of the uptake of CL-6TAMRA, TP2-6TAMRA maleimide, Arg9-5FAM, and CL-5FAM in MDCKII cells after 1-hour incubation at 37 °C.

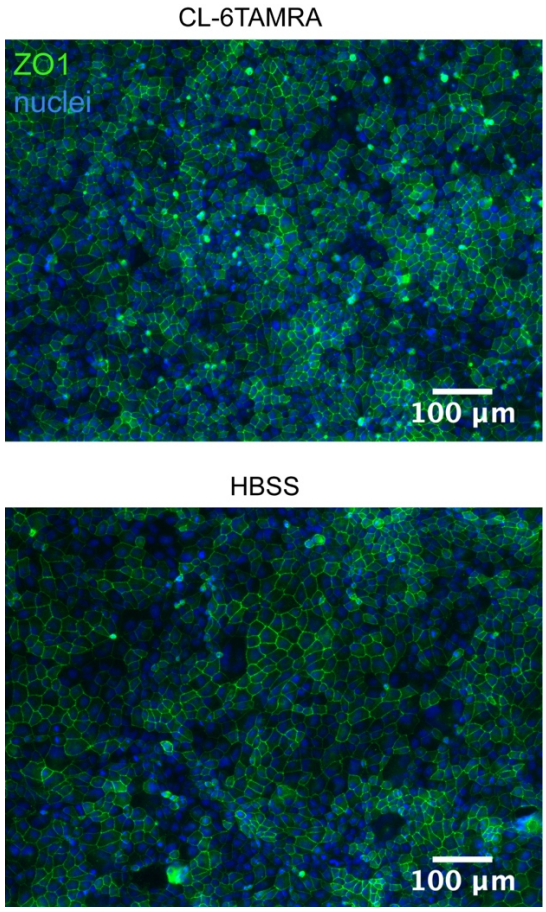


Figure S8. Immunofluorescent staining of tight junction protein, Zona occludens-1 (ZO-1), after one-hour treatment with 2.5 µM CL-6TAMRA conjugate and HBSS buffer. The cells were fixed immediately after the treatment. We observe no difference in ZO-1 junction expression after CL-6TAMRA treatment, when compared to the HBSS control. The results show that junctional integrity is not compromised by the peptide-cargo conjugate.

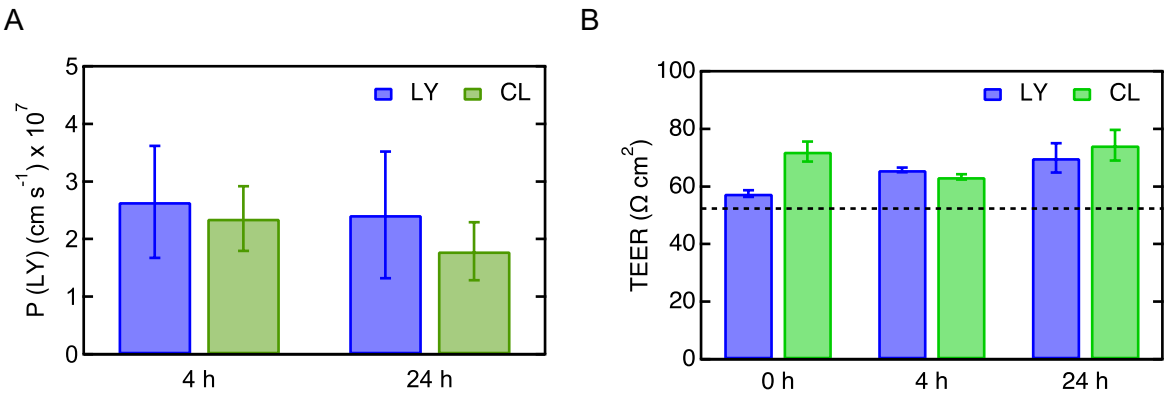
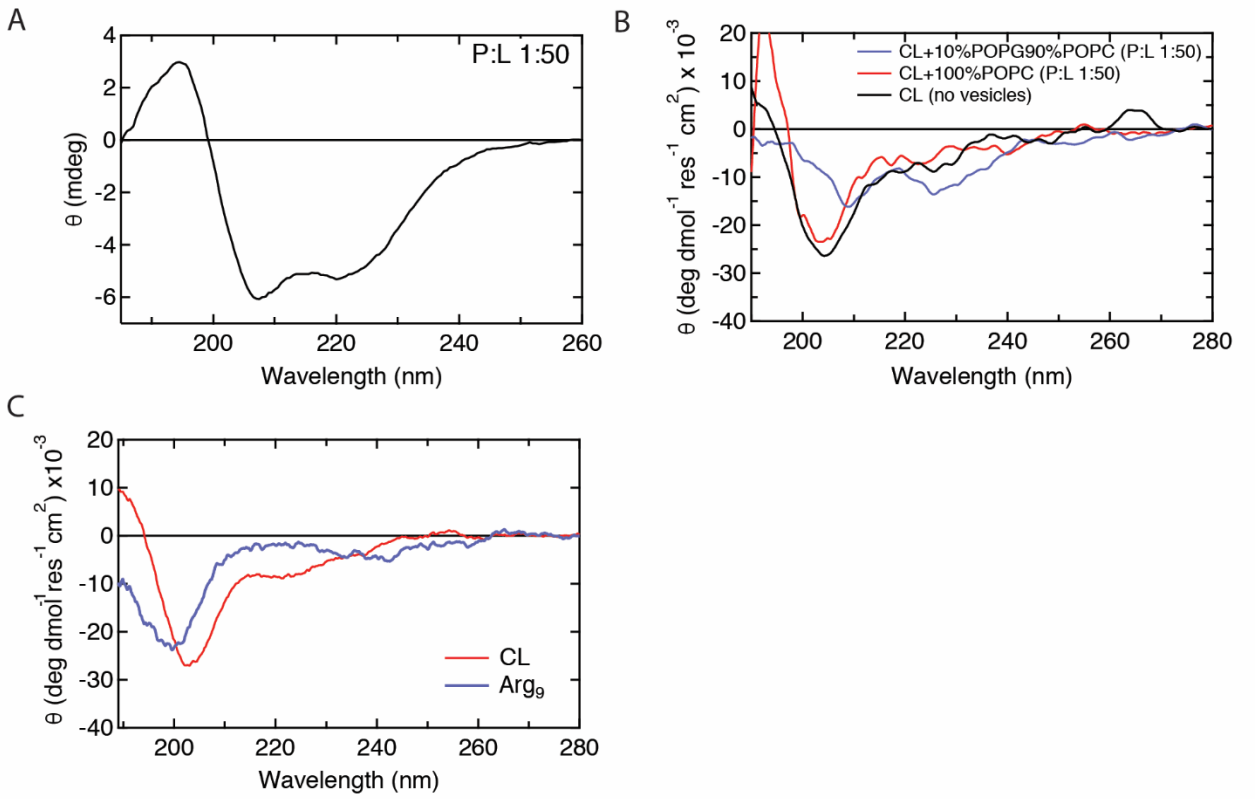


Figure S9. The effect of CL-cargo conjugate on epithelial junctional integrity over time. **A.** Measured permeability of Lucifer yellow (LY) . **B.** Measured transepithelial electrical resistance (TEER) 4 hours and 24 hours after a one hour incubation of cells with the CL peptide, LY, and HBSS control at 37 °C. The apical concentrations were 2.5 μM for the CL peptide and 100 μM for LY. The dashed line in B represents the mean initial TEER measurement prior to the treatments. LY is commonly used as a barrier integrity probe, and the measured LY permeability in a range of 1 – 3 x 10^{-7} cm s^{-1} confirms barrier integrity. TEER measured in the range of 60 – 70 Ω cm^2 further confirms that the CL-cargo conjugate did not induce long-term decrease in junctional integrity. The lack of short- and long-term effect on junctional integrity suggests that CL-cargo conjugate is not cytotoxic after standard permeability treatments. Data represent mean ± SD. Sample size n represents the number of transwell replicates: LY (n=3), CL (n=3), HBSS (n=4).



Figures S10. Circular dichroism characterization of the CL-cargo conjugate. **A.** Oriented circular dichroism of the CL-cargo conjugate in the presence of stacked POPC lipid bilayers at 1:50 peptide-to-lipid (P:L) ratio. The lipid bilayers were hydrated through the vapor. The peptide acquires alpha helical structure. **B.** CD spectra of the CL-cargo conjugate in the presence of 100% POPC vesicles (P:L 1:50), 10%POPG/90%POPC (P:L 1:50), or in the absence of vesicles in 10 mM sodium phosphate buffer, pH 7. **C.** Circular dichroism spectra of 10 μM Arg_9-cargo and CL-cargo conjugates in 10 mM sodium phosphate buffer, pH 7.

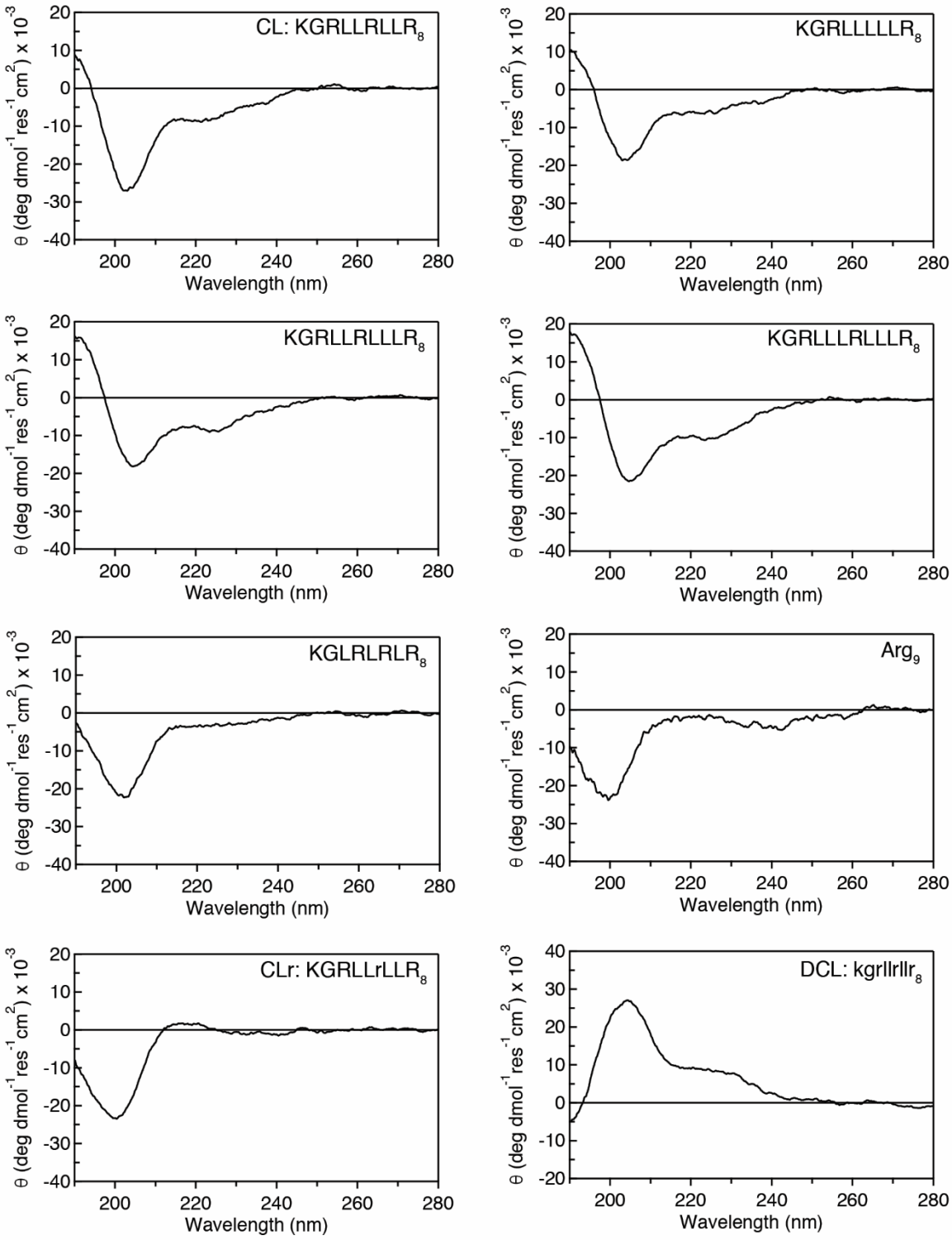


Figure S11. Circular dichroism spectra of 10 μM peptide-cargo conjugates in 10 mM sodium phosphate buffer, pH 7.

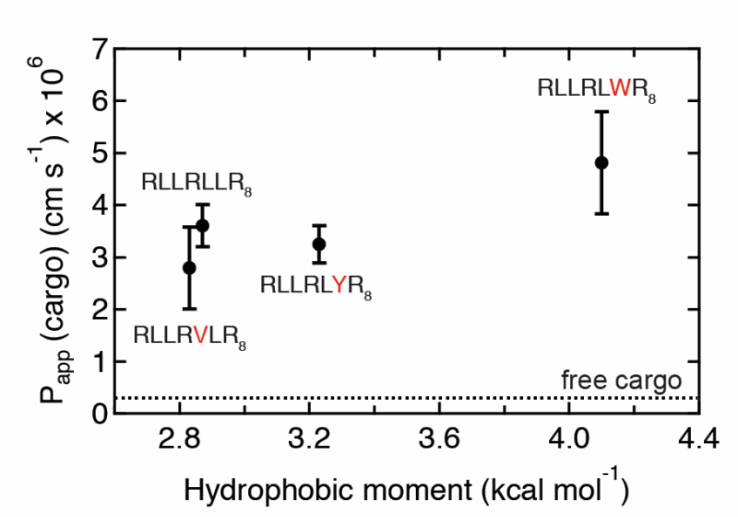


Figure S12. The permeability versus theoretical hydrophobic moment of CL-6TAMRA variants with single leucine substitutions to either tryptophan (W), tyrosine (Y) or valine (V). The position of the leucine substitution (shown in red in the sequences) was chosen to maximize the theoretical hydrophobic moment. The dashed line represents P_{app} for 5(6)TAMRA. The apical concentrations were 2.5 μM for the peptide-cargo conjugates and 20 μM for 5(6)TAMRA. Sample size n represents the number of transwell replicates: n=12. Data represent mean ± SD. The hydrophobic moment was calculated based on the Wimley-White scale for peptide partitioning from water into the lipid bilayer interface (MPEX 3.2.15). The partial correlation between the alpha helix hydrophobic moment and P_{app} of peptide-cargo is not statistically significant when controlling for the hydrophobicity ($r=0.08$, $p=0.60$, Pearson's partial correlation on individual measurements).

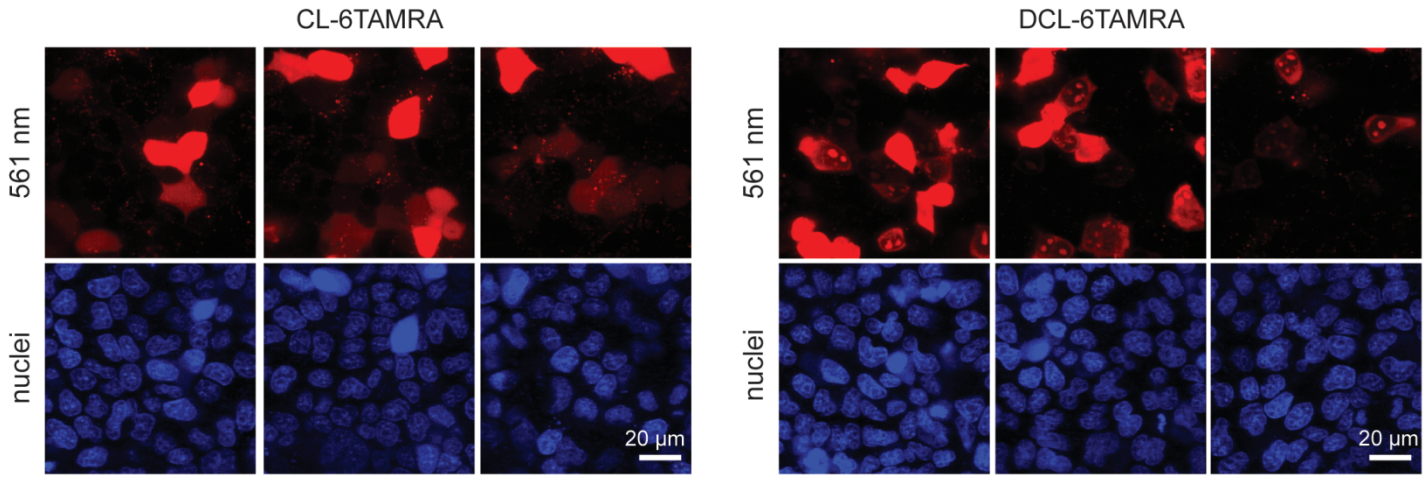


Figure S13. Confocal microscopy images of the uptake of 2.5 µM CL-6TAMRA and DCL-6TAMRA by MDCKII cells after 1-hour incubation at 37 °C.

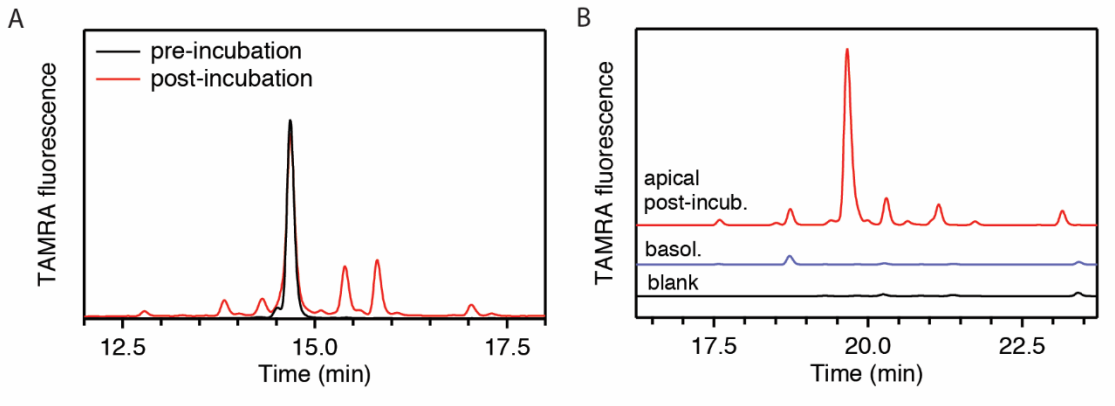


Figure S14. HPLC spectra of apical and basolateral samples after the permeability experiment in a transwell assay. **A.** The apical solution before and after 1-hour incubation of CL-6TAMRA with MDCKII cells in a transwell assay. The spectra are normalized relative to the peak with maximum fluorescence intensity. TAMRA ex/em: 542 nm/566 nm. Gradient (0-28 min: 5-95% acetonitrile). **B.** The apical and basolateral solutions after the permeability experiment with CL-6TAMRA. The HPLC spectra were measured at a shallower acetonitrile gradient (0-28 min: 5-60% acetonitrile). The apical sample contains a peak corresponding to the basolateral product. This suggests that the digested product exits cells in both the apical and the basolateral directions.

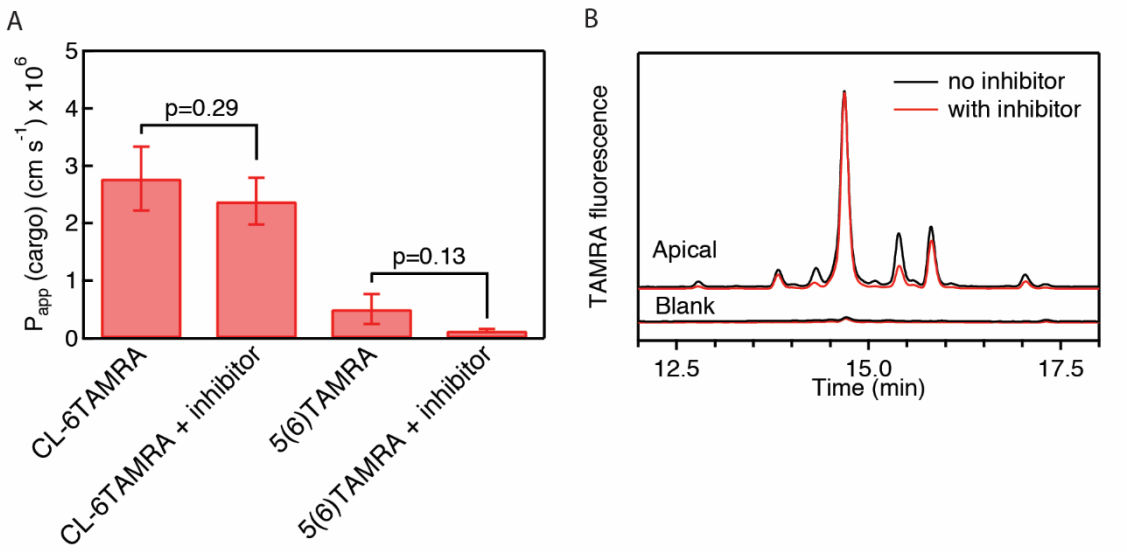


Figure S15. The effect of inhibitor cocktail treatment on the permeability and digestion of CL-6TAMRA. **A.** The permeability of CL-6TAMRA conjugate across MDCKII cells in a transwell assay with or without a protease inhibitor cocktail treatment. The inhibitor solution was prepared by dissolving one tablet of Roche Complete Mini, EDTA-free (Cat. no. 11836170001, Sigma-Aldrich) in 10 mL of HBSS buffer and filter-sterilizing the solution. For the inhibitor treatment, the cells were preincubated with the inhibitor solution for 30 minutes at 37 °C, followed by the addition of the solutes diluted in the inhibitor solution. The apical concentrations were 2.5 μM for CL-6TAMRA and 20 μM for 5(6)TAMRA. Sample size n represents the number of transwell replicates: 5(6)TAMRA (n=3), all other treatments (n=4). Data represent mean ± SD. There is no statistically significant difference between the treatments with the inhibitor and without the inhibitor (Student's t-test, two-tailed, unequal variance). **B.** HPLC spectra of the apical CL-6TAMRA samples after 1-hour incubation with MDCKII cells with or without the inhibitor treatment. The spectra are normalized relative to the peak with the maximum fluorescence intensity.

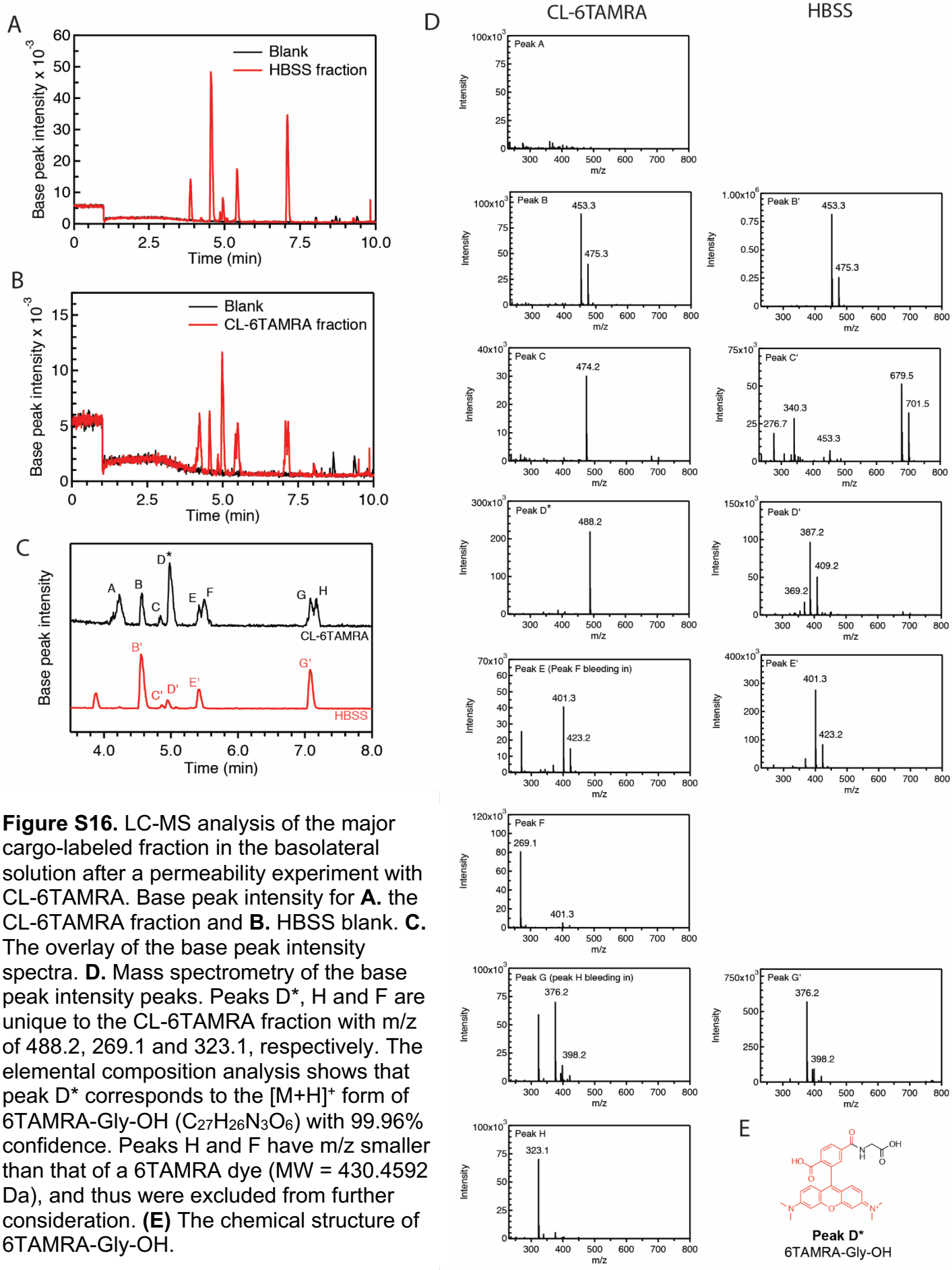


Figure S16. LC-MS analysis of the major cargo-labeled fraction in the basolateral solution after a permeability experiment with CL-6TAMRA. Base peak intensity for **A.** the CL-6TAMRA fraction and **B.** HBSS blank. **C.** The overlay of the base peak intensity spectra. **D.** Mass spectrometry of the base peak intensity peaks. Peaks D*, H and F are unique to the CL-6TAMRA fraction with m/z of 488.2, 269.1 and 323.1, respectively. The elemental composition analysis shows that peak D* corresponds to the $[M+H]^+$ form of 6TAMRA-Gly-OH ($C_{27}H_{26}N_3O_6$) with 99.96% confidence. Peaks H and F have m/z smaller than that of a 6TAMRA dye (MW = 430.4592 Da), and thus were excluded from further consideration. **(E)** The chemical structure of 6TAMRA-Gly-OH.

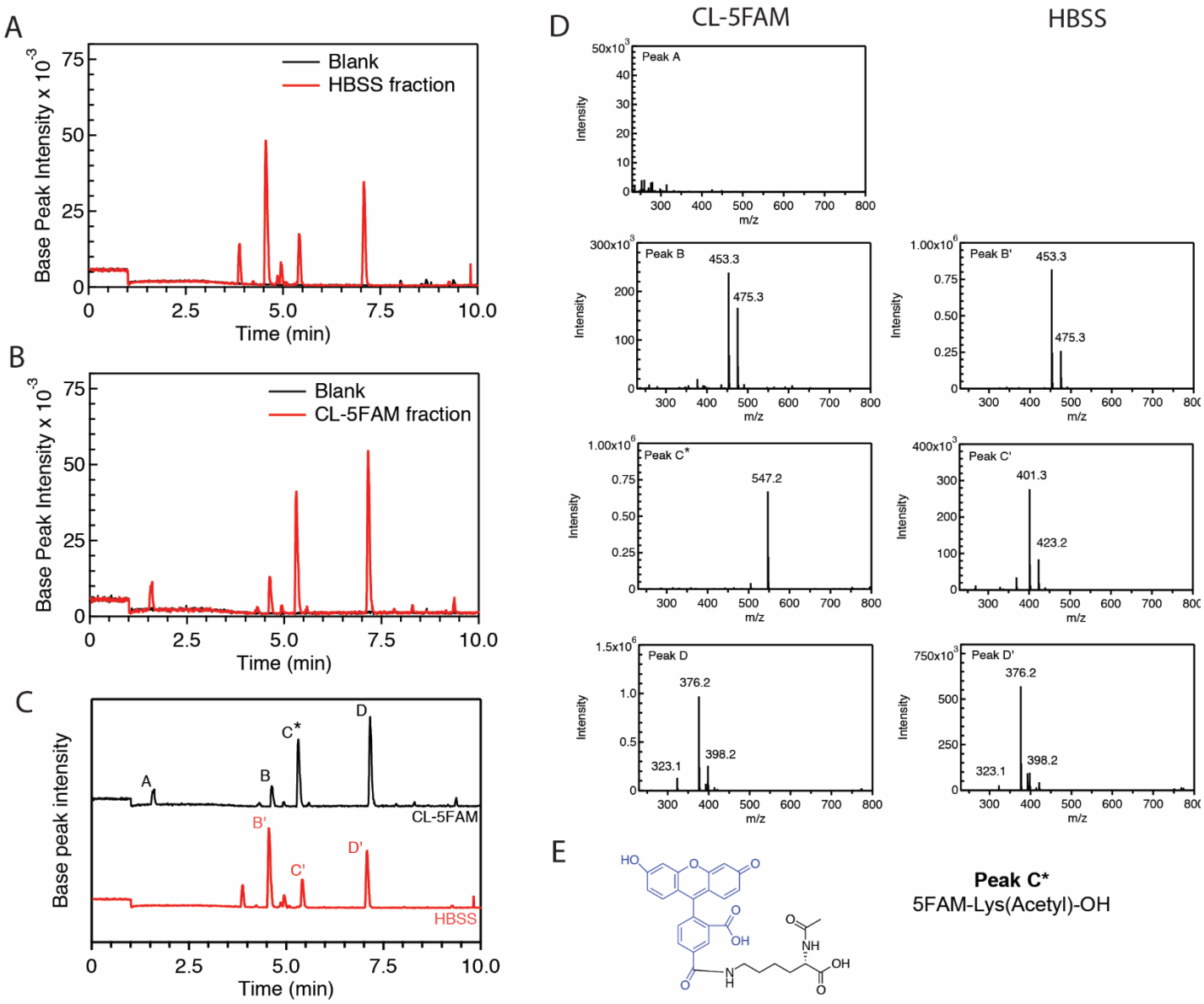


Figure S17. LC-MS analysis of the major cargo-labeled fraction in the basolateral solution after a permeability experiment with 2.5 μM CL-5FAM. Base peak intensity for **A.** the CL-5FAM fraction and **B.** HBSS blank. **C.** The overlay of the base peak intensity spectra. **D.** Mass spectrometry of the base peak intensity peaks. Peak C* is unique to the CL-5FAM fraction with m/z of 547.2 Da. The elemental composition analysis shows that peak C* corresponds to $[M+H]^+$ form of 5FAM-Lys(Acetyl)-OH ($C_{29}H_{26}N_2O_9$) with 99.86% confidence. **E.** The chemical structure of 5FAM-Lys(Acetyl)-OH.

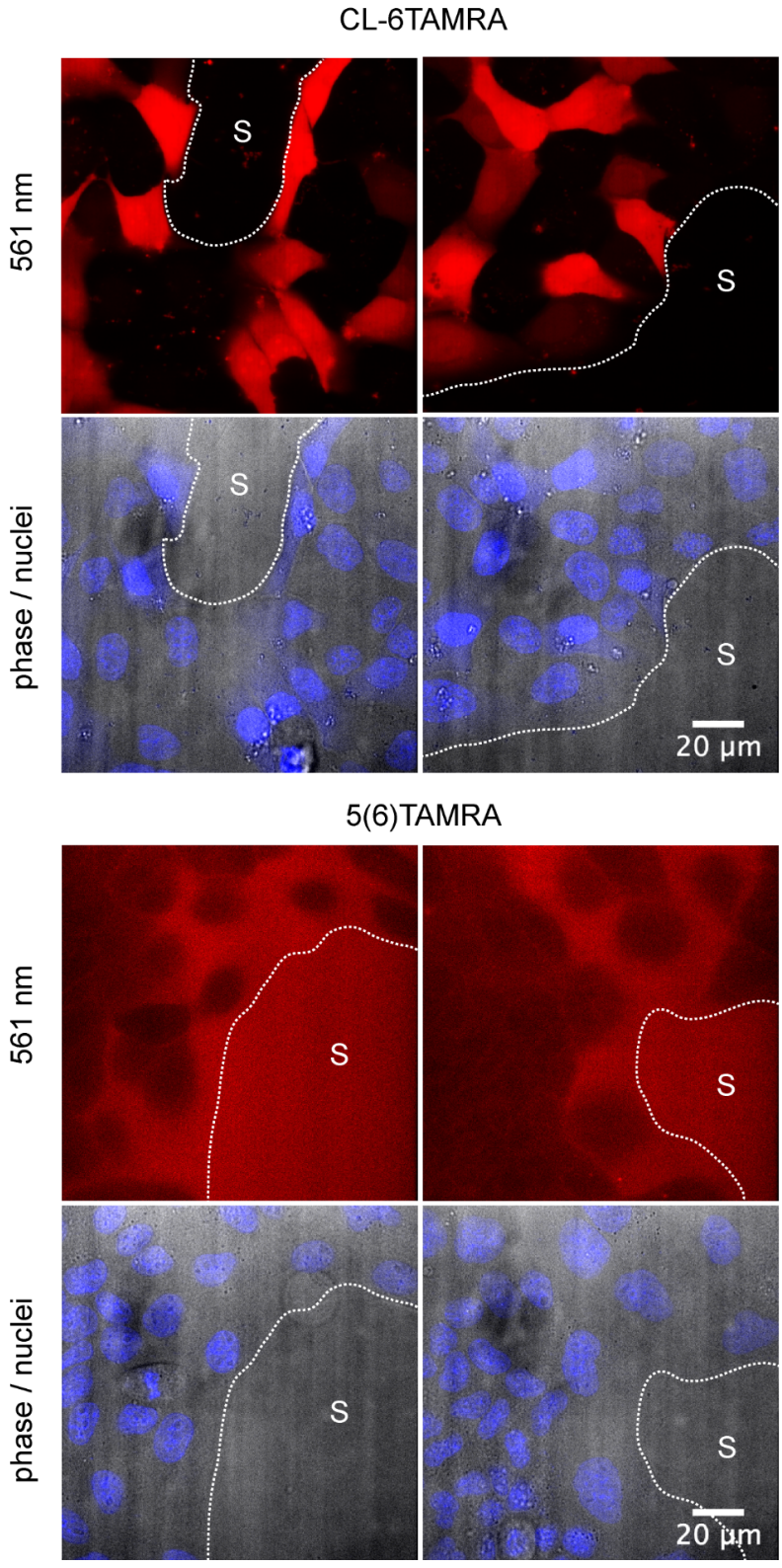


Figure S18. Confocal microscopy images of sub-confluent MDCKII monolayers incubated with 2.5 µM CL-6TAMRA or 5(6)TAMRA. The peptide-cargo conjugate and dye were not washed away from the cells and are present in solution. Dashed line is drawn to outline borders between the cell monolayers and the solution (indicated by "S"). LUTs between CL-6TAMRA and 5(6)TAMRA are not the same and were adjusted for 5(6)TAMRA to demonstrate that the dye does not enter the cells. We observe that CL-6TAMRA significantly accumulates inside cells and the accumulation is heterogeneous. Images were taken after 30 minutes of incubation at 37°C.